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PhD

Insights into the mechanisms of zinc tolerance and accumulation in *Solanum nigrum* L.

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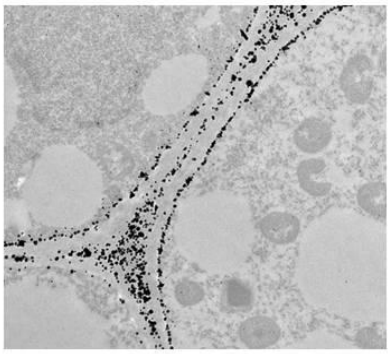

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PhD

Aos meus pais, terra firme

E à Júlia

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SUMMARY

Anthropogenic activities, associated with economic and population growth, are a continuous source of organic and inorganic contaminants causing environmental deterioration. Certain plants, known for their ability to degrade and/or accumulate contaminants, show potential for environmental clean-up in a green-technology designated as phytoremediation. This is a growing area of biotechnological interest and research, as can be inferred by the increasing number of patents registered these last years, presenting a broad range of creative solutions namely, plant growth enhancement, manipulation of the physico-chemical characteristics of the contaminated environments, or through genetically engineered plants to obtain improvements in key characteristics, such as the tolerance, uptake and accumulation of contaminants.

Solanum nigrum L. plants, known to accumulate zinc, hyperaccumulate cadmium and endure combined metal contamination, have been acknowledged as promising candidates in phytoremediation. This is a vigorous and persistent plant species that is vastly distributed in the globe and possesses characteristics favouring interspecific competition. However, much is to be revealed about the mechanisms involved in Zn accumulation in *S. nigrum*. This PhD project was aimed at disclosing mechanisms into *S. nigrum* tolerance and accumulation of Zn.

With the aim of identifying the specific tissue, cell and subcellular compartments of Zn sequestration in roots, stems and leaves of *S. nigrum* plants challenged with Zn at 0.025 g L^{-1}, Zn localization was evaluated by autometallography (AMG). Zinc concentration in the plants was highest in the roots, 666 mg kg^{-1} f.w., and lower in the stems and leaves, 318 and 101 mg kg^{-1} f.w (fresh weight), respectively. A generalized Zn distribution associated with the cell walls was revealed by light microscopy through AMG in all tissues of the roots, stems and leaves. Conspicuous Zn deposits were detected in the vacuoles of cortical parenchyma of the root and stem, with particular intensity in the starch sheath. Further detail of Zn localization was revealed by electron microscopy. In the vascular tissues, Zn was observed at the level of the plasma membrane – cell wall complex of vascular parenchyma and conducting elements. The Zn distribution observed suggests that Zn flux through the plant occurs via the xylem, phloem and their associated parenchyma until it is conducted to the apoplast and vacuoles of parenchyma cells of the root, stem and the leaf mesophyll which emerge as important sequestration sites.

Aiming to further unveil the mechanisms of Zn tolerance and accumulation in *S. nigrum* plants, the involvement of organic acids and differentially expressed proteins

were also evaluated at different stages of plant development. Interestingly, pre- and post-flowering *S. nigrum* plants, when challenged with Zn concentrations lethal to plantlets, 0.10 g L^{-1}, showed an increase in tolerance from pre-flowering to post-flowering, which was accompanied by a reduction of Zn accumulation in the aerial plant parts. Furthermore, organic acid concentrations also varied between plant organs and developmental stages. Some of the organic acids identified by HPLC, namely malic and citric acids, may be involved by participating in Zn root-to-shoot transport, subcellular sequestration and also in the mitigation of the effects of Zn on plant metabolism by providing metabolites for respiration. In addition, the increases observed in shikimic acid suggest the activation of secondary metabolism through which important metabolites such as chelators, signalling molecules and cell wall constituents are produced. The differential expression of proteins in the roots of these plants, where higher accumulation of Zn was observed, was assessed by two-dimensional electrophoresis. The results showed 19 induced or highly up-regulated proteins in response to Zn treatment with distinct biochemical assignment suggesting a pleotropic Zn response in *S. nigrum* roots recruiting several metabolic pathways. In fact, while a number of the these proteins were engaged in energy metabolism, namely enolase, malic enzyme and alcohol dehydrogenase, indicating a higher energy demand in Zn treated *S. nigrum* plants, another well represented group of proteins identified are acknowledged as key players in abiotic and biotic stress defense, proteolysis and oxidative stress responses. The identification of an alpha-L-arabinofuranosidase, a protein involved in cell wall modification, highlights the role of the cell wall in tolerance and accumulation of Zn in this plant.

The results lead to the conclusion that Zn tolerance and accumulation in *S. nigrum* are growth dependent and that several mechanisms are involved. Metal flux through the plant occurs through both vascular tissues while the apoplast and cellular vacuoles stand out as key sequestration sites. Organic acids are also relevant in this response as vacuolar ligands, in long-distance transport or possibly as respiratory substrates. This last hypothesis is supported by increase in the expression of proteins involved in energy metabolism. The “damage control” of metal toxicity also takes relevance and is indicated by the increase of enzymes involved in proteolysis and antioxidative stress response. Lastly, an important role is likely played by secondary metabolites, as suggested by the increases observed in shikimic acid and in defense proteins activated by these metabolites or involved in secondary metabolism.

All together these results offer insight into the mechanisms of Zn tolerance and accumulation in *S. nigrum* and further contribute to the notion of a complex network of mechanisms involved in metal response in plants.

Keywords:

Solanum nigrum L., zinc, tolerance, accumulation, zinc sequestration, plant development, organic acids, proteomics, phytoremediation.

RESUMO

As atividades humanas, associadas ao crescimento económico e populacional, são uma fonte contínua de contaminantes orgânicos e inorgânicos que resultam na degradação do ambiente. Determinadas plantas, reconhecidas pela sua capacidade de degradar e/ou acumular contaminantes, demonstram ter potencial para a remediação ambiental numa tecnologia designada por fitorremediação. Esta é uma área de crescente interesse biotecnológico e de investigação, como pode ser inferido pelo crescente número de patentes registadas ao longo dos últimos anos, que propõem uma larga variedade de soluções para a aplicação desta tecnologia incluindo a promoção do crescimento das plantas e a manipulação das características físico-químicas dos ambientes contaminados, até à manipulação genética das plantas para melhorar características importantes, como a tolerância, a absorção e a acumulação dos contaminantes.

As plantas de *Solanum nigrum* L., caracterizadas pela sua capacidade de acumular zinco, hiperacumular cádmio e tolerar contaminação combinada por vários metais, têm sido reconhecidas pelo seu potencial em fitorremediação. Estas plantas vigorosas e persistentes, apresentam uma distribuição global e possuem características que favorecem a competição interespecífica. No entanto, os mecanismos envolvidos na acumulação de zinco em plantas de *S. nigrum* estão ainda pouco esclarecidos. Este projeto de doutoramento teve como principal objetivo contribuir para melhor conhecer os mecanismos de tolerância e acumulação de zinco em plantas de *S. nigrum.*

Com o objetivo de identificar os tecidos e compartimentos celulares envolvidos na sequestração do zinco, foram realizados estudos de autometalografia (AMG) em raízes, caules e folhas de plantas de *S. nigrum* expostas a zinco na concentração de 0,025 g L^{-1}. A concentração de zinco nestas plantas foi mais elevada na raiz, 666 mg kg^{-1} p.f. (peso fresco), e mais baixa no caule e folhas, com valores de 318 e 101 mg kg^{-1} p.f., respetivamente. Observações de microscopia ótica mostraram, de forma geral, uma distribuição de zinco associada às paredes celulares em todos os tecidos da raiz, caule e folha. Depósitos zinco foram também observados nos vacúolos do parênquima cortical da raiz e do caule, com particular intensidade na bainha amilífera. Maior detalhe da localização de zinco foi fornecido pela observação dos tecidos por microscopia eletrónica. Curiosamente, nos tecidos vasculares, o zinco foi observado a nível do complexo da membrana plasmática – parede celular no parênquima vascular e nos elementos condutores. Esta distribuição sugere que o fluxo de zinco ocorre através da planta pelo xilema e floema e parênquima associado, até ser depositado a

nível do apoplasto e dos vacúolos de parênquima cortical da raiz, caule e o mesófilo, que surgem como locais preferenciais de sequestração.

Com o objetivo de melhor compreender os mecanismos de tolerância e acumulação de zinco em plantas de *S. nigrum,* foi também estudada a participação de ácidos orgânicos e a expressão diferencial de proteínas em resposta ao metal em diferentes estádios do desenvolvimento das plantas. Estes estudos permitiram verificar que plantas de *S. nigrum* em fase de pré- e pós-floração sujeitas a concentrações letais de zinco para plântulas, e.g. 0,10 g L^{-1}, demonstraram um aumento de tolerância na fase de pós-floração que foi acompanhada por uma redução de acumulação de zinco na parte aérea da planta. Adicionalmente, foram observadas variações na concentração de ácidos orgânicos entre órgãos bem como entre as fases de desenvolvimento. Alguns dos ácidos orgânicos identificados por HPLC, nomeadamente os ácidos málico e cítrico, poderão estar envolvidos no transporte de zinco da raiz para a parte aérea da planta, na sequestração subcelular e também contribuir para a mitigação dos efeitos de zinco no metabolismo da planta fornecendo metabolitos para a respiração. Aumentos observados na concentração do ácido xiquímico em resposta ao zinco sugerem uma ativação do metabolismo secundário através do qual podem ser sintetizados metabolitos secundários, entre os quais quelantes, moléculas sinalizadoras e constituintes da parede celular. A expressão diferencial de proteínas em resposta ao tratamento de zinco nas raízes destas plantas, analisada por eletroforese bidimensional, revelou a indução ou sobre-expressão de 19 proteínas com funções bioquímicas distintas sugerindo uma resposta pleotrópica ao zinco nas raízes de plantas de *S. nigrum* envolvendo várias vias metabólicas. De facto, verificou-se que enquanto várias das proteínas identificadas, nomeadamente enolase, enzima málica, e álcool desidrogenase, estão envolvidas no metabolismo energético, o que sugere um aumento dos requisitos energéticos das plantas tratadas com zinco, outras proteínas identificadas têm como funções a defesa a stresse abiótico e biótico, proteólise e a resposta ao stresse oxidativo. Foi ainda identificada uma alpha-L-arabinofuranosidase que é uma proteína que participa na modificação da parede celular, salientando o papel da parede celular na tolerância e acumulação do zinco em *S. nigrum*.

Os resultados conduzem à conclusão que a tolerância e a acumulação de zinco em *S. nigrum* são dependentes do desenvolvimento da planta e derivam de vários mecanismos. O fluxo do metal através da planta ocorre por ambos os tecidos vasculares, enquanto o apoplasto e os vacúolos se evidenciam como principais locais de sequestração. Os ácidos orgânicos são relevantes na resposta ao zinco na medida

em que podem atuar como quelantes ao nível do vacúolo ou no transporte para a parte aérea, ou ainda, como substratos para a respiração. Esta última hipótese é também sustentada pelo aumento da expressão de proteínas envolvidas no metabolismo energético, que sugere processos pela ativação do metabolismo energético. A atenuação da toxicidade resultante do metal também assume relevância, como sugerido pelo aumento da expressão de enzimas envolvidas na proteólise e em resposta ao stresse oxidativo. Por último, um papel importante poderá ser representado por metabolitos secundários, como sugerido pelo incremento na concentração de ácido xiquímico e de proteínas de defesa ativadas por esses metabolitos ou envolvidas no metabolismo secundário.

No seu conjunto, estes resultados constituem uma contribuição para o esclarecimento dos mecanismos de tolerância e acumulação de zinco em *S. nigrum* e corroboraram a noção de uma complexa “network” de mecanismos que está envolvida na resposta das plantas aos metais.

Palavras chave:

Solanum nigrum L., zinco, tolerância, acumulação, sequestração de zinco, desenvolvimento vegetal, ácidos orgânicos, proteómica, fitorremediação.

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ABBREVIATIONS

2-DE – Two-dimensional electrophoresis

AMG – Autometallography

ANOVA – Analysis of variance

APX – Ascorbate peroxidase

ATCC – American type culture collection

ATP – Adenosine triphosphate

CDF – Cation diffusion facilitator

CDTA - Trans-1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid

Cys – Cysteine

DNA – Deoxyribonucleic acid

DTPA - Diethylenetriaminepentaacetic acid

DTT - Dithiothreitol

EDDS – Ethylenediaminedisuccinic acid

EDTA – Ethylenediaminetetraacetic acid

EEA – European Environmental Agency

EGTA - Ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N-tetraacetic acid

GLDA – Glutamic acid diacetic acid

Glu – Glutamyl

Gly – Glycine

GMO – Genetically modified organism

GS-X – Glutathione S-conjugate export

HEDTA - N-hydroxyethylenediaminetriacetic acid

HGA – Homogalacturonan

His – Histidine

HMA – Heavy metal-transporting P-type ATPase

HPLC – High performance liquid chromatography

IEF – Isoelectric focusing

IPG - Immobilized pH gradient

IRT – Iron regulated transporter

LAP – Leucine aminopeptidase

MALDI-TOF/TOF - Matrix-assisted laser desorption/ionization-tandem time of flight

ME – malic enzyme

MGDA – Methylglycinediacetic acid

MRP - Multidrug resistance-associated protein

MT – Metallothionein

MTP1 - Metal tolerance protein 1

NA – Nicotianamine

NAD - Nicotinamide adenine dinucleotide

NADP - Nicotinamide adenine dinucleotide phosphate

NRAMP - Natural resistance associated macrophage protein

NTA – Nitriloacetic acid

OAS-TL – O-acetylserine (thiol) lyase

PAH – Polycyclic aromatic hydrocarbon

PC – Phytochelatin

PCB – Polychlorinated biphenyl

PGPR – Plant growth promoting rhizobacteria

pI – Isoelectric point

PM – CW - Plasma membrane – cell wall

PPO – Polyphenol oxidase

PS – Phytosiderophore

RNA – Ribonucleic acid

ROS – Reactive oxygen species

SAT – Serine acetyltransferase

SDS - Sodium dodecyl sulfate

TCA – Tricarboxylic cycle

TCE – Trichloroethylene

UV – Ultraviolet

YCF –Yeast cadmium factor

YSL – Yellow-stripe 1-like

ZIF1 – Zinc induced facilitator

ZIP – Zinc-regulated transporter, iron regulated transporter-like protein

ZRT - Zinc-regulated transporter

CHAPTER I – GENERAL INTRODUCTION

1.1 ENVIRONMENTAL CONTAMINATION: ZINC

The continuous contamination of the environment by numerous anthropogenic activities has as consequences the reduction of arable land availability, biological production, ecosystem sustainability, biodiversity and poses a serious threat to human health. A report of the European Environmental Agency refers that a striking number of three million contaminated sites are estimated to exist in the European Union of which at least 250000 require urgent attention and it is estimated that 52 million hectares of soil in Europe are, to some degree, contaminated (Peuke and Rennenberg 2005; Gheorghe *et al.* 2007; Memon and Schroder 2009).

In this scenario, zinc has been pointed out as one of the most important inorganic pollutants (Raskin, Smith, and Salt 1997) and Singh *et al.* (2003) indicate that in the previous five decades 1,350,000 t of Zn had been released into the environment. Zinc is a transition metal and a natural constituent of the earth's crust, however, great quantities are released into the environment due to activities such as mining, smelting, electroplating, gas exhaust, energy production and waste, and these are estimated to be in excess of 20 fold the natural inputs of Zn in the environment (Broadley *et al.* 2007; Saraswat and Rai 2011). From a biological perspective, Zn is an essential element in the cells where it is found in all enzyme classes and other proteins, membrane lipids, DNA and RNA molecules, and its deficiency in plants can lead to severe symptoms such as root apex necrosis, interveinal chlorosis and internode shortening (Mengel and Kirkby 2001; Broadley *et al.* 2007). Plants obtain Zn from the soil solution mainly in the form of Zn^{2+}, however, the metal can also be absorbed in complexes with organic ligands (Broadley *et al.* 2007). Adequate leaf Zn concentrations for plant growth are in the range of 15-20 mg kg^{-1} d.w. (Broadley *et al.* 2007). Although Zn is an essential micronutrient for plant growth, proven as such in 1926, excess Zn has consequences on plant physiology and development, affecting mineral absorption, antioxidant defenses and photosynthesis, among other important metabolic processes (Jones 2003; Atici, Agar, and Battal 2005; Khudsar *et al.* 2008; Wang *et al.* 2009; Sagardoy *et al.* 2010; Xu *et al.* 2010; Sagardoy *et al.* 2011). Visual symptoms of Zn toxicity include chlorotic and necrotic leaf tips, interveinal chlorosis and stunted growth (Mengel and Kirkby 2001; Jones 2003; Broadley *et al.* 2007). The levels indicated in the literature for the toxic levels of Zn vary, most likely due to different levels of sensitivity presented by plants, however, in general concentrations above 100-200 mg kg^{-1} d.w. plant tissue may cause toxicity symptoms (Mengel and Kirkby 2001; Jones 2003; Broadley *et al.* 2007).

Although several physicochemical techniques are available for the remediation of contaminated soils, namely, soil washing, soil vapor extraction, soil flushing, solidification, stabilization/immobilization, vitrification, electrokinetics, thermal desorption and encapsulation, it is acknowledged that these involve high costs and are often destructive, rendering the site inadequate for plant growth or human use (Prasad and Freitas 1999; Arthur *et al.* 2005; Marques, Rangel, and Castro 2009). Consequently, there is a need to develop environmentally friendly and cost effective remediation technologies.

1.2 THE USE OF PLANTS FOR ENVIRONMENTAL CLEAN-UP

Phytoremediation, the use of the natural capability of plants to remove, destroy or sequester hazardous substances from the environment (Glick 2003), is emerging as a promising green remediation technology. It is suitable for both organic and inorganic contaminants and different substrates (Salt, Smith, and Raskin 1998; Pilon-Smits 2005). However, while organic contaminants may be degraded by plants, inorganics cannot and are stabilized or sequestered by the plant tissues (Pilon-Smits 2005). Phytoremediation is a highly interdisciplinary area where soil chemistry, plant biology, ecology, microbiology and environmental engineering cross paths (Ali, Khan, and Sajad 2013). Numerous reviews have been published over the past twenty years on the general topic of phytoremediation where the main principles and types of remediation techniques are discussed (Chaney *et al.* 1997; Salt *et al.* 1998; Pilon-Smits 2005; Dickinson *et al.* 2009; Marques *et al.* 2009; Ali *et al.* 2013). The techniques include: phytoextraction, the accumulation of the contaminants into harvestable parts of the plant; phytodegradation, in which organic contaminants are degraded by plants and phytostimulation when this process is carried out by plant associated microorganisms; rhizofiltration, adequate for aqueous media where contaminants are adsorbed or absorbed into plant roots; phytostabilization, the reduction of the bioavailability of the contaminants and phytovolatilization, the release of contaminants by the plant in volatile form (Pilon-Smits 2005; Pilon-Smits and Freeman 2006). Phytoremediation is very appealing due to its low costs comparatively to other remediation methods, for example, as little as 5% of alternative methods (Prasad 2003). The commercial application of this technology is more advanced in the USA, where in the last decades numerous companies have been formed, than in Europe (Conesa *et al.* 2012). According to Pilon-Smits (2005), in 2005 the phytoremediation market reached 100-

150 million dollars per year, contributing with 0.5% to the remediation market in the United States, having grown 2-3 fold in comparison with 1999. On a world-wide basis, the phytoremediation market is estimated to be in the order of 15-18 billion dollars per year (Memon and Schroder 2009). The field trials carried out in Europe were reviewed in detail by Mench *et al.* (2010) and Vangronsveld *et al.* (2009) and recognize the need to further expand knowledge in the area and reach out to policy makers and stakeholders. A number of developments in the mechanisms and methods of phytoremediation have given origin to patents worldwide (Samardjieva *et al.* 2011). In fact, a search carried out on the 7th of April of 2014 on the Web of Science for patents with the keyword "phytoremediation" retrieved 131 results distributed over the last 17 years (Fig. 1.1). Interestingly, an increase is observed from 2010 onwards relative to previous years, and this must reflect an increased interest in this technology (Fig 1.1). It might also be hypothesized that the current economic crisis is a contributing factor escalating the pursuit of cost-effective remediation technologies. Other advantages, aside the low costs of phytoremediation due to being solar driven, include low levels of maintenance, being environmentally friendly and socially well accepted (Ali *et al.* 2013). However it is endowed with some limitations. Namely, it is a lengthy process that is dependent on the bioavailability of the contaminants, it is not adequate for contaminants present in high concentrations and may result in food chain contamination (Ali *et al.* 2013).

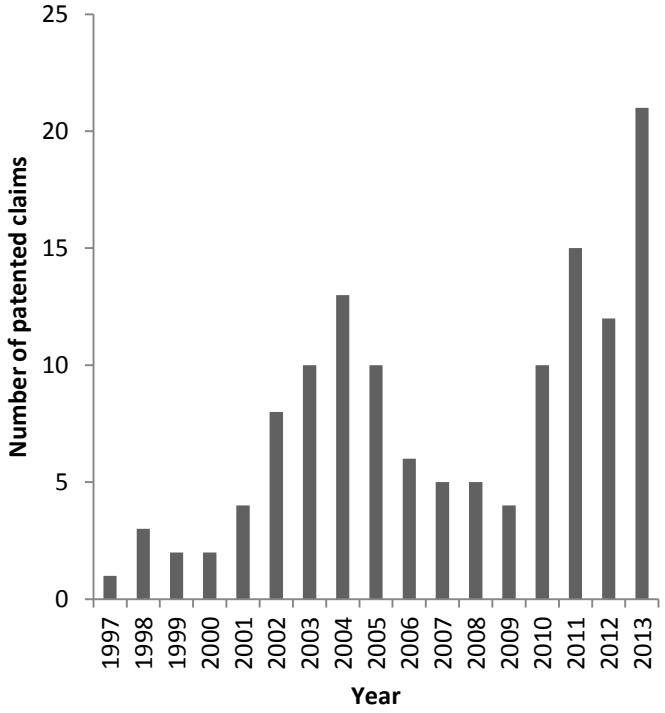

Fig. 1.1 – Patent numbers retrieved on the 7th of April of 2014 from the Web of Science with the keyword "phytoremediation" over the last 17 years.

.According to Pilon-Smits (2005), the plants used for phytoremediation should be tolerant to contaminants, be fast growing, high biomass, competitive and hardy plants. In respect to the metal accumulation and translocation to above ground tissues, plants can be classified as accumulators, indicators or excluders (Baker 1981). Accumulators concentrate metals in their above ground tissues at low or high metal soil levels, the concentration found in the above ground tissues of indicators reflect the concentration of the metal in the soil, and, excluders maintain low concentrations in the shoot (Baker 1981). Certain plants are known to accumulate abnormally high concentrations of metals in their above ground parts, and these are known as hyperaccumulators (Marques *et al.* 2009). These plants are able to accumulate metals such as Zn, nickel, manganese or selenium in their above ground tissues to more than 1% of their dry weight (Salt *et al.* 1998). Other characteristics of hyperaccumulators include a bioconcentration factor and a shoot to root ratio greater than one (McGrath and Zhao 2003). About 450 plant species have been identified as hyperaccumulators, however, often these plants are characterized by low biomass and slow growth (Pilon-Smits 2005; Rascio and Navari-Izzo 2011).

Numerous studies of the mechanisms of plant metal accumulation and tolerance have employed hydroponic approaches. This type of plant growth set-up is particularly suitable since it allows a better control of the culture environmental conditions, increases contaminant availability, creates a less complex root-zone environment (Nzengung 2007) and also insures a complete retrieval of plant roots for analysis. However, as phytoremediation will eventually be applied in field conditions care must be taken in extrapolating from data obtained from hydroponics, pot experiments, spiked soils, etc. especially concerning the phytoextraction capacity of plants (Dickinson *et al.* 2009; Vangronsveld *et al.* 2009).

1.3 *SOLANUM NIGRUM* PLANTS AND PHYTOREMEDIATION POTENTIAL

Solanum nigrum plants are annual dicotyledonous that can reach 70 cm in height, produce white flowers and berries, dull black or green, containing numerous seeds (Tutin *et al.* 1972; Edmonds and Chweya 1997). These plants produce taproot systems that facilitate plant removal from the soil. *Solanum nigrum* are vigorous and persistent plants, vastly distributed throughout the globe and possess characteristics favouring interspecific competition (Edmonds and Chweya 1997; Chao *et al.* 2005;

Henriques *et al.* 2006). Moreover, various molecular and tissue culture tools have been presented for *S. nigrum* plants (Hassanein and Soltan 2000; Schmidt *et al.* 2004)

Solanum nigrum is included in exhaustive lists of plants and other organisms characterized by potential in metal accumulation or tolerance with the indication that these may be useful in phytoremediation experimentation and technology (Prasad and Freitas 1999; Prasad and Freitas 2003). *Solanum nigrum* plants, collected from a heavy metal polluted site in Northeast Portugal, characterized by a high predominance of Zn, were shown to contain Zn up to 1130 mg kg^{-1} d.w (Marques, Rangel, and Castro 2003). Additionally, a screening for cadmium hyperaccumulators published in 2005 identified *S. nigrum* plants as new Cd-hyperaccumulators able to accumulate in the stems and leaves, 103.8 and 123.6 mg kg^{-1} d.w., respectively, values above the 100 mg kg^{-1} d.w. defined as the threshold for Cd hyperaccumulation (Wei *et al.* 2005). Previously it had been shown that *S. nigrum* can endure Cd, lead, copper and Zn combined contamination (Wei *et al.* 2004). Until the present date, a number of reports have been published regarding cadmium accumulation in this plant, and light has been shed on the involvement of organic acids, growth stage, antioxidative defenses, proline and phytochelatins, exogenous chelators and bacterial endophytes (Sun, Zhou, and Jin 2006; Wei, Zhou, and Koval 2006; Pinto *et al.* 2009; Sun *et al.* 2009; Xu, Yin, and Li 2009; Gao *et al.* 2010; Luo *et al.* 2011; Gao *et al.* 2012; Xu *et al.* 2012). Zinc tolerance and accumulation in *S. nigrum* plants have received less attention and the mechanisms responsible are largely unknown. It was reported that Zn accumulation was enhanced due to inoculation with the mycorrhizae *Glomus claroideum* and *Glomus intraradices* and the application of exogenous chelating agents such as EDTA (ethylenediaminetetraacetic acid) and EDDS (ethylenediaminedisisuccinic acid) (Marques *et al.* 2006; Marques *et al.* 2007, 2008b). These studies also gave indication of Zn accumulation sites such as the apoplast and vasculature, additionally, in these studies Zn was also detected intracellularly in several tissues with a high intensity in the starch sheath (Marques *et al.* 2007, 2008b). The supplementation with amendments, in particular manure, was shown to reduce Zn percolation and improved *S. nigrum* biomass yields, suggesting that this plant may be useful in phytostabilization techniques (Marques *et al.* 2008a). Additionally, Wei *et al.* (2006) indicate that the shoot biomass production by *S. nigrum* is superior to Zn and Cd hyperaccumulators

Thlaspi caerulescens[1] and *Arabidpsis halleri*. Consequently, there is ample evidence of the potential of *S. nigrum* in phytotechnologies.

1.4 ZINC ACCUMULATION AND TOLERANCE MECHANISMS

The tolerance to and accumulation of metals is a complex phenomenon and should be looked upon as a network of contributing mechanisms (Sinclair and Kraemer 2012; Viehweger 2014). Although essential metals such as Zn are necessary for normal plant growth, their concentration in the cytoplasm must be regulated in order to avoid a toxic build up and consequences such as oxidative stress and enzyme inactivation (Martinoia *et al.* 2012). The segregation of toxic elements in compartments less metabolically active than the cytosol, such as the cell wall or vacuole, the chelation with ligands such as organic acids, amino acids, peptides, and the activity of metal transporters on cell membranes appear to be the main plant mechanisms involved and have been periodically reviewed (Cobbett and Goldsbrough 2002; Hall 2002; Callahan *et al.* 2006; Haydon and Cobbett 2007; Kramer, Talke, and Hanikenne 2007; Krzeslowska 2011; Rascio and Navari-Izzo 2011).

1.4.1 Sequestration in the apoplast and cell vacuole

The sequestration of metals in specific tissues and cell compartments, such as the apoplast and vacuole, is proposed as a mechanism for the protection of the more metabolically active cell sites from metal toxicity (Krzeslowska 2011; Rascio and Navari-Izzo 2011).

The involvement of the cell wall where metals may accumulate via the uptake of water or due to efflux from the protoplast has been recently reviewed in detail by Krzeslowska (2011). Polysaccharides rich in carboxyl groups, for example homogalacturonans (HGA), play an essential role in binding divalent and trivalent metals (Krzeslowska 2011). Metal binding to HGA results in the formation of interactions between HGA molecules and this may lead to the stiffening of the cell wall and ultimately to the inhibition of cell elongation and consequently, plant growth

[1] It is indicated the *Thlaspi caerulescens* should be referred to as *Noccaea caerulescens* (Koch and German 2013), however, in order to avoid confusion, throughout this dissertation, the plant species will be referred to by the nomenclature used in each report cited.

(Krzeslowska 2011). Several modifications of the cell wall content and structure have been proposed by Krzeslowska (2011) as a plant tolerance strategy to metals, for example, the increase in pectin content, cell wall thickening and the deposition of callose that may isolate the protoplast from metals.

The plant vacuole is a membrane enveloped compartment which can occupy up to 90% of the volume of the cell (Taiz 1992). This compartment has many functions in plant cell metabolism, for example, storage of sugars and organic acids, ionic homeostasis, accumulation of bitter tasting phenolic compounds for defence, pigmentation due to anthocyanins and toxic avoidance by accumulation of harmful compounds (Taiz 1992). The vacuole is also a sink for metals and active transport to this cellular compartment is one of the mechanisms behind metal tolerance in plants (Hall 2002; Memon and Schroder 2009; Maestri *et al.* 2010). The vacuole was indicated to be the preferential compartment of Zn sequestration in the leaves of hyperaccumulators *Potentilla griffithii* and *T. caerulescens* (Kupper, Zhao, and McGrath 1999; Ma *et al.* 2005; Hu *et al.* 2009; Qiu *et al.* 2011). A study into the compartmentation of Zn in hyperaccumulator *Sedum alfredii* indicated the cell wall and the vacuole as sites for Zn sequestration (Li *et al.* 2006). The *MTP1* (*Metal Tolerance Protein 1*) genes, of the Cation Diffusion Facilitator (CDF) family of metal transporters, from the hyperaccumulators *A. halleri*, *T. caerulescens* and *Thlaspi goesingense* are believed to be involved in Zn influx into the vacuole, increasing Zn sequestration (Colangelo and Guerinot 2006; Maestri *et al.* 2010). As another example, the expression of *Oryza sativa Zn Transporter 1* (*OZT1*), encoding a CDF family protein, was found to be induced by Zn and OZT1 was located to the vacuole (Lan *et al.* 2013). The relevance of compartmentalization in the apoplast and the vacuole as a tolerance mechanism was also demonstrated by the comparison of hyperaccumulating and non-hyperaccumulating populations of *S. alfredii* where Zn was found to be sequestered in these compartments to a much higher degree in the hyperaccumulating population (Li *et al.* 2006).

1.4.2 Organic acids

Plant metal accumulation and tolerance, particularly to Zn, are likely to be dependent on organic acid production (Broadley *et al.* 2007; Haydon and Cobbett 2007). Organic acids are found in high concentrations in plants, where they participate

in several processes such as energy production, amino-acid biosynthesis, osmotic adjustment, alleviation of nutrient deficiencies, metal tolerance and plant-microbe interactions (Lopez-Bucio *et al.* 2000). Although organic acids are produced chiefly in the mitochondria, these metabolites are stored in the cell vacuole and it has been indicated that the acidic pH of this cellular compartment favors the formation of metal-organic acid complexes (Lopez-Bucio *et al.* 2000; Haydon and Cobbett 2007). It is known that organic acids may be excreted into the apoplast and transported through the phloem, or transported through the xylem together with the transpiration stream, and their presence in this vascular tissue has been correlated with the transport of micronutrients such as Zn (Lopez-Bucio *et al.* 2000). For example, increases of citric and malic acids in the xylem were observed after Zn treatment in sugar beet plants (Sagardoy *et al.* 2011). Moreover, in two known Zn hyperaccumulator plants *T. caerulescens* and *S. alfredii,* 21% and 36.7-42.3%, respectively, of the Zn detected in the xylem was coordinated with citrate (Salt *et al.* 1999; Lu *et al.* 2013). The exudation of organic acids from the roots may also be perceived as a mechanism for tolerance or accumulation, and it was shown, in a comparison between the organic acid exudation of the roots of hyperaccumulator *S. nigrum* and non-hyperaccumulator *Solanum lycopersicum* when exposed to Cd, that the hyperaccumulator exuded a higher amount of organic acids (Bao, Sun, and Sun 2011).

Organic acid concentrations vary between plant species, developmental stages, plant tissues and are also subject to diurnal variations (Lopez-Bucio *et al.* 2000). For example, in the roots of hyperaccumulator *T. caerulescens* Zn induced an increase in citric and malic acids while no such pattern was observed in the shoots (Zhao *et al.* 2000). However, another study in Zn accumulation in *T. caerulescens* showed that shoot malate and citrate concentrations were increased in response to Zn treatment (Wojcik, Skorzynska-Polit, and Tukiendorf 2006). Also in *T. caerulescens* plants it was reported that 38% of the Zn in the shoot was coordinated with citrate (Salt *et al.* 1999). The differential tissue response to metal accumulation is evident in *T. caerulescens* plants where the higher concentration of Zn detected in the epidermis was most likely associated with organic acids such as malic and citric acid, while the Zn detected at lower concentrations in the mesophyll was associated with nicotianamine (Schneider *et al.* 2013). In the leaves of Zn hyperaccumulator, *A. halleri*, Zn was predominantly complexed to malate (Sarret *et al.* 2002). In *S. nigrum* plants constitutive concentrations of malic and citric acids were higher than in non-hyperaccumulator *Solanum torvum* plants and *S. nigrum* plants responded to Cd with an increase in citric acid contrary to the non-hyperaccumulator plants (Xu *et al.* 2012). Therefore, organic

acids, namely malic and citric acids, appear to be important players in plant metal homeostasis by participating in their transport and sequestration.

1.4.3 Amino acids and peptides: histidine, glutathione, phytochelatins and metallothioneins

Other important mechanisms involve the amino acid histidine and also several peptides indicated to act as ligands or as reactive oxygen species scavengers. The free amino acid histidine (His) is involved in metal tolerance by forming metal-His complexes and there is ample evidence that it is involved in the hyperaccumulation of nickel (Callahan *et al.* 2006). For example, nickel exposure elicited a 36 fold increase in histidine content in the xylem sap of *Alyssum lesbiacum* plants where the metal was shown to be complexed with histidine, additionally, supplying histidine to the non-hyperaccumulator *Alyssum montanum* plants increased tolerance to the nickel and the rates of metal xylem transport (Kramer *et al.* 1996). In hyperaccumulator *T. caerulescens* plants 70% of the Zn accumulated in the root was shown to be coordinated with histidine (Salt *et al.* 1999).

Glutathione, a low molecular weight thiol, is a tripeptide with the sequence γ-Glu-Cys-Gly that is a key in maintaining cellular redox balance and metal detoxification (Rouhier, Lemaire, and Jacquot 2008; Memon and Schroder 2009). This tripeptide is found in cells in a reduced and oxidized state and can participate in antioxidative metabolism by being oxidized by certain reactive oxygen species (Rouhier *et al.* 2008). Glutathione can also bind xenobiotics that are posteriorly transferred to the vacuole by ATP-dependent GS-X pumps (Rouhier *et al.* 2008). A very important role for glutathione is being a precursor for the synthesis of phytochelatins (PCs) (Rouhier *et al.* 2008; Memon and Schroder 2009). The chelation of metals with metallothioneins (MTs), cystein-rich polypeptides, and PCs, cystein rich peptides, is also referred to be a mechanism contributing to metal tolerance (Cobbett and Goldsbrough 2002). Both MTs and PCs are characterized by a high percentage of cysteine sulfhydryl groups that bind metals in stable complexes (Karenlampi *et al.* 2000). Importantly, PC synthesis from glutathione by phytochelatin synthase is known to be activated by metal ions and it has been observed that PC-Cd complexes are sequestered in the vacuole (Cobbett and Goldsbrough 2002) reinforcing the importance of this cell compartment in metal tolerance and that multiple mechanisms are interconnected and responsible for the

tolerance and accumulation of metals in plants. The importance of MTs in Zn tolerance and accumulation has been reported and authors have also suggested a role for MTs in metal detoxification by participating in antioxidative defense response (Yang *et al.* 2009).

1.4.4 Proteins involved in metal tolerance and accumulation

Membrane transporters are responsible for the control of the concentration of metals in the cytoplasm and can be differentially expressed to regulate the uptake, efflux, translocation and sequestration. A number of transporter families have been described, examples are the P_{1B}-ATPases, cation diffusion facilitator (CDF) family, the natural resistance associated macrophage protein (NRAMP) and the zinc-regulated transporter, iron-regulated transporter-like protein (ZIP) families (Colangelo and Guerinot 2006; Kramer *et al.* 2007).

A proteomic approach has been employed by several researchers to identify key proteins in metal tolerance and accumulation and their findings have been thoroughly reviewed (Ahsan, Renaut, and Komatsu 2009; Hossain and Komatsu 2012; Visioli and Marmiroli 2013). A recent review of the application of proteomic approaches to unravel mechanisms involved in hyperaccumulation has identified several classes of proteins, which change in abundance in response to metal exposure (Visioli and Marmiroli 2013). The group including proteins involved in energy and carbohydrate metabolism contributes with close to 40% of the proteins identified and this indicates that hyperaccumulation is an energy demanding phenomenon (Hossain and Komatsu 2012; Visioli and Marmiroli 2013). Interestingly, the root and shoot have contributed with similar percentages of proteins to the classes of energy and carbohydrate metabolism, cellular metabolism and regulation and signal transduction, however, the root contributes with a higher percentage of proteins involved in stress and antioxidant response while the shoot was the source of proteins involved in defense and metal chelators and transporters (Visioli and Marmiroli 2013). Metal concentration varies in plant tissues and it is to be expected that proteins will also be differentially expressed in these tissues. Accordingly, a comparison of the protein content of the epidermis and the mesophyll tissue, characterized by higher and lower Zn content, respectively, of *Noccaea* (formerly *Thlaspi*) *caerulescens*, showed that proteins involved in stress

protection, metal transport and chelation are differentially expressed in these tissues (Schneider *et al.* 2013).

It is apparent that metal accumulation and metal tolerance in plants are a complex phenomenon, dependent on the plant and the metal characteristics, and resulting from several mechanisms. Ultimately, these mechanisms are interconnected and, they must be considered as a network in order to allow phytotechnologies to evolve into a truly viable alternative in environmental remediation.

1.5 THESIS FRAMEWORK AND OBJECTIVES

Since the time when this PhD project was initiated, the awareness of the problems resulting from environmental contamination has increased and the current context of financial crisis may have further fostered the need for more cost-effective remediation methods. Phytoremediation is a technology that offers a potential solution to the problem, however, it is apparent that the knowledge of the mechanisms allowing for tolerance and accumulation of metals that are essential for the development of phytotechnologies, is still lacking.

Advances in the knowledge and tools available for phytotechnologies are necessary on several levels. Ultimately, environmental contamination is complex and conditions for plant growth are likely to be harsh, therefore an investment in understanding metal tolerance and accumulation mechanisms is pertinent particularly in robust plants known to tolerate combined contamination such as *S. nigrum*. Importantly, it is most likely that the tolerance and accumulation of metals in plants is the result of a combination of mechanisms and therefore must be analyzed through that prism.

In that sense, this PhD project was aimed at the identification and understanding of potential mechanisms of Zn tolerance and accumulation in *S. nigrum* plants. The general objective of this PhD project was to study aspects of Zn tolerance, transport and accumulation in *S. nigrum* plants at the structural, biochemical and molecular levels. The effects of Zn accumulation on *S. nigrum* plant growth, histology, ultra-structure and biochemistry were evaluated. Particular emphasis was given to Zn accumulation and localization in the tissues, to the production of organic acids and differences in abundance of proteins in specific organs. Therefore, the main objectives of this PhD project were to:

- Identify areas of specific interest for the development of phytotechnogies by review of the most recent patents conceded in the field.
- Determine the detailed histological an ultra-structural localization of Zn in *S. nigrum* plants.
- Evaluate the influence of *S. nigrum* plant development on the degree of tolerance and accumulation of Zn.

- Characterize the production of organic acids in *S. nigrum* plants as a potential mechanism of tolerance and accumulation.
- Identify proteins that would allow hypothesizing probable pathways related to Zn tolerance, transport or accumulation in *S. nigrum* plants.

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CHAPTER II – INSIGHTS INTO PHYTOREMEDIATION SOLUTIONS FOR ENVIRONMENTAL RECOVERY

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ABSTRACT

Our environment is contaminated with organic and inorganic compounds released by anthropogenic activities that cause negative impacts on biological productivity and ecosystem sustainability and place human health at risk. Within the available remediation technologies, phytoremediation has emerged with high potential due to its reduced environmental impacts and economic costs. The research into phytoremediation has developed through a wide array of approaches, which also pertains to its inherent interdisciplinary characteristics, towards enhancing the potential of the technology for application in the field. Numerous patents present molecular solutions through which plants can be engineered to display improvements in key characteristics, such as the tolerance, uptake and accumulation of contaminants. The manipulation of plant growth and of the physico-chemical characteristics of the contaminated environments in order to enhance the remediation potential has also been the focus of several issued patents. This review attempts to highlight the most relevant patented advances in phytoremediation and to emphasise recent research efforts through which this green technology might be expected to develop into a commercially competitive alternative to other remediation methods.

2.1 INTRODUCTION

Anthropogenic activities generate large amounts of organic and inorganic non-biodegradable compounds that are frequently released into the environment and cause severe disturbances and negatively impact both biological productivity and ecosystem sustainability. Common environmental pollutants include organic compounds, which include the largely spread chlorinated solvents, petroleum hydrocarbons and polyaromatic hydrocarbons, inorganic compounds, mainly consisting of heavy metals, such as lead, zinc and cadmium, and radioactive elements, such as uranium (Glick 2003). These compounds have biotoxic properties that directly affect biodiversity or enter the food chain, through which they become biomagnified to lethally toxic levels, ultimately threatening human health (Streit 1992; Kelly *et al.* 2007). Although the mechanisms of toxicity of many of these molecules are poorly known, the carcinogenicity of numerous contaminants is widely acknowledged (Pilon-Smits 2005; Galanis, Karapetsas, and Sandaltzopoulos 2009). Over the last decades, the awareness of the finiteness of natural resources has increased and has motivated a need to reassure the sustainability of environment services and to remediate already-polluted sites. According to the European Environmental Agency (EEA), 80,000 contaminated sites have been remediated in the last 30 years and another 240,000 sites are in need of reclamation (Gheorghe *et al.* 2007). The main contaminating activities in Europe are industrial production and commercial services, with the oil industry, waste treatment and disposal and power plants being the main polluters (Gheorghe *et al.* 2007). These activities have occurred in a total of three million sites where investigation is still needed in order to determine whether remediation is necessary (Gheorghe *et al.* 2007). The classic physico-chemical environmental remediation methods include volatilisation, vitrification, excavation, soil washing, soil incineration, chemical extraction, solidification and landfill (Prasad and Freitas 1999; Arthur *et al.* 2005). Even though these methods have been successfully used in numerous interventions, it is undeniable that many are expensive and invasive, and they should remain as a last option (Prasad and Freitas 1999; Arthur *et al.* 2005). This scenario has driven research to develop economic and environmentally friendly remediation methods. In the early nineties, the use of the natural capability of plants to remove, convert or sequester hazardous substances from the environment has emerged as a promising remediation technique known as phytoremediation. This environmentally friendly and low-cost remediation strategy has been the focus of numerous studies aimed at optimising its efficiency, the number and diversity of the targeted pollutant compounds and its suitability for use in a wide range of sites.

Currently, the diverse phytoremediation approaches, Fig. 2.1, that are available include the following: phytoextraction, which consists of the removal of contaminants from the soil and into aerial plant parts; phytostabilisation, in which the contaminants are immobilised in the rhizosphere or in plant roots; phytodegradation, concerning the metabolic conversion of organic pollutants by plants; phytovolatilisation, which is the release into the atmosphere of soil contaminants; rhizodegradation, also called phytostimulation, in which the exudates released by the plant into the rhizosphere stimulate the degradation activity of microorganism; and rhizofiltration where contaminants in water are filtered by plant roots (Pilon-Smits 2005; Pilon-Smits and Freeman 2006). The chemical heterogeneity of contamination, often characterised by a mixture of inorganic and organic contaminants, poses an additional difficulty concerning the application of phytoremediation methods.

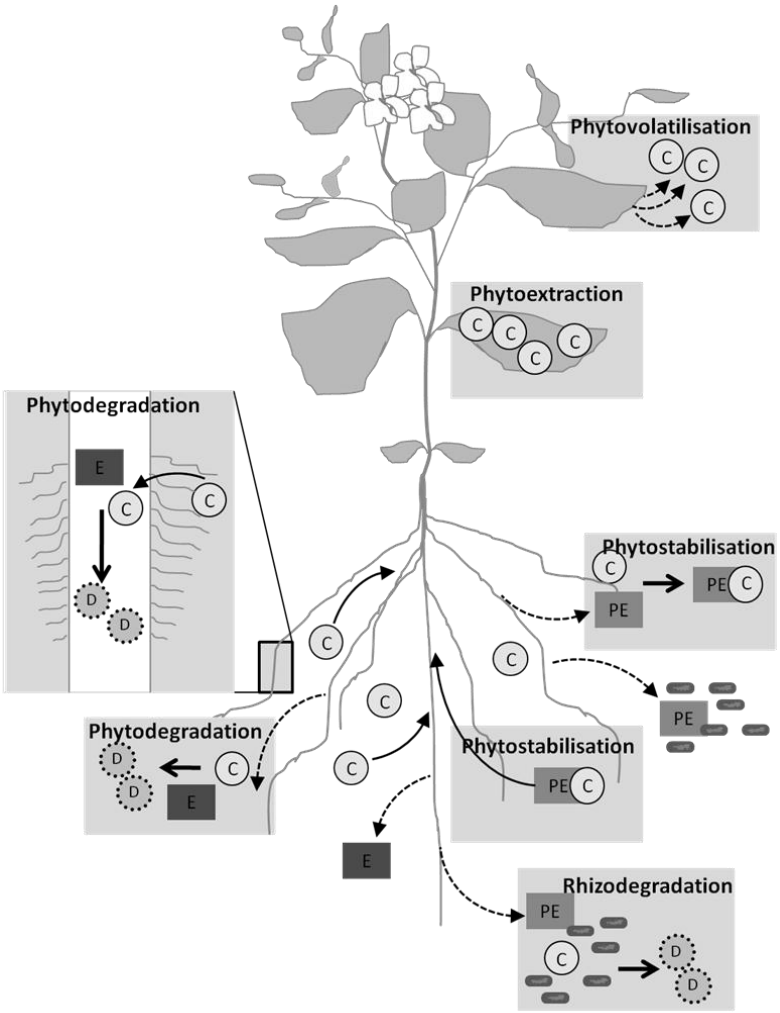

Fig. 2.1 - A schematic summary of the various phytoremediation technologies available. Shown is the path followed by the inorganic and/or organic contaminants (C) in the plant, and their interaction with plant exudates (PE), forming chelated complexes (PEC). Plant exudates also enhance the activity of rhizospheric bacteria in degrading organic contaminants (D). Enzymes (E) produced by the plants act by degrading the organic contaminants within the plant or in the rhizosphere.

For plants to be potentially useful in any of these phytoremediation techniques, they must be tolerant to the targeted pollutants and should, ideally, be fast growing and produce a large amount of biomass. Accumulators, excluders and indicators have long been nomenclatural references in phytoremediation to distinguish plant species that are able to tolerate environmental toxicity (Baker 1981). While accumulator plants are characterised by their ability to concentrate a high concentration of toxic compounds in their cells, organs and tissues, making them particularly useful for phytoextraction remediation strategies, the term 'excluders' has been attributed to tolerant plants characterized by low or neglected shoot accumulation of contaminants (Baker 1981). However, the phytoremediation potential of excluder plants is primarily academic and has focused on the understanding of physiological and molecular tolerance mechanisms. Lastly, indicator plants can be likened to the ecological meaning of 'bioindicators', and refer to plants in which there is a direct proportionality between the concentration of soil contaminants and their uptake and accumulation by these plants.

Aside from the intrinsic remediation capacity of several plants, research has elucidated the ways in which plants avoid toxicity and their tolerance mechanisms to contaminants, whether by exclusion or by specific tolerance mechanisms. A thorough understanding of the physiological and molecular mechanisms underlying the tolerance, transport, accumulation and degradation of contaminants by plants is essential to foster the optimisation of the phytoremediation potential of plants. The identification of key traits that, through genetic manipulation, may be transformed into different plants is essential in order to reconstruct in a single transgenic plant the efficient tolerance mechanisms with a higher growth, biomass and enhanced adaptability to a wide range of bioclimatic and edaphic conditions.

In the last two decades, research on phytoremediation has resulted in an endless number of claims described in numerous patents. This review is not intended to address exhaustively these claims, many are only vaguely or indirectly related to phytoremediation, but rather to identify the patents that we consider most relevant, ranging from those addressing the physiological and molecular mechanisms of plant tolerance and accumulation to promising plants for phytoremediation and innovative phytoremediation methods.

2.2 TOLERANCE MECHANISMS

The transport and sequestration of contaminant compounds into the cell wall (Sousa et al. 2008) or vacuole (Liu *et al.* 2009) are important tolerance mechanisms that are dependent on cellular trafficking. The chelation of the pollutant with ligands, such as organic acids, namely malate, citrate and oxalate (Tolra, Poschenrieder, and Barcelo 1996; Verkleij *et al.* 2009), glutathione (Dietz and Schnoor 2001; Verkleij *et al.* 2009), metallothioneins (Guo, Meetam, and Goldsbrough 2008), phytochelatins (Selvam and Wong 2008), and amino acids (histidine and nicotianamines) (Kramer *et al.* 1996; Kim *et al.* 2005), has been identified as an important intervention in tolerance. Furthermore studies have suggested the involvement of organic acids (Callahan *et al.* 2006), phytochelatins (Cobbett and Goldsbrough 2002) and glutathione in vacuolar compartmentalisation (Yadav 2010).

Not surprisingly, this knowledge has led to several patents (Table 2.1) alleging the use of plants rich in these metabolites or claiming the isolation, characterisation and expression of enzymes engaged in the biosynthesis of these molecules and their impending utility for phytoremediation.

Table 2.1 Main attributes of patents regarding tolerance and transport of contaminants.

Patent #	Title	Contaminant[a]	Plant[a]	GMO[a]	Mechanism [a]
TOLERANCE					
US6489537-B1	Phytochelatin synthases and uses therefor.	Inorganic (metal)	Not defined	+	Phytochelatin synthase
WO2004020628-A1	Method for genetically engineering plant-derived nucleic acid sequences comprising gene shuffling and selective mutagenesis.	Inorganic and organic	Not defined	+	Glutathione S-transferases
WO200248335-A2	Metal resistant plants and phytoremediation of environmental contamination.	Inorganic (metals)	Putatively any spermatophyte	+	Arsenate reductase Phytochelatin biosynthetic enzyme
US7034202-B1	Heavy metal phytoremediation.	Inorganic (metals)	*Populus angustifolia* *Nicotiana tabacum* *Silene cucubalis*	+	γ-glutamylcysteine synthetase
WO200260939-A2	Method for increasing the sulphur-compound content in plants.	Not defined	Not defined	+	Serine acetyltransferase
WO200272834-A1	Plant that is resistant to heavy metal-contaminated media.	Inorganic (metals)	Brassicaceae Cruciferae	+	O-acetylserine (thiol) lyase
US6974896-B1	Trace element phytoremediation.	Inorganic (metals)	Brassicaceae	+	Sulfate adenylyltransferase
US6657106-B2	Removal of metals from contaminated substrates by plants.	Inorganic (metals)	Brassicaceae	+	Histidine biosynthesis enzyme
WO9960107-A2	Nicotinamine synthase genes, isolation and use thereof.	Not defined	Monocotyledonous Dicotyledonous	+	Nicotianamine synthase
US20070191666-A1	Method of cleaning up lead-contaminated soil.	Inorganic (metals)	Polygonaceae Oxalidaceae Chenopodiaceae Araceae Begoniaceae Musaceae	-	Oxalic acid

TRANSPORT					
WO200170989-A2	Genetically modified plants and plant cells comprising heterologous heavy metal transport and complexation proteins.	Inorganic (metals)	Not defined	+	P-type ATPases ABC transporters Cation Diffusion Facilitator proteins
WO200281707-A1	Genetic modification of plants for enhanced resistance and decreased uptake of heavy metals.	Inorganic (metals)	Not defined	+	P-type ATPase
WO2005093078-A1	Genetically modified plants and their applications in phytoremediation.	Inorganic (metals)	*Brassica juncea* *Nicotiana tabacum* *Poplar* spp.	+	P-type ATPase
WO2004078905-A2	Agents for phytoremediation.	Inorganic (metals)	*Arabidopsis thaliana* Brassicaceae Caryophyllaceae tobacco	+	P-type ATPase
WO200240688-A2	Maize *yellow stripe1* and related genes.	Inorganic (metals)	*Amaranthus* spp. *Brassica* spp. *Raphanus* spp. *Sinapis* spp.	+	Yellow stripe 1 Yellow-stripe 1-like transproters
US6166290	Glutathione-S-conjugate transport in plants.	Not defined	Not defined	+	ABC transporters

[(a)] As indicated in the claims of the patent

Glutathione has been seen as a core molecule in molecular approaches to improve the remediation ability of plants. In addition to its anti-oxidant role (Rouhier, Lemaire, and Jacquot 2008), which may be important as a response to oxidative stress that is indirectly induced by diverse pollutants, this tripeptide acts as a metal chelator and is the substrate for phytochelatin (PC) synthesis (Rauser 1995; Verkleij *et al.* 2009). PCs, often induced in plants after exposure to cadmium, zinc, copper, silver, arsenic, mercury and lead (Maitani *et al.* 1996), are synthesised from glutathione by PC synthases (Grill *et al.* 1989). Rea and co-workers (2002) report the isolation and identification of PC synthase-encoding genes, Fig. 2.2, from *Arabidopsis thaliana* and *Triticum aestivum* and described a method to obtain transgenic heavy metal-resistant plants by the expression of these genes. Dixon and Edwards (2004) have presented a

method, consisting of a combination of random gene shuffling and selective mutagenesis, for engineering a DNA sequence that codes for a protein, glutathione S-transferase, with enhanced detoxification activity.

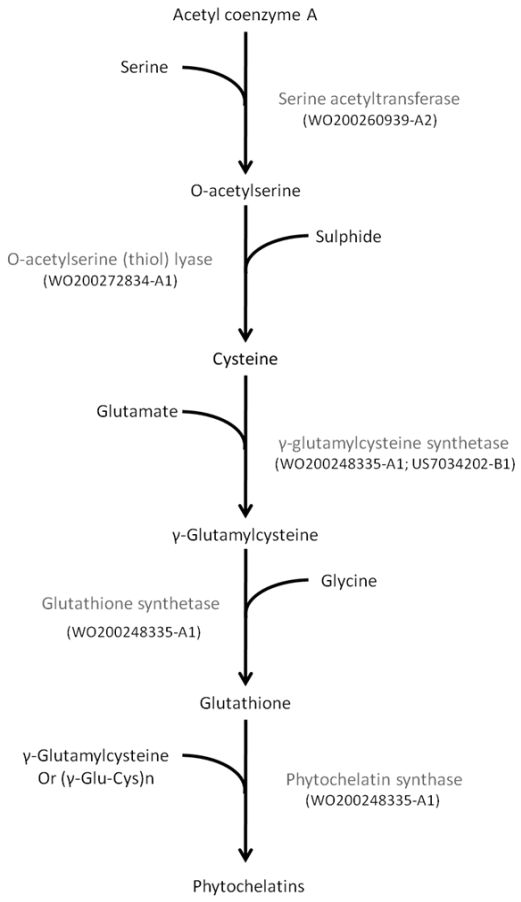

Fig. 2.2 -The phytochelatin biosynthetic pathway indicating the relevant patents concerning the enzymes involved.

Meagher and Li (2002) have presented methods for engineering transgenic plants that express at least one gene of the phytochelatin biosynthetic pathway, namely the genes for γ-glutamylcysteine synthetase, glutathione synthetase and phytochelatin synthase, which catalyse the biosynthesis, respectively, of γ-glutamylcysteine, glutathione and phytochelatins, Fig. 2.2. This patent also included the disclosure of the expression of arsenate reductase, *arsC*, which resulted in plants that were able to educe Cd(II) to Cd(0) and exhibited an increased resistance to cadmium. In this description, evidence was shown that plants transgenic for γ-glutamylcysteine synthetase showed resistance to 250 μM of arsenate, a concentration lethal to the wild type. The overexpression of γ-glutamylcysteine synthetase to achieve an enhanced tolerance and accumulation to a variety of metals, such as cadmium, chromium,

molybdenum, tungsten and mercury, has also been shown in a taxonomically broad spectrum of plants, including *Brassica juncea, Populus angustifolia*, *Nicotiana tabacum* and *Silene cucubalis* (Terry, Pilon-Smits, and Zhu 2006).

The biosynthesis of cysteine, an essential amino acid necessary for glutathione synthesis, Fig. 2.2, is mediated by serine acetyltransferase (SAT) and O-acetylserine (thiol) lyase (OAS-TL) (Hell et al. 2002). Wirtz and co-workers showed that the overexpression of an enzymatically inactive SAT increased the cysteine, glutathione and methionine levels by up to 30-, 8- and 2-fold, respectively (Wirtz, Berkowitz, and Hell 2002). It is hypothesised that the transgenic inactive SAT outcompetes the cytosolic SAT, and this is arguably followed by the upregulation of the mitochondrial SAT, which would act as a compensatory mechanism leading to an increase of cysteine levels (Wirtz and Hell 2007). The overexpression of OAS-TL in *A. thaliana*, described by Dominguez-Solis and co-workers (2002), resulted in up to a 300% increase of cysteine, cysteine-rich peptides and an enhanced tolerance and accumulation of cadmium, arsenic and mercury. Sulphide is a substrate of OAS-TL and, therefore, essential for cysteine synthesis; when the concentrations of sulphide are limiting, the reaction catalysed by OAS-TL stops (Hell et al. 2002). In this regard, a method of phytoextraction with plants that were genetically modified to overexpress a sulphate assimilation pathway gene was presented by Terry and co-workers (2005) leading to an improved tolerance and accumulation of selenium and cadmium.

Histidine is considered to be the most important free amino acid for chelation, and forms complexes with nickel, cadmium and zinc (Verkleij *et al.* 2009). An increase in histidine has been observed for *Alyssum* hyperaccumulator species in response to nickel and cobalt and *Thlaspi caerulescens* in response to nickel and zinc exposure (Smith, Kramer, and Baker 2003). The authors present evidence that nickel is coordinated with histidine in the tissues of *Alyssum lesbiacum*. Moreover, supplying histidine as a foliar spray or to the root medium of a non-tolerant, non-accumulator plant, such as *Alyssum montanum*, during exposure to toxic concentrations of nickel increased the tolerance to and the accumulation of nickel. The authors of this patent (Smith *et al.* 2003) also provided a method for producing transgenic Brassicacea plants that harboured a gene encoding a histidine biosynthesis enzyme obtained from *Escherichia coli*; these plants should produce more histidine and possess an improved accumulation performance compared to nontransgenic plants.

A number of other molecules, such as nicotianamine (NA) and oxalic acid, have been the subject of patents due to their role in metal tolerance and transport (Baeumlein *et al.* 1999; Watanabe, Yamada, and Uchida 2007).

2.3 TRANSPORT

There are various families of metal transporters in plants that have been straightforwardly classified by Colangelo and Guerinot (2006) into the following two groups according to their general function:

a) The efflux transporters, such as the P-type ATPases and the members of the cation diffusion facilitator (CDF) family, which transport cations from the cytoplasm to the exterior of the cell or into organelles, and
b) The metal-uptake transporters, including the yellow-stripe 1-like (YSL) protein transporter, the natural resistance associated macrophage protein (NRAMP) family and the ZIP transporters (ZRT and IRT-like proteins), which transport metals into the cytoplasm across the plasma membrane or from cell organelles.

The heavy metal-transporting P-type ATPase (HMA) family members transport cations out of the cytoplasm across biological membranes (Kramer, Talke, and Hanikenne 2007) and have been the focus of intense research regarding the optimisation of phytoremediation strategies (Table 2.1). A higher resistance to cadmium was accomplished by the transformation of *N. tabacum* with a *CadA* (a P-type ATPase) gene from *Staphylococcus aureus* (Borremans *et al.* 2001). Lee and co-workers (2002) claimed the production of plants transformed with a P-type ATPase, ZntA, which conferred resistance to heavy metals. The authors demonstrated that *A. thaliana* plants transformed with ZntA from *E. coli* showed a higher resistance to cadmium and lead, accompanied by a low accumulation of these metals when compared to wild type. Transformed plants overexpressing one or more of P-type ATPases, namely HMA1-4, have been claimed by Verret and co-workers (2005) to be useful in the phytoextraction of Cd, Zn, Pb and Co due to the accumulation of these metals in the aerial parts of the plant. The authors also showed that *A. thaliana* plants overexpressing AtHMA4 accumulated more Zn and were more tolerant to Zn and Cd than wild type. Verbruggen and Bernard (2004) have presented the identification of a truncated form of a putative HMA4 ATPase of *T. caerulescens* whose higher expression is suggested to result in a higher cadmium phytoremediation fitness. The YSL transporters have been suggested

to be involved in the uptake of metals complexed with phytosiderophores (PS) and NA (Colangelo and Guerinot 2006). A patent by Walker and Dellaporta (2002) referred to the characterisation of the maize YS1 (Yellow stripe 1) and *Arabidopsis* YSL transporters and claimed that transgenic plants expressing these transporters will be efficient in the phytoremediation of iron, copper and other metals. Metal-chelated complexes with glutathione (GS-X) have been reported to be transported across membranes by a GS-X pump transporter (Rea, Lu, and Li 2000). In their patent, Rea and co-workers showed that the yeast gene, *YCF1* (Yeast cadmium factor), encodes a vacuolar GS-X pump and confers resistance to cadmium. Furthermore, two plant homologs of YCF1, AtMRP1 and AtMRP2 (multidrug resistance-associated proteins), have been identified in *A. thaliana* (Rea *et al.* 2000). The authors have also shown that AtMRP1 can substitute for YCF1 as a GS-X pump in YCF1 deficient strain of yeast.

To the best of our knowledge, despite its relevance and importance for phytoremediation, the NRAMP protein family has not been addressed in any filed patents.

2.4 USEFUL PLANTS FOR THE PHYTOREMEDIATION OF KEY METAL CONTAMINANTS

Plants exhibit tolerance to a variety of inorganic and organic contaminants. These plants can be generally classified into three groups, as mentioned above: metal excluders, indicators, and accumulators. Plants that are able to accumulate considerable concentrations in their above-ground tissues are called hyperaccumulators. These plants accumulate metals in their shoots in concentrations 100-fold higher than non-accumulating plants (Lasat 2002). Consequently, a plant must accumulate at least 0.001% mercury, 0.01% cadmium, 0.1% copper and chromium and 1% zinc and nickel to dry weight in order to be classified as a hyperaccumulator (Lasat 2002). Additionally, the ability of the plants to absorb and transport metals is evaluated by two factors: the ratio between the metal concentration in the plant shoot and in the soil, called the bioconcentration factor, and the shoot-to-root ratio (McGrath and Zhao 2003), which is also designated as the translocation factor. Both of these ratios should be greater than 1 in a hyperaccumulator.

These characteristics are found in many naturally occurring plants. In the past decade, numerous patents have been granted for plants showing tolerance and an accumulation of contaminants, particularly arsenic, zinc, cadmium, nickel, lead and copper (Table 2.2). Some of these elements, such as copper, zinc and iron, are essential for plant growth, while others, such as arsenic (Zhao *et al.* 2009), have no known biological function.

Table 2.2 Main attributes of patents regarding plants, associations with microorganisms and the degradation of organic contaminants

Patent #	Title	Contaminated medium[a]	Contaminant[a]	Plant[a]	Observations
			PLANTS		
US7049492-B1	*Thlaspi caerulescens* subspecies for cadmium and zinc recovery.	Soil	Cadmium Zinc	*Thlaspi caerulescens*	Indicate methods for recovering metals from the plant tissues
US5927005	Phytoremediation of heavy metals with creosote plants.	Soil	Heavy metals	Creosote bushes	Indicate methods for recovering metals from the plant tissues
US5720130	Removal of soil contaminates.	Soil	Calcium, Selenium, Boron, Sodium, Chlorine	*Hibiscus* spp.	
US6280500-B1	Method for removing pollutants from contaminated soil materials with a fern plant.	Soil	Arsenic, Metals, Trace element, Phosphorus.	Pteridaceae	Indicate methods for recovering metals from the plant tissues
US7065920-B2	Contaminant removal by additional ferns.	Soil Groundwater Surface water Wetland	Arsenic	*Pteris vittata* *P. biaurita* *Chielanthes sinuta* *Adiantum raddianum* *A. hispidulum* *Polystichum acrostichoides* *P. polyblepharum* *Actiniopteris radiate* *Pellaea rotundifolia* *Nephrolepis cordifolia* *N. exaltata* *Dennstaedtia punctilobula* *Dryopteris flixmas* *Didynachlaena trunvatula*	Indicate methods for recovering metals from the plant tissues

US6313374-B1	Method of using *Pelargonium* sp as hyperaccumulators for remediating contaminated soil.	Soil, Sand, Sludge Compost, Artificial soil	Metals Organic compounds	*Pelargonium* spp.	Refer the concomitant extraction of aromatic oils
PHYTO- AND RHIZOREMEDIATION					
US7169965-B2	Transgenic plant expressing secretory laccase and use thereof.	Water	Diphenols and related compounds	Putatively any seed plant	Transgenic plants expressing a secretory laccase
US6369299-B1	Transgenic plants expressing bacterial atrazine degrading gene AtzA.	Not defined	S-triazines	Dicotyledonous	Transgenic plants expressing an atrazine chlorohydrolase
US6613961-B1	Plants capable of metabolizing drugs and use thereof.	Not defined	Environmental load	Gramineous Solanaceous plants	Transgenic plants transformed with a P450 monooxigenase and a P450 reductase gene
WO2004050882-A1	Bioremediation with transgenic plants.	Not defined	Heavy metal Oil hydrocarbons	*Nicotiana tabacum* *Arabidopsis thaliana*	Transgenic plants expressing an enzyme with rhamnosyltransferase activity
US6087547	Method for decomposing toxic organic pollutants.	Soil	Halo-organic compounds	*Spartina* spp.	Transgenic plants expressing a dehaloperoxidase
ASSOCIATION WITH MICROORGANISMS					
US5809693	Microbial isolates promote phytoremediation.	Soil Aquatic environment	Metals	Brassicaceae Turfgrasses Spinach, Sorghum Tobacco, Corn Sunflower	Plants cultivated in association with *Pseudomonas* spp. and *Bacillus* spp.
US7214516-B2	Bacterial effects on metal accumulation by plants	Soil	Metals	*Alyssum* spp.	Plants cultivated in association with *Sphingomonas macrogoltabidus*, *Microbacterium liquefaciens*, *M. arabinogalactanolyticum*
US20030126632-A1	Method for improving phytoremediation treatment of a contaminated medium.	Soil Aqueous medium	Organic pollutants Heavy metals Radionuclides	Putatively any vascular plant	Plants cultivated in association with a genetically modified endophytic organism
US20040101945-A1	Method and system for plant/bacterial phytoremediation.	Soil, Sludge Sediment Wastewater	Organic pollutants	Alfalfa and wheat	Plants cultivated in association with *Burkholderia* spp., *Sphingomonas* spp.

Zinc is an essential element necessary for plant development and has roles in carbohydrate and protein metabolism and enzyme and plant hormone activities. Normal concentrations of zinc in the environment are in the range of 17-160 mg kg^{-1}, and its bioavailability is dependent on the pH (Mengel and Kirkby 2001). In plants, zinc is normally found in concentrations of 20 mg kg^{-1} dry weight (Jones 2003). However, high concentrations of zinc, on the order of 150-200 mg kg^{-1} dry matter, are toxic to most plants and results in chlorotic and necrotic leaves and retarded growth of the plants (Mengel and Kirkby 2001; Jones 2003). **Cadmium** is a non-essential element (Jones 2003) and may be taken up by plants because of the chemical similarities it has with zinc. It is found in low concentrations in the environment; normal concentrations in non-contaminated soils are lower than 1 mg kg^{-1} soil (Mengel and Kirkby 2001). Sources of cadmium contamination are the electroplating industry, plastics and batteries (Jones 2003). Cadmium is toxic at very low concentrations; for example, in leaf tissue, at concentrations of 0.05-0.2 mg kg^{-1} dry weight, toxicity is evident by symptoms of chlorosis, reddish veins and petioles, brown and stunted roots and deterioration of the xylem tissue (Jones 2003). As mentioned above, certain plants can tolerate and/or accumulate high concentrations of metals. Li and co-workers (2006) have described a genotype of *T. caerulescens,* a known zinc and cadmium hyperaccumulator, which was capable of accumulating 1800 mg kg^{-1} of cadmium and 18,000 mg kg^{-1} of zinc (dry shoot tissue); this genotype showed the highest Zn:Cd ratio. As soil cadmium concentrations tend to be much lower than zinc concentrations, a high ratio of Cd:Zn in hyperaccumulating plants allows a more efficient removal of cadmium from the soil.

In plants, **copper** is a structural element of various enzymes, and it is involved in carbohydrate and nitrogen metabolism. Normal plant concentrations of copper range from 5 to 20 mg kg^{-1} dry weight; above this value, copper can be toxic, affecting the uptake or the metabolic displacement of other important ions, such as iron, which causes chlorosis and inhibiting root growth (Mengel and Kirkby 2001). High concentrations of copper have been reported for *Larrea tridentata* (Gardea-Torresdey *et al.* 1999); although the concentration of copper in the leaf material, 493 mg kg^{-1} dry weight, is not high enough for the plant to be considered a hyperaccumulator, the authors found that that 47% of the copper was found in the aerial parts. The same pattern was observed for cadmium (61%) and nickel (55%).

Boron is mainly involved in the metabolism and transport of carbohydrates, flavonoid synthesis, nucleic acid synthesis, phosphate utilisation and polyphenol

production; it is normally found in concentrations of 20 mg kg^{-1} dry weight (Jones 2003). When in excess, the leaf tips and margins become chlorotic, causing leaf necrosis (Jones 2003). Bost (1998) has described a *Hibiscus laevis* var "Guadalupe", which is capable of accumulating boron up to 1126 mg kg^{-1} of the leaf dry weight. In this plant, boron was found to be highly concentrated in the leaf tissues, a trait important for the process of phytoextraction.

Arsenic may be essential for carbohydrate metabolism in algae and fungi, however it is non-essential and toxic to plants at concentrations of 1-1.7 mg kg^{-1} dry weight (of leaf tissue) (Jones 2003). Anthropogenic sources of arsenic in the environment are pesticides, insecticides, wood preservatives, coal and petroleum wastes and mine tailings (Jones 2003; Zhao *et al.* 2009). Arsenic toxicity is evidenced in plants by the appearance of red-brown necrotic spots on older leaves, the yellowing and browning of roots and the wilting of new leaves (Jones 2003). Ma and co-workers (2001) described arsenic hyperaccumulation by *Pteris vitatta*. Plants of this species were able to accumulate high concentrations of As in its leaves, up to 7526 mg kg^{-1} dry weight. Other potential arsenic hyperaccumulators, including other *Pteris* species, have been suggested by Ma and co-workers (2006).

Nickel is found in the soil in concentrations lower than 100 mg kg^{-1}, and its toxicity is related to the replacement of essential elements in biomolecules (Mengel and Kirkby 2001). Nickel is generally not considered an essential nutrient, but it has been shown to be important for the germination of certain plants; possible roles for nickel are in the actions of hydrogenase and the translocation of nitrogen (Mengel and Kirkby 2001; Jones 2003). A method of metal remediation using *Pelargonium* sp. has been described by Krishnaraj and co-workers (2001), where these plants were shown to accumulate cadmium, lead and nickel, up to 456, 3005 and 1195 mg kg^{-1} dry weight, respectively, in the shoots and 27,043, 60,986 and 21,141 mg kg^{-1} dry weight in the roots. The authors have further shown that the plants also showed a tolerance and accumulation to the metals when supplied as mixtures.

2.5 METHODS TO IMPROVE PHYTOREMEDIATION

Several factors, such as the root access and contaminant bioavailability, influence the efficiency of phytoremediation (Lestan 2006). Various techniques have been patented with methods aimed at reducing these limitations (Table 2.3).

Table 2.3 Main attributes of patents related to phytoremediation methods.

Patent #	Title	Contaminated medium[a]	Contamination[a]	Plant[a]	Method[a]
MANIPULATION OF THE PHYSICO-CHEMICAL CHARACTERISTICS OF THE ENVIRONMENT					
US6159270	Phytoremediation of metals.	Soil	Inorganic (metals)	Not defined	pH adjustment Addition of chelating agents
US7268273-B2	Recovering metals from soil.	Soil	Inorganic (metals)	*Alyssum* spp.	pH adjustment
US6786948-B1	Method for phytomining of nickel, cobalt and other metals from soil.	Soil	Inorganic (metals)	*Alyssum* spp.	pH adjustment Addition of chelating agents
US7829754-B2	Method of cleaning heavy metals-containing soil.	Soil	Inorganic (metals)	Polygonaceae	Addition of chelating agents
US5944872	Method for phytomining of nickel, cobalt and other metals from soil.	Soil	Inorganic (metals)	*Alyssum* spp.	pH adjustment Addition of chelating agents
WO9734714-A1	Method for hyperaccumulation of metals in plant shoots.	Soil	Inorganic (metals)	Brassicaceae	pH adjustment Addition of chelating agents
US5785735	Phytoremediation of metals.	Soil	Inorganic (metals)	Brassicaceae	pH adjustment Addition of chelating agents Application of electric field.
US5928406	Conversion of metal oxidation states by phytoreduction.	Soil	Inorganic (metals)	Brassicaceae	Application of electric field pH adjustment
US6145244	Methods for enhancing phytoextraction of contaminants from porous media using electrokinetic phenomena.	Soil, sludge, composted material	Inorganic	Not defined	Application of electric field
WO9826881-A1	*In situ* remediation of contaminated soil.	Soil	Organic	Not defined	Intoduction of a co-metabolite Application of electric field
MANIPULATION OF THE ROOT SYSTEM					
US5829191	Method of growing and harvesting vegetation for use in remediating contaminated soil and/or groundwater.	Soil or groundwater	Not defined	Tree	Controlling root growth by physical constraints
US5829192	Method for focusing the growth of a vegetative root system to target a contaminated area.	Aquifer	Not defined	Tree	Controlling root growth by physical constraints

US6360480-B1	Method and system to facilitate deep phytoremediation.	Not defined	Not defined	Tree	Controlling root growth by physical constraints Addition of growth-enhancing substances
US20030196375-A1	Method of producing deep-rooted trees for phytoremediation applications.	Not defined	Not defined	*Populus* spp., *Salix* spp.	Controlling root growth by physical constraints Addition of growth-enhancing substances
US6303844-B1	Method of decontaminating medium containing polychlorinated biphenyls or dioxins.	Soil, lake, marsh	Organic	Solanaceae, Cruciferae, Umbelliferae, Clenopodiaceae, Leguminosae, Compositae and Saxifragaceae.	Induction of hairy roots by *Agrobacterium rhizogenes* Ri plasmid
OPTIMIZATION OF ROOT ABSORPTION BY ENGINEERED STRUCTURES					
US5876484	Method for removing soluble metals form an aqueous phase.	Aqueous solution	Inorganic (metals) Organic	Brassicaceae, turfgrasses, sunflower, pea, rye, bean, spinach, sorghum, tobacco, corn	Growing plants in a receptacle with incorporated nutrients and allowing the roots into the nutrient-lacking metal-containing solution
WO200048755-A1	Remediation of contaminated groundwater.	Groundwater	Organic Inorganic (metals)	Phreatophyte trees, poplars, grasses, evergreen plant species	Extraction of groundwater and recycling the extracted water through a vegetative stand
US6406627-B1	Method for removing pollutants from water.	Water	Inorganic	Wetland vegetation	Establishment of aerobic and anaerobic zones Variable water levels
US5853576	Phytorecovery of metals using seedlings.	Solution	Inorganic (metals)	*Brassica napus*, *B. rapa*, *B. juncea*, *Medicago sativa*, *Oryzae sativa*	Biomass of plant seedlings
US6727091-B2	Room air cleansing using hydroponic plants.	Air	Not defined	Lamiaceae, aromatic culinary herbs, ferns, mosses, orchids, baby's tears, croton	Apparatus to draw contaminated air through plant roots

[(a)] As indicated in the claims of the patent

2.5.1 Manipulation of the physico-chemical characteristics of the environment.

It has been acknowledged that only a fraction of the metals present in the soil or other medium are available to be taken up by plant roots (Marques, Rangel, and Castro 2009). Among the factors known to influence metal bioavailability, the pH emerges as a key factor (Wang et al. 2006). In fact, soil acidification has been shown to result in an increase of bioavailable aluminium, cadmium, manganese and zinc (Wang et al. 2006). Moreover a higher accumulation of lead was observed in *Lemna minor* cultivated at pH 4.5 than at pH 6.0 (Uysal and Taner 2009). Raskin and co-workers (2000) claimed that the addition of acetic or citric acid to the soil in order to adjust the pH to less than 5.0 increases the metal availability and, therefore, metal accumulation. However, the effect of pH is not always straightforward, as was described in the patent by Chaney and co-workers (2007), where it was shown that elevating the pH of the medium favoured nickel accumulation. In this patent, the authors proposed that metals may be selectively accumulated by adjusting the pH to different levels.

The presence of chelating agents, such as EDTA (ethylenediaminetetraacetic acid), to complex the soluble metals also has been shown to facilitate the mobilisation of insoluble forms of metals (Blaylock *et al.* 1997). The use of chelating agents to increase metal availability to plants is supported by patents by Raskin and co-workers (Raskin *et al.* 2000) and Chaney and co-workers (1999; 2004), who indicate the use of nitriloacetic acid (NTA) and EDTA as preferable agents for nickel phytoextraction. Tamura and co-workers (2010) have evaluated the increase of lead dissolved out of a contaminated soil sample by 26 different chelating agents. Highest results were obtained for EDTA, methylglycinediacetic acid (MGDA), ethylenediaminedisuccinic acid (EDDS) and glutamic acid diacetic acid (GLDA).

Phytoextraction can be specifically limited by the amount of uptake and the root-to-shoot transport of metals and enhanced by the addition of mobilising agents to the contaminated medium (Garbisu and Alkorta 2001; Lestan 2006). A concentration of 38,601 mg kg^{-1} Pb was obtained in *Fagopyrum esculentum* cultivated in Pb contaminated soil with addition of MGDA (Tamura *et al.* 2010). However, stress symptoms, such as yellow leaves, spotting, discoloration of leaves and leaf drop were observed. An increase in lead and uranium accumulation in the shoots of *B. juncea* in the presence of EDTA and citric acid, respectively, has been exemplified by Ensley and co-workers (1997). In the method claimed by the authors, the inducing agent of metal hyperaccumulation is a chelator selected from the group EDTA, EGTA (ethyleneglycol-

bis(β-aminoethyl ether)-N,N,N′,N-tetraacetic acid), DTPA (diethylenetriaminepentaacetic acid), CDTA (trans-1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid), HEDTA (N-hydroxyethylenediaminetriacetic acid), NTA, citric acid, salicylic acid, malic acid; also claimed is the application of an inducing agent to promote the hyperaccumulation of other metals. The effectiveness of some of these chelators, and the enhancement of the accumulation of cadmium, copper, nickel and zinc by EDTA has been demonstrated by Blaylock and co-workers (1997). Furthermore, Ensley and co-workers (1997) showed an additional improvement of lead accumulation and transport in *B. juncea* shoots, up to concentrations of 1.7% of dried tissue, by the combination of soil acidification and the addition of EDTA as a chelating agent.

Other remediation techniques, such as electrokinetics, have been proposed for use in combination with phytoremediation in order to enhance this process. Electrokinetics is a soil remediation technique, in which an electric current is passed through the soil resulting in the movement of fluid (electro-osmosis), charged chemicals (electro-migration) and charged particles (electrophoresis) (Mulligan, Yong, and Gibbs 2001). The increased contaminant mobility can contribute to a higher availability of metals, as claimed by Raskin and co-workers (1998), and to a higher availability of organic pollutants for remediating plants (Iyer 2001). Salt and co-workers (1999) described a method for the phytoreduction of chromium (VI) into chromium (III) by members of the Brassicaceae family and the manipulation of the mobility of chromium (VI) in the soil by the application of an electric field. The increase in lead accumulation by the application of an electric current was demonstrated in *B. juncea* by Hodko and co-workers (2000). The authors also showed that the combination of electrokinetics with phytoremediation increases the depth of soil that can be remediated by creating a counter-gravitational movement of the contaminants to the root zone. However, it was shown that the pH variations induced in the vicinity of the electrodes can be extreme and that polarity reversal was the most efficient method of pH stabilisation. The application of an electric current in combination with the exudation of co-metabolites from plant roots was proposed by Ho (1998) to enhance the biodegradation of a contaminant by rhizospheric microorganisms.

2.5.2 Manipulating the root system

The root system of the plants used for phytoremediation should ideally be deep and dense (Cunningham and Ow 1996), as the root depth is one of the main limitations to phytoremediation (Pilon-Smits 2005). Various patents present methods for

increasing and directing the growth and depth of roots. Gatlif (1998a) has presented a method of growing trees for transplantation that are engineered to have a long and narrow root system by cultivation in a deep hole that has walls lined with an impermeable material, so that the root growth is directed downward. Alternatively trees can be planted at the site of a contaminated aquifer in a hole with lined walls; the tree roots continue to grow downward towards the contaminated aquifer and, thus, extract the contaminants (Gatliff 1998b). In the method described by Christensen (2002), trees are planted more deeply than usual, and an irrigation tube is installed to provide water, nutrients and gas exchange to the areas where root growth is desired; additionally, the authors state that the root growth in these areas could also be enhanced by coating the trunk of the tree with vitamin B1 and mycorrhizae spores. However, Ferro (2003) has pointed out that methods that include planting in a lined hole would limit the lateral root growth and make the tree susceptible to windfall. Instead, the author described a method where the hole for the tree would be prepared by direct push technology, which is more economical than drilling and does not produce waste. A drip irrigation line should be used to transport nutrients, auxins for root growth and microbes to the root system. Moreover, irrigation rates could be manipulated to enhance the root growth into deeper soil layers.

The formation of “hairy roots”, which is promoted by *Agrobacterium rhizogenes* infection, results in an increased root density that is advantageous for phytoremediation. Morita and co-workers (2001) presented a method of remediating polychlorinated biphenyl- (PCB) and dioxin-contaminated media by hairy root cultures or regenerated plantlets obtained from hairy roots. The authors have shown that the hairy roots of *Atroppa belladonna, B. juncea* var. *multiceps*, *B. juncea* var. *cernua*, *B. juncea* var. *rapa* and *Daucus carota* absorbed and decomposed more PCBs than the roots of uninfected control plants. In their patent, the authors highlighted the accumulation and decomposition of PCBs and dioxin by the hairy root cultures and regenerated plantlets of *A. belladonna*.

2.5.3 Optimisation of the root absorption of pollutants by engineered structures

The remediation of contaminated sites can be enhanced by the design and implementation of structures aimed at improving the contact between contaminated solutions and plant roots, while assuring adequate plant growth. Contaminated waters are usually poor in nutrients (Gerhardt *et al.* 2009) and unsuitable for plant growth, which may hamper the efficiency of phytoremediation techniques. Raskin and co-

workers (1999) have described a system for the remediation of metal-contaminated water in which plants were cultivated in a receptacle that was placed at the interface of the air/solution; the receptacle contained plant nutrients and allowed the main root biomass to grow into the external, contaminated nutrient-free solution. A different approach has been proposed by Farmayan and co-workers (2000), where contaminated ground water, inaccessible to the plant root system, was pumped and injected into the plant root zone for decontamination by the plants.

Wallace (2002) has described a rhizofiltration-constructed wetland system for the remediation of waste water, in which enhancing root depth and creating aerobic and anaerobic zones, facilitates the growth of rhizobacteria that promote a more efficient degradation of organic pollutants.

A structure for the phytorecovery of metals from contaminated waters has also been described by Kapulnik and co-workers (1998). In the proposed method, the concentration of cadmium in a solution was reduced from 0.6ppm to 1ppb by *B. juncea* seedlings enclosed in a chamber. Contrary to other methods, this one required neither a plant culture medium nor an energy source because the seedlings were dependent on the reserves contained in the seeds. Furthermore, the plant biomass was easily collected for disposal.

Plants may be used to remediate the air, as well as water and soil (Salt, Smith, and Raskin 1998). In fact, Darlington (2004) has presented a method for refreshing indoor air by plant roots. In the proposed structure, plants are grown on a vertical panel, nutrients are supplied by a hydroponic solution, which circulates down the panel, and air is drawn through the plant roots by a fan. The author states that both volatile organic compounds and organic dust particles are removed from the air by the plant roots and associated microorganisms.

2.6 PHYTO- AND RHIZODEGRADATION

The capacity of plants and their associated rhizospheric microorganisms to decompose organic contaminants into inert molecules is termed phytodegradation and rhizodegradation, respectively. As photosynthetic primary producers, plants obtain their anabolic precursors from inorganic chemical forms and have a negligible capacity to absorb organic compounds. On the contrary, soil chemo-organotrophic

microorganisms, which rely on the oxidation of organic compounds to obtain energy and possess a broad range of metabolic capacities, are suitable candidates to carry out biodegradation processes. Not surprisingly, the research on the biodegradation of organic xenobiotics has identified numerous bacteria and characterised several enzymes able to metabolise organic contaminants (Abhilash, Jamil, and Singh 2009). Accordingly, it has been suggested that plants have an indirect role in degradation, mainly through the mediation by root exudates, which modulate and promote the enrichment of soil microbial communities that are able to metabolise organic pollutants. Therefore, to accomplish a sustainable phytodegradation of organic contaminants, a fine equilibrium between the rhizospheric microorganisms, the plants and their respective tolerance to different pollutants needs to be reached.

Over the last decade, efforts have been made to genetically engineer microbial biodegradation traits in plants tolerant to organic xenobiotics, and some of these innovations have been patented (Table 2.2). A patent filed by Iimura and Katayama (2007) discloses a method of producing genetically engineered plants with roots able to secrete a laccase from *Trametes versicolor*, a basydiomycete fungi formerly known as *Coriolus versicolor*. Laccase is a phenoloxidase enzyme able to oxidatively decompose chlorophenols, polycyclic aromatic hydrocarbons, alkyl phenol, nitro compounds and agricultural chemicals.

An important source of organic contaminants is herbicides. Sadowsky and co-workers (2002) have described a method to obtain transgenic plants that are able to degrade S-triazine herbicides, which are slowly biodegradable in soil. Through the expression of a *Pseudomonas* atrazine chlorohydrolase enzyme, the transgenic plants were able to convert atrazine to hydroxyatrazine, an inactive compound, which led to a decrease of S-atrazine in the soil and, consequently, limited the leaching of these herbicides into the groundwater. Another invention aiming to degrade herbicides was described by Ohkawa and co-workers (2003). Using a fusion protein of a cytochrome P450 monooxygenase and a P450 reductase, to provide electrons, the authors claimed that transgenic plants expressing these fusion proteins were able to absorb, metabolise and decompose agrochemicals, including various herbicides, depending on the P450 molecular species.

Contamination with oil hydrocarbons is frequently at the centre of bioremediation concerns. In a patent by Sorokin and co-workers (2004), the authors

claimed that transgenic plants expressing rhamnosyltransferases acted as biosurfactants and were able to enhance the phytodegradation of oil hydrocarbons.

The enzymatic mineralisation of aliphatic and aromatic halogenated organic compounds is generally carried out by bacteria. Nevertheless, dehalogenating enzymes have been discovered in the halophytic plant, *Spartina alternaria*. Marton and co-workers (2000) have described a method for decomposing toxic organic pollutants using the phytodegradation potential of plants that were genetically transformed to express the *Spartina alternaria* dehaloperoxidase genes.

2.7 ASSOCIATIONS WITH MICROORGANISMS

Plant root exudates influence the characteristics of the soil, resulting in a particular zone called the rhizosphere, which is characterised by intense microbial activity. Research over the last few years has shown the bioremediation potential of rhizospheric bacteria in pure culture and their contribution to improve phytoremediation (Gerhardt *et al.* 2009). Rhizospheric microorganisms affect contaminant bioavailability, confer protection against plant pathogens, degrade contaminants and enhance plant growth. Currently, numerous patents addressing the enhancement of phytoremediation potential by microorganism/plant interactions have been applied for and issued (Table 2.2).

The isolation and application of soil microorganisms capable of increasing non-essential metal bioavailability for metal-accumulating plants has been described by Chet and co-workers (1998), where members of the *Pseudomonas* and *Bacillus* genera were indicated as useful. These bacteria were isolated from the rhizosphere of plants collected from a contaminated site and, through bioassays, shown to increase the cadmium concentrations in *B. juncea* shoots. Angle and co-workers (2007) have isolated bacteria from the rhizosphere of *Alyssum murale* plants growing on a nickel-rich soil and have suggested that other criteria, in addition to nickel tolerance, are important for the selection of enhancing bacteria, such as thriving in phosphorus, a tolerance to low pH and the production of chelating agents. In this patent, a method of enhancing the nickel extracted from contaminated soil by *A. murale* plants with the addition of these rhizobacteria to the soil or plant seeds was evaluated. Of the selected microorganisms, *Sphingomonas macrogoltabidus, Microbacterium liquefaciens* and *Microbacterium arabinogalactanolyticum* were shown to increase nickel accumulation

in the shoot by 17, 24 and 32%, respectively when added to the seeds. The use of endophytic organisms in the remediation of inorganic and organic contamination has been described by Van Der Lelie and co-workers (2005). The roots of *Lupinus luteus* plants inoculated with the nickel-resistant *Burkholderia cepacia* L.S.2.4::ncc-nre accumulated more nickel than the control plants. In this patent application, the authors also proposed that the inoculation of endophytic microorganisms capable of degrading organic contaminants would reduce the volatilisation of dangerous pollutants, namely toluene and trichloroethylene (TCE). Certain rhizospheric microorganisms enhance plant growth and are collectively known as plant growth-promoting rhizobacteria (PGPR) (Lugtenberg and Kamilova 2009). Bogan and co-workers (2004) have described the isolation of three polycyclic aromatic hydrocarbon (PAH)-resistant bacterial strains, *Burkholderia* ATCC No. PTA-4755, *Burkholderia* ATCC No. PTA4756, and *Sphingmonas* ATCC No. PTA 4757. The inoculation of alfalfa with these strains resulted in an improvement of seedling health and an increase in biomass. Furthermore, these bacterial strains conferred protection against pathogenic fungi, and the combination of the alfalfa and bacterial association showed a greater removal of PAHs than the removal by the bacteria or plants alone. The enhanced plant growth, namely the root hair area and the root and shoot length of maize plants, promoted by *Trichoderma harzianum* T-22 has been demonstrated by Harman and co-workers (2004); furthermore, root growth at adequate nitrogen levels and the yield of soybean when cultivated in combination with *T. harzianum* T-22 and *Bradyrhizobium japonicum* were also shown to be enhanced.

2.8 DISPOSAL OF CONTAMINATED PLANT MATERIAL

Certain organic contaminants may be degraded to harmless forms, whereas others may be volatilised; in many cases, the pollutants are accumulated in the plant tissues. However, the risk of returning the contaminants to the substrate by the falling of leaves or plant decay may result from this accumulation. In many of the proposed methods of phytoremediation, the plants are collected and disposed of as hazardous material, or they may be used for metal recovery provided they have commercial value (Rugh 2004). Different methods have been proposed as a solution.

The creation of a protective layer on the surface of the soil to receive the falling leaves of woody plants and to bind the pollutants that may be released by their decay has been described by Wenzel and Adriano (Wenzel and Adriano 2004). This layer

may be usable for twenty years and reduces costs by eliminating the need for frequent harvesting. In order to make phytoremediation more attractive financially, various strategies of adding value to the resulting plant material have been proposed. One example of an economic return is the extraction of aromatic oils from *Pelargonium* sp. (Krishnaraj *et al.* 2001). In another approach, it was suggested that value could be added to the contaminated plant material by using it as combustible material for energy production, a process which also would reduce the biomass (Rugh 2004). The reclamation of lead from plant material has been addressed by Cunningham (1994), where it was indicated that the plant material could be smelted directly, or the lead could be concentrated by biomass reduction through different techniques, namely by aerobic and anaerobic digestion, acid digestion, incineration and composting.

Volume reduction by drying and acid digestion for the recovery of metals have also been addressed by Gardea-Torresdey and co-workers (1999), where the separation of metals from the plant biomass by the use of chelators, such as EDTA, cyclic polyamine chelator compounds and polyethers, was suggested. The authors claimed that the extraction of metals from the plant tissues is accomplished by acid oxidation of the metals followed by collection of the metal oxides.

In order to achieve an economically viable recovery of metals, after the reduction of the plant material to ash by drying and incineration, metals, such as nickel, can be recovered by roasting, sintering, smelting, acid dissolution or electrowinning (Chaney *et al.* 1999; Chaney *et al.* 2004; Chaney *et al.* 2007). According to Chaney and co-workers (1999), recovery is cost-effective at metal concentrations of 2.5 to 5.0% in the above-ground tissues.

2.9 CURRENT AND FUTURE DEVELOPMENTS

In a changing world, demands for products to meet an increasing population and the needs of emerging economies, frequently supported by unsustainable industrial and agricultural practices, are likely to result in the increase of contaminated sites. There is a growing awareness of the adverse effects on biodiversity and human health that result from environmental contamination. Phytoremediation, the use of plants to remove, convert or sequester hazardous substances from the environment, has been the subject of many scientific publications and patents during the past decade.

The plants best suited for phytoremediation should be fast growing, possess extensive root systems and produce a large amount of biomass. However, many plants identified as hyperaccumulators lack these characteristics. Moreover, the optimal growth of plants used for remediation can be further hindered by nutrient deficiencies and other sources of stress that frequently characterise contaminated sites. The unique nature of each case of contamination and the soil and climatic conditions are additional constraints that should be taken into account. A better understanding of the labile pools of, and interaction between, contaminants is necessary in order to design more efficient phytoremediation strategies. This should be complemented with further research into plant remediation mechanisms.

Insights into the complex interactions between plants, microorganisms and physico-chemical factors may contribute to a better understanding of the labile fraction of contaminants and its dynamics and aid in the improvement of parameters, such as plant biomass, root system size and depth. It is also imperative that we remain aware of the biomagnification potential of the contaminants up through the food-chain in order to avoid the contamination of higher trophic levels and, ultimately, food products. Further studies into the genetic, biochemical and structural traits of plants with regard to the uptake, translocation, accumulation and tolerance mechanisms for contaminants can result in practical improvements of phytoremediation techniques. Ultimately, this research effort should lead to the identification of important genes for phytoremediation, the characterisation of their expression patterns and the cellular localisation of their products, which is still largly unresolved for hyperaccumulators (Verbruggen, Hermans, and Schat 2009). The potential of phytoremediation may be enhanced by genetically engineering advantageous traits into fast-growing, high-biomass plants. However, this paradigm of phytotechnologies is limited by the present regulatory restrictions of genetically modified organism (GMO) use. Finally, it should be kept in mind that the disposal of the plant material is an inseparable part of the remediation process; careful consideration of the options available may render phytoremediation more economically attractive.

Phytoremediation is a promising and interdisciplinary area of research where plant biology, microbiology, soil science, genetic engineering, and environmental modelling converge. With our current knowledge and perspectives, we believe that phytoremediation will become a sustainable alternative and complement other remediation methods.

ABBREVIATIONS:

CDF – Cation diffusion facilitator

EDTA – Ethylenediaminetetraacetic acid

GMO **-** Genetically modified organism

HMA – Heavy metal-transporting P-type ATPases

MGDA – Methylglycinediacetic acid

NA – Nicotianamine

NRAMP – Natural resistance associated macrophage protein

NTA – Nitriloacetic acid

OAS-TL – O-acetyl (thiol) lyase

PAH – Polycyclic aromatic hydrocarbon

PCs – Phytochelatins

SAT – Serine acetyltransferase

YSL – Yellow-stripe 1-like

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CHAPTER III - HISTOLOGICAL AND ULTRASTRUCTURAL EVIDENCE FOR ZINC SEQUESTRATION IN *Solanum nigrum* L.

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ABSTRACT

The accumulation of contaminants in the environment due to anthropogenic activities is a matter of global concern. *Solanum nigrum* L. plants, able to accumulate zinc and hyperaccumulate cadmium, were challenged with 0.025g Zn L^{-1} during 35 days. The localization of Zn in roots, stems and leaves of *S. nigrum* plants was evaluated by autometallography (AMG) in order to determine the specific tissue, cell and subcellular compartments of Zn sequestration. This Zn concentration resulted in stunted plant growth but no other symptoms of Zn toxicity. Zinc concentration in the plants was highest in the roots, 666 mg Zn kg^{-1} f.w., and lower in the stems, 318 mg Zn kg^{-1} f.w., and leaves, 101 mg Zn kg^{-1} f.w. Roots of Zn treated plants showed an underdeveloped structure but additional layers of proliferating cortical parenchyma cells. AMG of *S. nigrum* roots, stems and leaves revealed a generalized Zn distribution associated with the cell walls in all tissues. In the vasculature (xylem and phloem) Zn was observed at the plasma membrane – cell wall complex of vascular parenchyma cells and conducting elements. Conspicuous Zn deposits were detected in the vacuoles of cortical parenchyma and starch sheath, as well as in the tonoplast of the mesophyll cells. Our results suggest that Zn flux through the plant occurs via the xylem and phloem and associated parenchyma until it is conducted to permanent storage sites, namely the apoplast and vacuoles of cortical parenchyma cells of the root, stem and the leaf mesophyll.

3.1 INTRODUCTION

Environmental contamination is recognized as a serious problem for our and future generations, and much effort has been given to its mitigation. A great variety of organic and inorganic pollutants find their way into the environment due to anthropogenic activities and in this context, phytoremediation, the use of plants for environmental cleanup, emerges as a cost effective and environmentally friendly solution (Pilon-Smits 2005; Marques, Rangel, and Castro 2009). Among the inorganic pollutants, zinc is considered to be one of the most important contaminants and although it is an essential micronutrient in plants, high concentrations can cause toxic effects and severely hamper plant growth (Raskin, Smith, and Salt 1997; Rout and Das 2003; Broadley *et al.* 2007).

A number of plants have been characterized for their ability to tolerate and accumulate high concentrations of metals (Prasad and Freitas 2003; Broadley *et al.* 2007). Among these, *Solanum nigrum* L. plants have been shown to accumulate Zn and hyperaccumulate cadmium (Wei *et al.* 2005; Marques *et al.* 2007, 2008). Recently we have shown that Zn tolerance and accumulation in *S. nigrum* are growth dependent and have suggested the involvement of several organic acids (Samardjieva *et al.* 2014), although the cellular compartmentalization of this metal remains to be elucidated. *Solanum nigrum* is a plant species vastly distributed in the globe, possess characteristics favouring interspecific competition and has been proposed as a model system for plant tissue and protoplast culture (Edmonds and Chweya 1997; Hassanein and Soltan 2000; Chao *et al.* 2005).

Plant tolerance and accumulation of metals have been addressed in various plant species, including *S. nigrum*, and studied on various levels, namely by assessing the contribution of amino acids, organic acids and peptides as metal chelators, by understanding the role of membrane transporters for subcellular sequestration, and by characterizing the synergistic effect of mycorrhiza and external chelators (Callahan *et al.* 2006; Sun, Zhou, and Jin 2006; Haydon and Cobbett 2007; Marques *et al.* 2007, 2008; Sun *et al.* 2009; Gao *et al.* 2012). The sequestration of metals in specific tissues and cell compartments, such as the cell wall and vacuole, is proposed to be a mechanism for protection of the more metabolically active cell sites from metal toxicity (Krzeslowska 2011; Rascio and Navari-Izzo 2011). Zinc localization in plants has been studied using several techniques among which autometallography (AMG) emerges as

a high-resolution localization method with the great advantage of preserving the cellular ultrastructure (Heumann 2002; Wu and Becker 2012).

Determining the location of the metals considering the whole plant may help to elucidate the routes of metal transport in the plant, and therefore contribute to unveil organ- and tissue-dependent differences of plant tolerance and accumulation of metals. Although several studies have addressed metal localization, the detailed histological and subcellular distribution of Zn remains unclear. In order to shed light on the importance of subcellular sequestration for Zn tolerance and accumulation in *S. nigrum,* the histological and cellular location of Zn, revealed by AMG, was investigated by bright field and transmission electron microscopy. To our knowledge, this is the first study to detail Zn localization in *S. nigrum* at the tissue, cell and ultrastructural level, contributing to clarify the flux and sequestration sites that may be involved in Zn accumulation and tolerance in this plant species.

3.2 MATERIAL AND METHODS

3.2.1 Plant material, culture conditions and biometric analysis

Solanum nigrum seeds, collected from the Porto district (Portugal) were supplied by the Department of Biology of the University of Porto. Seeds were surface sterilized with 1% NaClO (2 min) followed by 75% ethanol (2 min), washed thoroughly with sterile H_2O after each disinfectant and germinated on moist filter paper. Seedlings with fully expanded cotyledonary leaves were transferred to plastic containers containing Hoagland nutrient solution and polypropylene granules (Taiz and Zeiger 1998; Battke, Schramel, and Ernst 2003). The nutrient solution was composed of 607 mg L^{-1} KNO_3; 945 mg L^{-1} $Ca(NO_3)_2.4H_2O$; 230 mg L^{-1} $NH_4H_2PO_4$; 246 mg L^{-1} $MgSO_4.7H_2O$; 3.73 mg L^{-1} KCl; 1.55 mg L^{-1} H_3BO_3; 0.338 mg L^{-1} $MnSO_4.5H_2O$; 0.576 mg L^{-1} $ZnSO_4.7H_2O$; 0.124 mg L^{-1} $CuSO_4.5H_2O$; 0.08 mg L^{-1} H_2MoO_4 and 23.5 mg L^{-1} NaFeEDTA (Taiz and Zeiger 1998). The nutrient solution was renewed weekly to avoid over-concentration due to evapotranspiration and to prevent nutrient deficiency of essential elements. Plants were grown in a chamber with 16 h day length, 19-22 ºC and light intensity of 70 µMol m^{-2} s^{-1}. Plants were divided in two groups. The control group (n=8) was cultured in the aforementioned conditions and another group (n=8) was cultured in nutrient solution supplemented with Zn at 0.025 g L^{-1} (382 µM) (supplied as $ZnSO_4.7H_2O$). This Zn concentration was chosen

considering previous experiments that have shown that this was the highest Zn concentration which, although affecting development, allowed the plants to grow. All plants were harvested after 35 days. At harvest, plants were carefully collected and the roots were washed with deionized water. Root and stem length and the biomass of roots, stems and leaves were measured. Organs of individual plants were frozen in liquid nitrogen and stored at -80 ºC until further analysis or fixed for AMG.

3.2.2 Zinc concentration in plant tissues

Zinc concentrations were determined in roots, stems and leaves. Individual plant organs were ground with a pestle and mortar in liquid nitrogen and the concentration of Zn was spectrophotometrically determined with zincon by the method described by Macnair and Smirnoff (1999). Absorbance at 606nm was measured using a Shimadzu UVmini-1240 spectrophotometer.

3.2.3 Autometallography

Portions of roots, stems and leaves of four control and four Zn treated plants were fixed in glutaraldehyde (2.5 % v/v) in phosphate buffer (0.1M, pH 7.3) and Na_2S (0.1 % w/v) during 2h at room temperature and 12h at 4ºC (Danscher 1981; Heumann 2002). Samples were washed in phosphate buffer, dehydrated in increasing concentrations of ethanol, namely 30%, 50%, 70%, 90% and 100%, followed by propylene oxide. Samples were impregnated with increasing proportions of EMBed-812 (Embedding Kit, EMS) in propylene oxide, namely 1:3, 2:3 followed by 100 % of EMBed-812. Finally the plant portions were embedded in EMBed-812. Semi-thin sections of 3 µm were cut with a glass knife on an Ultramicrotome Leica Reichert SuperNova and mounted on glass slides for light microscopy, or glass coverslips for reinclusion in EMBed-812 for electron microscopy. Sections on glass slides and coverslips were immersed in physical developer, prepared according to Danscher and Zimmer (1978), during 60min in the dark and washed with deionized water. The sections on glass slides were photographed on a light microscope (Olympus CX31 coupled with a DP-25 Camera). When visualized under bright field microscopy the silver precipitates, formed due to the presence of Zn may range from yellow to black, depending on the size of the precipitate (Holm *et al.* 1988). Sections on glass coverslips were re-embedded on top of EMBed-812 blocks and the glass was removed

by immersion in hydrofluoric acid for 60min. Ultrathin sections were prepared using a diamond knife on a Ultramicrotome Leica Reichert SuperNova and mounted on copper grids. Sections were contrasted with uranyl acetate (15 min), observed on a Transmission Electron Microscope Jeol JEM-1400 (80kV) and digitally recorded using a GATAN SC 1000 ORIUS CCD camera.

3.2.4 Statistics

Data of organ length and mass were analysed for statistically significant differences (95% confidence interval) by the Student's *t* test. Significant differences in Zn concentration between organs and Zn treatment were analysed by two-way analysis of variance (ANOVA). All statistical analyses were performed using the SPSS for Windows version 22 software package.

3.3 RESULTS

3.3.1 Effect of Zn on S. nigrum

S. nigrum control and Zn treated plants were collected after 35 days of growth to assess the effect of Zn on the length and mass of roots, stems and leaves. (Fig. 3.1). Root and stem length were significantly reduced in Zn treated plants, indicating that a concentration of Zn of 0.025 g L^{-1}, over a period of 35 days and at the initial stages of development of *S. nigrum* plants results in stunted growth (Fig. 3.1A). The effects of Zn were also evident by the reduction in mass of the Zn treated plant roots, stems and leaves relative to their controls (Fig. 3.1B). Although plant growth was significantly reduced, no other visual signs of toxicity were observed (data not shown). Zinc accumulation was significantly higher in Zn treated plants organs when compared to the controls (Fig. 3.2). Accumulation was highest in the roots of Zn treated plants, followed by the stems and leaves with lower concentrations (Fig. 3.2).

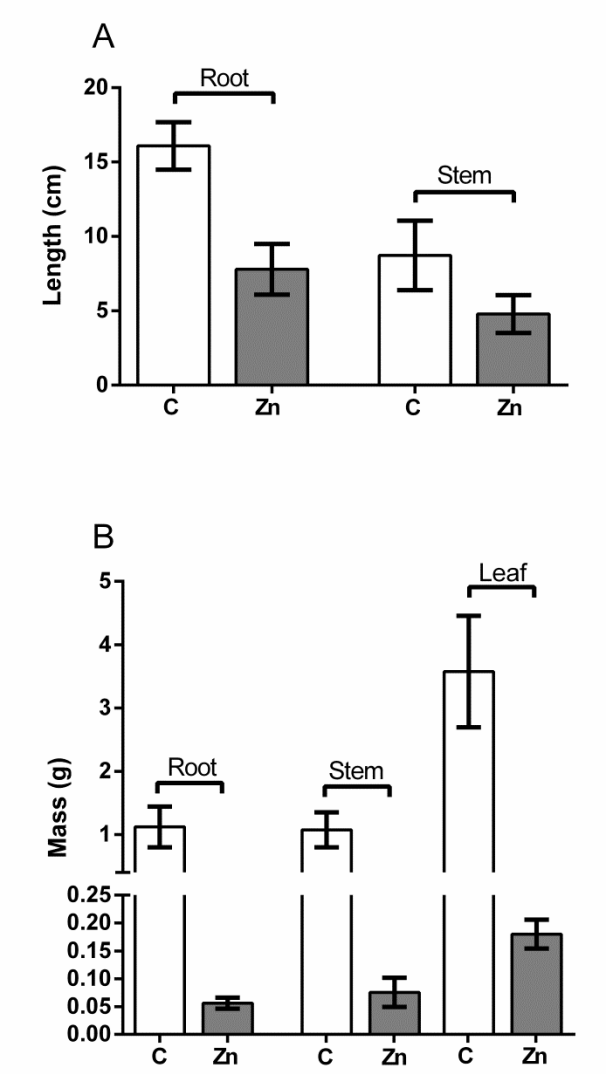

Fig. 3.1 Biometric analysis of *S. nigrum* plants cultivated with Zn at micronutrient and 0.025 mg L^{-1} concentrations of Zn. A) Root and stem length of control (C) and Zn treated (Zn) plants. B) Mass of roots, stems and leaves of control and Zn treated plants. Values are expressed as means ± SD (n=8). Data were checked for normal distribution, homogeneity of variance and the appropriate Student's *t* test was applied. Statistical analysis by the Student's *t* test at $P < 0.05$ showed significant differences between control and Zn treatedment for all comparisons.

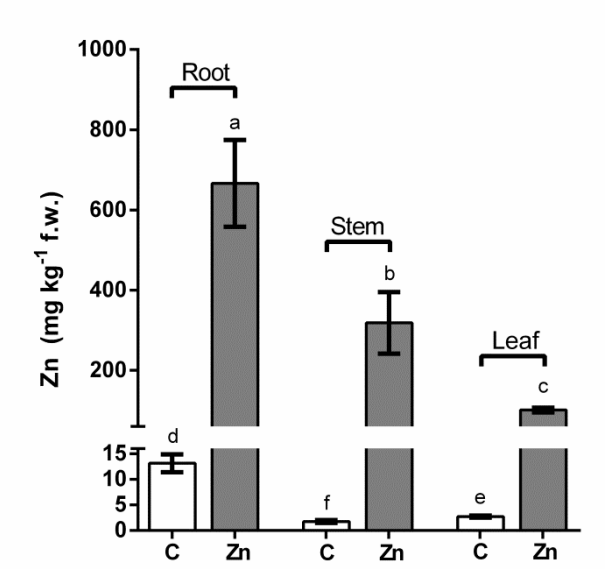

Fig. 3.2 - Zinc concentration in *S. nigrum* control (C) and Zn treated (Zn) plant roots, stems and leaves. Values are expressed as means ± SD (n=4). Data were checked for normal distribution and homogeneity of variances and log transformed prior to two-way ANOVA analysis. Zinc concentration varied significantly, $P < 0.001$, with plant organ and Zn treatment. Due to the detection of significant interaction, $P < 0.001$, between organ and Zn treatment a one-way ANOVA analyses was performed. Significant differences, $P < 0.05$, of Zn concentration in the plant organs, determined by the Bonferroni post hoc test, are represented in the figure by different letters.

3.1.1 Autometallography

3.1.1.1 Patterns of Zn accumulation in organs and tissues

Autometallography was carried out on the root, stem and leaf sections of control and Zn treated *S. nigrum* plants. Yellow to black precipitates formed due to the presence of Zn were observed in Zn treated plant roots, particularly associated with the cell walls of all tissues (Fig. 3.3 B) contrary to the control sections (Figs. 3.3 A and D). At a higher magnification of the vascular cylinder (Fig. 3.3 E) a more intense labelling of the area of the phloem in comparison with the xylem was observed. Black precipitates were also present in the vacuoles of the root cells of the inner and outer cortex of Zn treated plants (Figs. 3.3 C, E and F). Although the root sections were sampled from developmentally equivalent zones of control and Zn treated plants, i.e. the oldest region above the zone of histological differentiation, important structural differences were observed. While the control plant roots showed a normal secondary growth with secondary xylem and phloem originating from a well organized and developed cambium (Figs. 3.3 A and D) the Zn treated plant roots showed a stunted growth with a delayed cambium activity resulting in a reduction in xylem and phloem differentiation. Moreover, the cortex of Zn treated plant roots showed layers of

proliferating parenchyma cells, similar to a deep phellogen development and phellem production, possibly due to a precoce and intense exfoliation of the epidermis and disintegration of outer cortical cell layers (Fig. 3.3 C).

Zinc treated plant stems also presented a generalized AMG staining in all the tissues contrary to the controls (Figs. 3.3 G and J). At low magnification AMG staining was observed associated to the cell walls of stem medullary parenchyma, vascular tissues, cortical parenchyma and epidermis (Fig. 3.3 H). The stem structure of *S. nigrum* has bicollateral vascular bundles, characterized by an internal and external phloem. At higher magnification it was noticable that the Zn deposits of the internal phloem and xylem parenchyma were more conspicuous than at the xylem tracheary elements (Fig. 3.3 K). The interfascicular parenchyma cells presented black precipitates in the vacuoles (Fig. 3.3 K) indicative of Zn accumulations in these compartments. Large Zn deposits were observed in the vacuoles of starch sheath cells and, in the vascular cylinder, an intense AMG staining appeared to be associated with the cell walls of both the cambium and external phloem (Fig. 3.3 I). Smaller deposits were also present in the vacuoles of outer cortical parenchyma cells (Fig. 3.3 L).

Control leaf sections showed no AMG staining (Figs. 3.3 M and P). In Zn treated plant leaves AMG deposits were observed associated with the cell walls of the epidermis, mesophyll and vascular tissues (Fig. 3.3 N) where the internal and external phloem showed a higher density of Zn deposits in comparison to the xylem tissue (Fig. 3.3 Q). The large cells of the palisade and spongy parenchyma also presented AMG Zn staining associated with the cell walls and, interestingly, contouring the areas typically occupied by chloroplasts, which suggests that Zn deposits occur in the cytoplasm or tonoplast (Figs. 3.3 O and R).

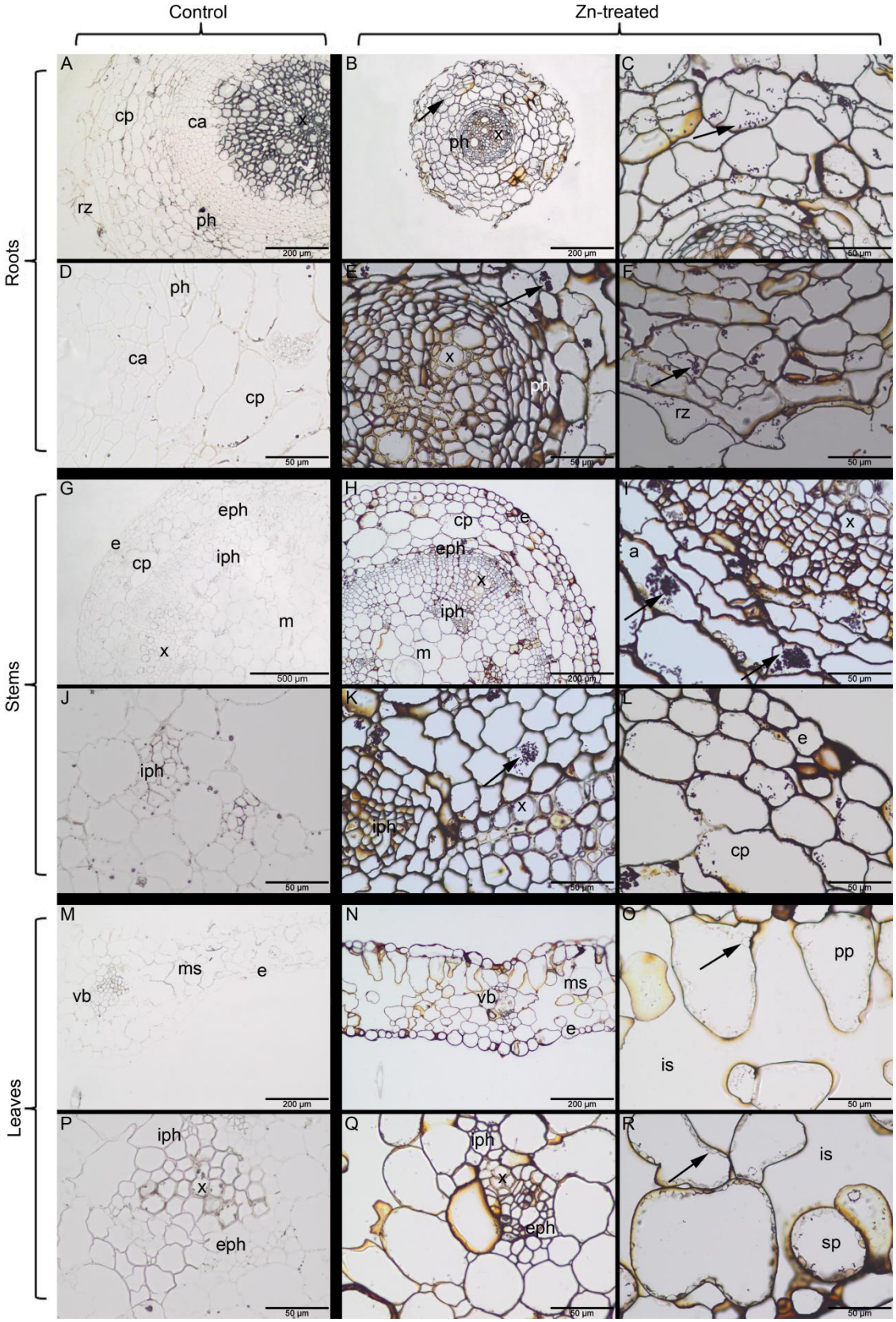

Fig. 3.3 – Light AMG of *S. nigrum* control and Zn treated plants. A) Control root section. B) Zn-treated root section showing layers of proliferating parenchyma cells (arrow). C) Zn treated plant root cortex with Zn deposits (arrow) and layers of proliferating parenchyma cells. D) Magnification of control root vascular cylinder and cortex. E) Detail of root vascular cylinder of a Zn-treated plant. F) Outer cortex of the root of a Zn treated plant showing exfoliating rhizodermis

and Zn deposits (arrow). G) Section of a control plant stem. H) Section of the stem of a Zn treated plant. I) Zn treated plant cambial zone, external phloem, cortical parenchyma and starch sheath with intense Zn deposits (arrow). J) Detail of a control plant stem section showing internal phloem and medullary parenchyma cells. K) Internal phloem, xylem and interfascicular parenchyma of a stem section. L) Detail of the outer cortex and epidermis of a Zn treated plant. M) Section of a control plant leaf. N) General structure of a Zn treated plant leaf. O) Palisade parenchyma and epidermis of Zn treated plant leaf, intracellular Zn deposits indicated by arrow. P) Detail of a bicolateral vascular bundle of a control plant leaf showing the phloem and xylem. Q) Detail of a bicolateral vascular bundle of a Zn treated plant leaf. R) Spongy parenchyma of Zn treated plant leaf, intracellular Zn deposits indicated by arrow. Plant tissues are indicated by lowercase letters as follows: a – starch sheath; ca – cambium; cp - cortical parenchyma; e – epidermis; eph - external phloem; iph - internal phloem; is - intercellular space; m - medulla; ms - mesophyll; ph – phloem; pp - palisade parenchyma; rz - exfoliating rhizodermis and outer cortical cells; sp - spongy parenchyma; vb - vascular bundle; x – xylem.

3.3.1.1 Subcellular localization of Zn

Further detail into the localization of Zn in the tissues and cells of *S. nigrum* was revealed by transmission electron microscopy. Sections of *S. nigrum* control roots showed none or minute amounts of Zn as observed in the micrographs of cortical cells and xylem tracheary elements shown in Figs. 3.4 A – C. In Zn treated plant roots, AMG staining was detected in all cell types, however, the intensity of the staining and the subcellular location varied. In the vascular tissues of the roots, Zn was detected in the xylem tracheary elements and at a higher intensity in the vascular parenchyma (Fig. 3.4 D). A more detailed observation of the xylem showed higher amounts of AMG labelling in the tracheary elements in the areas of primary cell wall, i.e. which had not been covered by a lignified secondary cell wall (Fig. 3.4 E). The Zn detected in the xylem parenchyma appears associated with the plasma membrane – cell wall (PM-CW) complex (Fig. 3.4 F). A similar pattern was observed for the phloem sieve tube elements, companion cells and associated parenchyma, where Zn was also deposited in the PM-CW complex (Figs. 3.4 G and H). In the vascular cambium Zn deposits were abundantly observed in the plasma membrane region, cell wall and also in the tonoplast, although in apparently smaller amounts (Fig. 3.4 I). This association of Zn with the tonoplast was also observed in cortical cells (Figs. 3.4 J and K) where Zn deposits were also observed in the cell walls, mainly in the regions contiguous to the intercellular spaces characteristic of these cells (Fig. 3.4 J).

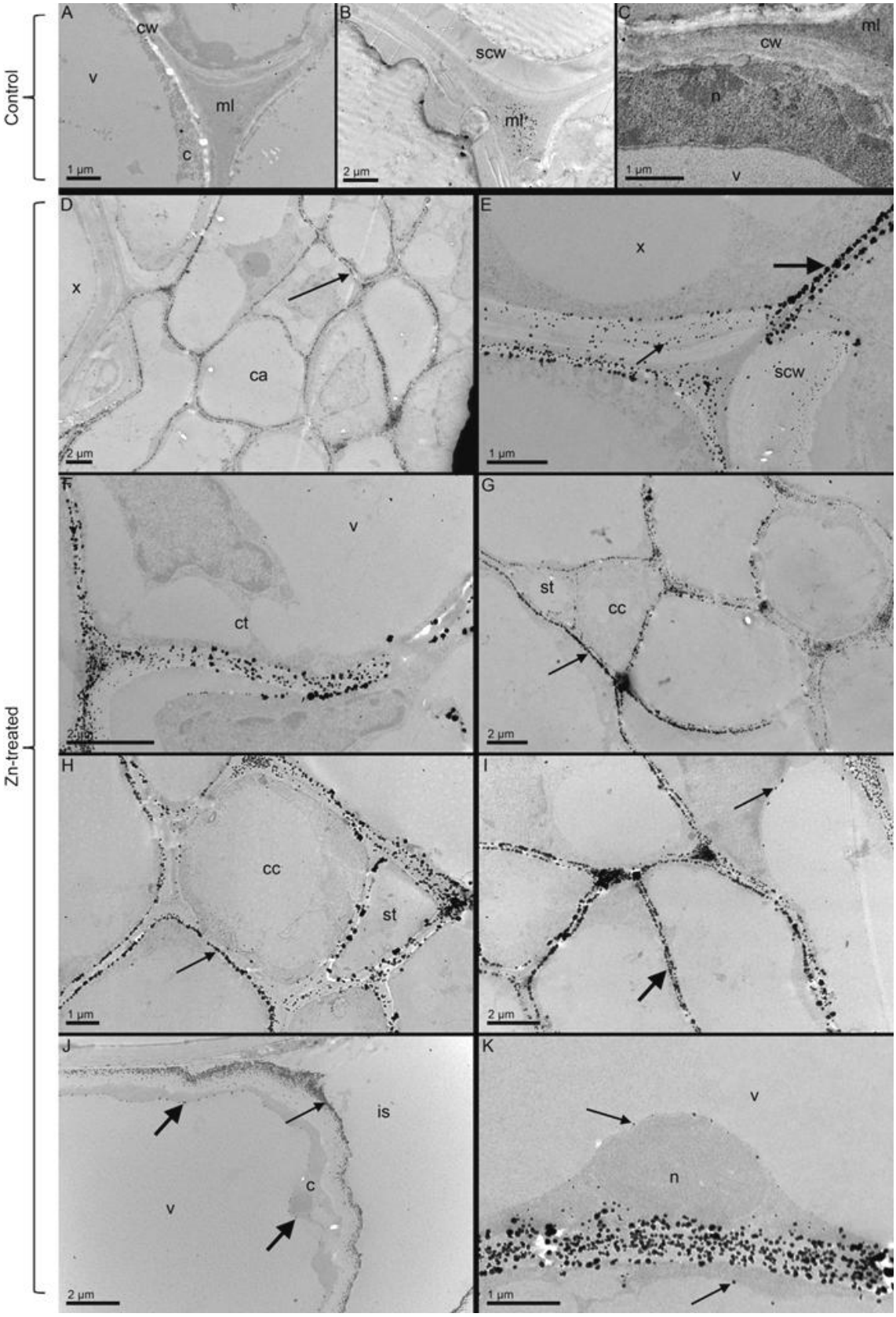

Fig. 3.4 – Transmission electron microscopy (TEM) of AMG treated control (A-C) and Zn treated plant roots (D-K). A) Detail of the contact region of three cortical cells showing vacuoles, cell walls and middle lamella. B) Xylem cells; contact region between tracheary cells, note the secondary wall thickenings. C) Cortical cell showing middle lamella, cell wall, nucleus and vacuole. D) Secondary xylem and cambium showing conspicuous labelling in the PM-CW complex

(arrow). E) Electron-dense granules at the secondary cell wall (thin arrow) and the primary cell wall (thick arrow) of the xylem tracheary elements. F) Vascular parenchyma cells showing labelling in the PM-CW complex. G) Phloem and associated parenchyma, electron-dense granules indicated by arrow in parenchyma cells. H) Detail of sieve tube element-companion cell complex and parenchyma cells; electron-dense granules appear at the PM-CW complex (arrow). I) Cambium cells with electron-dense granules associated with the PM-CW complex (thick arrow) and the tonoplast (thin arrow). J) Conspicuous AMG labelling in the cell wall of cortical cells facing the intercellular spaces (thin arrow), a smaller intensity is detected at the tonoplast (thick arrows). K) Detail of cortical cells with abundant electron-dense granules associated with the cell wall, the tonoplast (arrow) and in the cytoplasm. Cell types and structures are indicated by lowercase letters as follows: c – cytoplasm; ca – cambium; cc - companion cell; ct - cytoplasmic trabeculae; cw - cell wall; is - intercellular space; ml - middle lamella; n – nucleus; scw - secondary cell wall; st - sieve tube element; v – vacuole; x - xylem.

In stem sections of control plants, Zn was largely undetected in all the tissues surveyed, namely the vascular cambium, phloem and xylem tracheary elements shown in Figs. 3.5 A – C. However, as observed in the roots, stem sections of Zn treated plants presented electron-dense granules in all tissues, with various intensities and varied subcelular localizations. In the medullary parenchyma, Zn was found in the cell walls, preferably in the regions contiguous to intercellular spaces and also in the middle lamella (Fig. 3.5 D). In the internal phloem, Zn was associated with the plasma membrane region and the cell wall, with emphasis for the middle lamella of companion cells and sieve tube elements (Fig. 3.5 E) and phloem parenchyma cells (Fig. 3.5 F). In the tracheary elements Zn was observed in the secondary cell wall and prominently in the exposed areas of the primary cell wall (Fig. 3.5 G). The cambium presented an intense and uniform AMG labelling located at the PM-CW complex and cell corners (Figs. 3.5 H and I). The external phloem showed a pattern of Zn deposits similarly to what was observed for the internal phloem, i.e. the PM-CW complex of companion cells and sieve tube elements with the contiguous parenchyma cells presenting a comparatively higher amount of electron-dense granules at the level of the plasma membrane region (Fig. 3.5 J). Striking deposits of electron-dense granules, whose presence was already indicated by bright field AMG, were observed in the vacuoles of the starch sheath layer which is the innermost layer of the stem cortex (Fig. 5 K). Intense labelling was also evident in the PM-CW complex and cytoplasm of these cells (Fig. 3.5 K).

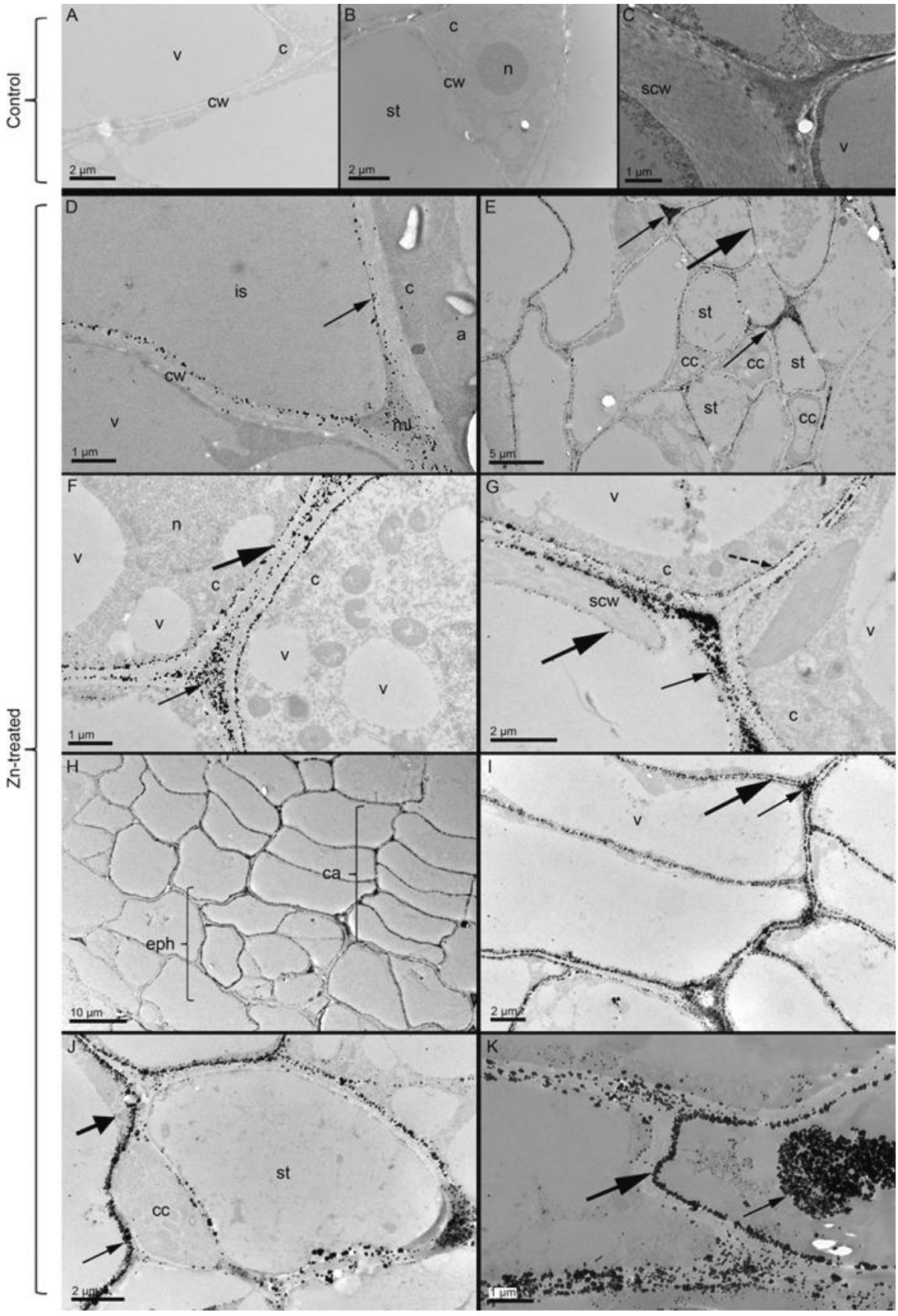

Fig. 3.5 - TEM of AMG treated control (A-C) and Zn treated plant stems (D-K). A) Cambium cells. B) Sieve tube elements and companion cell. C) Tracheary cell and xylem parenchyma cell. D) Medullary parenchyma cells of Zn treated plant stem, electron-dense granules are associated with the middle lamella and the cell wall facing the intercellular space (arrow). E) General view of an internal phloem bundle, where labelling can be seen in the PM-CW

complex (thick arrow) and deeply staining the middle lamella (thin arrows) contiguous to sieve tube elements. F) Detail of vascular parenchyma cells with electron-dense granules at the plasma membrane region (thick arrow) and middle lamella (thin arrow). G) Xylem showing electron-dense granules associated with the secondary cell wall (thick arrow), the primary cell wall of tracheary elements (thin arrow) and the PM-CW complex of the xylem parenchyma cells (dashed arrow). H) Cambium zone and external phloem. I) Detail of cambium cells showing electron-dense granules at the PM-CW complex (thick arrow) and in the cell corners (thin arrow). J) Detail of external phloem cells depicting electron-dense granules associated with the PM-CW complex and the cell membrane region of the phloem parenchyma cells (thin arrow). Week labelling was observed in the cytoplasm of these phloem parenchyma cells (thick arrow). K) Starch sheath cells showing accumulations of electron-dense granules within the vacuole (thin arrow), in the PM-CW complex (thick arrow) and more fainly in the cytoplasm. Cell types and structures are indicated by lowercase letters as follows: a – amyloplast; c – cytoplasm; ca – cambium; cc - companion cell; cw - cell wall; eph - external phloem; is - intercellular space; ml - middle lamella; n – nucleus; scw - secondary cell wall; st - sieve tube element; v – vacuole.

Concerning the localization of Zn deposits in the leaves, contrary to the leaf tissues of control plants where AMG staining was mostly undetected, as shown in the micrographs of phloem, vascular parenchyma and mesophyll cells (Figs. 3.6 A – C), the leaf tissues of Zn treated *S. nigrum* plants showed Zn deposit patterns similar to those observed for the other organs. The xylem tracheary elements evidenced a fainter deposition of electron-dense granules associated with the secondary cell wall when compared to their associated parenchyma cells where Zn was mainly detected at the the PM-CW complex (Figs. 3.6 D and E). The leaves of *S. nigrum* present, as in the stem, an external and internal phloem. In both cases, Zn was observed associated with the PM-CW complex of parenchyma cells and sieve tube elements (Figs. 3.6 F - H). In the mesophyll Zn was detected in the cell walls, particularly at the external part of the cell wall, i.e. contiguous to the intercellular spaces, and less markedly at the cytoplasm – vacuole interface, probably at the tonoplast (Figs. 3.6 I – K).

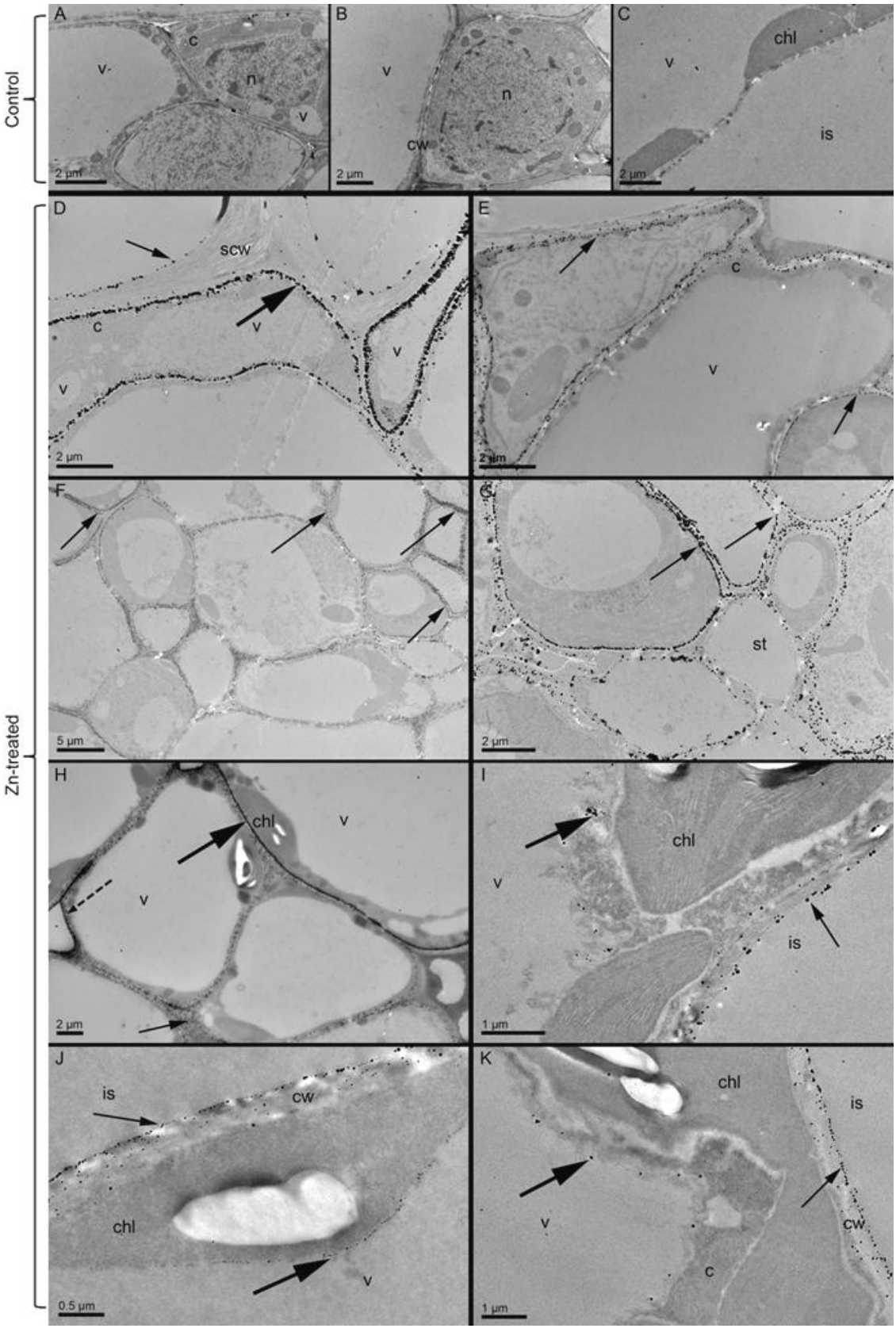

Fig. 3.6 - TEM of AMG treated control (A-C) and Zn treated plant leaves (D-K). Phloem conducting elements and associated parenchyma cells (A), vascular parenchyma cell (B) and in the leaf mesophyll cell (C). D) Xylem of Zn treated plant leaf with electron-dense deposits (thin arrow) at the surface of the secondary cell wall and the PM-CW complex of associated parenchyma cells (thick arrow). E) Vascular parenchyma cell with electron-dense granules

associated with the PM-CW complex (arrow). F) External phloem with electron-dense granules associated with the PM-CW complex (arrows). G) External phloem showing electron-dense granules associated with the PM-CW complex of conducting elements and parenchyma cells (arrows). H) Internal phloem showing electron-dense granules associated with the middle lamella (thin arrow), the intercellular spaces (dashed arrow) and the PM-CW complex of contiguous parenchyma cells (thick arrow). I-K) Details of mesophyll cells showing electron-dense granules at the cytoplasm – vacuole interface (thick arrows) and the cell wall (thin arrow). Cell types and structures are indicated by lowercase letters as follows: c - cytoplasm; chl – chloroplasts; cw - cell wall; is - intercellular space; n – nucleus; scw - secondary cell wall; st - sieve tube element; v – vacuole.

3.4 DISCUSSION

3.4.1 Effect of Zn on S nigrum growth

The ability of *Solanum nigrum* plants to tolerate and accumulate concentrations of Zn beyond their metabolic needs has been acknowledged and studies have emphasized *S. nigrum* capability to cope with Zn well above the physiological concentration and higher what is considered to be toxic for most plants (Marques *et al.* 2006; Marques *et al.* 2007, 2008). In the present work it was shown that Zn concentration of 0.025 g L^{-1} lead to a reduction of stem and root length and a decrease of the total plant mass, affecting plant growth. Recently Zn has been reported to have an effect on cell division and elongation which may explain the plant growth impairment observed (Seregin *et al.* 2011). Furthermore, a reduction of plant biomass was previously reported for *S. nigrum* when Zn was supplied to a sand matrix (Marques *et al.* 2006). Regardless of this growth reduction, the accumulation potential of *S. nigrum* plants is unquestionable considering that these plants are able to accumulate up to 3810 mg kg^{-1} d.w of Zn in the root without visual toxicity symptoms (Marques *et al.* 2006).

The effects of Zn on *S. nigrum* plant growth were also detected by histological analyses where notable differences in root and stem diameter and development are evident and in accordance with the reduction of length and biomass of the plant organs. Although Zn treatment has been shown to affect cell division, in the underdeveloped roots of the plants grown with Zn, a few layers of dividing parenchyma cells appeared in the inner cortex, probably to compensate for the large exfoliation of the epidermal and outer cortical dead cells in these roots. At the ultrastructural level, no damages to the cell structures were observed, contrary to what has been indicated by others in Zn stressed plants, namely damage to cell organelles, membranes and chromatin (Sresty and Rao 1999).

3.4.2 Zinc transport and sequestration

Autometallography of roots, stems and leaves of Zn treated *S. nigrum* plants allowed to disclose the tissues and cell compartments implicated in Zn sequestration, providing insights into the transport of the metal throughout the plant. Light microscopy AMG analysis root, stem and leaf transversal sections revealed that Zn deposits are present in all tissues particularly associated with the cell walls. In the vasculature of *S. nigrum* organs, a comparatively more intense staining was observed in the phloem tissues than the xylem, confirming previous results obtained for the stem of *S. nigrum* (Marques *et al.* 2008). A recent report has shown that in Zn hyperaccumulator *Sedum alfredii* Zn was remobilized from older to younger leaves through the phloem and in another study Zn was detected in the stem phloem tissues of *S. alfredii* with the Zn-fluorophore Zinpyr-1 (Tian *et al.* 2009; Lu *et al.* 2013). In *Arabidopsis thaliana* the high accumulation of cadmium in the phloem tissues is most likely a result of a redistribution of cadmium from the shoot to the root probably to avoid toxic effects on shoot tissues (Van Belleghem *et al.* 2007). Altogether these reports suggest that phloem is a key tissue in response to Zn stress, however the fate and transitorily of the Zn observed in the phloem is unclear.

In the vascular tissues of *S. nigrum* Zn was observed in association with the PM-CW complex of the vascular parenchyma cells. Interestingly, cytoplasmic membrane exclusion and complexation at the PM-CW interface has been pointed out as a potential tolerance mechanism (Hossain *et al.* 2012). For example, about 60% of copper in the roots of *Lolium multiflorum* and *Trifolium pratense* was bound by the cell walls and cytoplasmic membranes (Iwasaki, Sakurai, and Takahashi 1990). This association is consistent with indications that the plasma membrane is involved in metal tolerance, either by reduction of the uptake or by active efflux of the metals from the cytoplasm and several transporters participating in these processes have been identified and reviewed (Hall 2002; Kramer, Talke, and Hanikenne 2007; Kramer 2010; Maestri *et al.* 2010). The intense accumulations of Zn in the PM-CW complex in *S. nigrum* plants may constitute a tolerance mechanism in vascular parenchyma cells. The pattern of Zn localization observed suggests a constant process of influx from the

root cortex into the tracheary cells and upwards to the shoot and, importantly, also laterally to the phloem cells. This is also supported by the comparatively higher quantity of Zn associated with the primary cell wall of tracheary cells than with the secondary cell wall, which indicates that Zn is continuously imported and exported from the tracheary cells. Consequently it can be inferred that the Zn absorbed in the plant is translocated through the xylem, the phloem and their associated parenchyma.

If the vascular tissues seem to be decisive for the flux of Zn within the plants, the cell wall has been described as one of the main sinks for metals (Krzeslowska 2011). In the present study, the pattern of Zn distribution in the apoplast of *S. nigrum* was evident in tissues that have as common characteristics intercellular spaces and large vacuoles, as are the root cortical cells, the stem medullary and cortical cells and the leaf mesophyll cells. This is in agreement with previous reports of Zn localization by AMG in *S. nigrum* roots (Marques *et al.* 2007). The sequestration of Zn in the cell wall has been reported by several other authors. For example, in the hyperaccumulator *Thlaspi caerulescens,* where Zn is accumulated at higher concentrations in the above ground tissues, Zn localization was performed by energy-dispersive X-ray micro analysis (EDXMA) and was detected in the cell walls of epidermal and mesophyll cells of the leaves and the cortical cell walls of the roots (Frey *et al.* 2000). Moreover, cell wall biomass of *Solanum lycopersicum* cells in suspension culture and the Zn content in the walls were observed to increase with Zn treatment which was suggested to be a mechanism to limit metal entry into the cells (Muschitz, Faugeron, and Morvan 2009). In another report, *Hydrilla verticillata*, a Zn accumulator, was shown to sequester 43-54% of Zn in the cell wall and Zn detection by AMG showed densely packed particles in leaf the cell walls (Xu *et al.* 2013). However, deposits were also observed in the nucleus, chloroplasts and cytoplasm (Xu *et al.* 2013). In the root cortex of *Phragmites australis* Zn was detected at highest levels in the intercellular spaces and cell walls, i.e., the apoplast, and to lower concentrations in the vacuoles (Jiang and Wang 2008). In Zn hyperaccumulator *Potentilla griffithii*, Zn detection by light microscopy employing the silver-sulfide method and scanning electron microscopy combined with energy dispersive spectrometry in roots and leaves showed an organ dependent Zn distribution (Hu *et al.* 2009). In the roots Zn was detected in the cell walls of the epidermis, endodermis and xylem parenchyma while in the leaves the authors indicate the vacuoles of epidermal and bundle sheath cells as the main Zn sequestration sites (Hu *et al.* 2009). In *S. nigrum* although the apoplast is an important site for Zn

sequestration, Zn storage by large vacuoles of parenchyma tissues cannot be ignored. In fact prominent Zn deposits were also observed in the vacuoles of *S. nigrum* root and stem cortical cells and starch sheath, while minor deposits were detected in association with the tonoplast in certain root cortical and vascular cambium cells as well as leaf mesophyll cells. Therefore, the sequestration of Zn in vacuoles is possibly a tissue specific feature as shown by Zn detected in the epidermal layer of the leaves of hyperaccumulator *T. caerulescens,* contrarily to the vestigious levels of Zn observed in the vacuoles of mesophyll cells (Frey *et al.* 2000). This differential localization of Zn in *T. caerulescens* is also supported by data presented by Kupper, Zhao, and McGrath (1999) however, it is worth mentioning that these results are not conciliatory for the different hyperaccumulator species which have been studied over the last years, suggesting that Zn sequestration is, to some extent, a species dependent feature. In fact, in another well known hyperaccumulator, *Arabidopsis halleri,* higher Zn concentrations were found in the mesophyll tissue than in the epidermis (Zhao *et al.* 2000). The importance of compartmentalization of metals in the cell wall and vacuole as a tolerance mechanism was also demonstrated in the comparison of hyperaccumulating and non-hyperaccumulating populations of *S. alfredii* where a significantly higher proportion of Zn was sequestered in the cell wall and vacuole of the hyperaccumulating population (Li *et al.* 2006).

The present work, besides giving support to previous studies describing Zn tolerance and accumulation in *S. nigrum*, provides a holistic perspective for Zn distribution and accumulation in the same plant, by detailing Zn sequestration in the different organs, the different plant tissues and at the cellular level, therefore contributing to understand the overall plant response to Zn stress. Although 0.025 mg L^{-1} of Zn cause a hindering effect on *S. nigrum* plant growth and development, Zn is accumulated without causing other toxicity symptoms or ultrastructural damage. The pattern observed for the histological and cellular of Zn accumulation in the different organs of *S. nigrum* suggests that Zn taken up from the medium flows in from the root cortex into the xylem tracheary elements where it is transported upward through the stem to the leaves and simultaneously laterally through the vascular parenchyma to the phloem. The accumulation of Zn in the PM-CW complex of some of these cells may constitute a tolerance mechanism by limiting Zn entrance into the cytoplasm. Zinc flux also occurs from the vascular elements into the parenchyma cells of the cortex,

medulla and mesophyll conducting the Zn along the plant body to more permanent sites of sequestration, namely the apoplast and vacuoles of those tissues.

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CHAPTER IV - ZINC ACCUMULATION AND TOLERANCE IN *Solanum nigrum* ARE PLANT GROWTH DEPENDENT

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ABSTRACT

Zinc tolerance, accumulation and organic acid production by *Solanum nigrum*, a known Zn accumulator, was studied during pre- and post-flowering stages of development. The plants, when challenged with Zn concentrations lethal to plantlets, showed an increase in tolerance from pre-flowering to post-flowering, which was accompanied by a reduction of Zn translocation to the aerial plant parts. Treatment with Zn induced a differential response in organic acids according to the plant organ and developmental stage. In the roots, where Zn concentrations were similar in pre- and post-flowering plants, a general decrease in organic acid in pre-flowering roots contrasted with the increase observed in post-flowering plants. In the stems, Zn induced a generalized increase in organic acids at both growth stages while in the leaves, a slight increase in malic and shikimic was observed in pre-flowering plants and only shikimic acid levels were significantly increased in post-flowering plants. This work shows that Zn accumulation and tolerance in *S. nigrum* vary during plant development – an observation that may be important to improve the efficiency of phytoremediation approaches. Furthermore, the data suggest the involvement of specific organic acids in this response.

4.1 INTRODUCTION

Our environment is increasingly contaminated with toxic organic and inorganic compounds resulting from anthropogenic activities, adding to the natural inputs. The classic physico-chemical environmental remediation methods are expensive and invasive (Prasad and Freitas 1999; Arthur *et al.* 2005) and research has been directed at developing economic and environmentally friendly remediation methods. The use of the natural capability of plants to remove, convert or sequester hazardous substances from the environment has emerged as a promising low-cost remediation technique known as phytoremediation. However, much has yet to be clarified about plant mechanisms of tolerance and accumulation of contaminants.

Zinc is one of the most important environmental contaminants (Raskin, Smith, and Salt 1997) and by 2002 it was estimated that 1,350,000 t of Zn had been released into the environment (Singh *et al.* 2003). Anthropogenic sources of Zn in the environment, such as mining activities, fuel combustion, agricultural activities, among others, largely surpass natural inputs (Broadley *et al.* 2007). Although Zn is an essential micronutrient for plant growth, excess Zn has detrimental consequences on plant physiology and development, affecting mineral absorption, antioxidant defences and photosynthesis, among other important metabolic processes (Atici, Agar, and Battal 2005; Khudsar *et al.* 2008; Wang *et al.* 2009; Xu *et al.* 2010; Kabata-Pendias 2011; Sagardoy *et al.* 2011). Within the large diversity of plants acknowledged for their potential in phytoremediation (Prasad and Freitas 2003; Broadley *et al.* 2007), it has been shown that *Solanum nigrum* plants are capable of accumulating high levels of cadmium and Zn (Wei *et al.* 2005; Marques *et al.* 2007, 2008). This feature, together with their vast distribution (Edmonds and Chweya 1997) and interspecific competitiveness (Chao *et al.* 2005), make this species a promising model for phytoremediation.

Beyond the characterization of the phytoremediation potential of a given plant, a major challenge in recent studies has been to understand the main physiological processes and metabolites engaged in metal tolerance and accumulation. These studies emphasized the role of numerous metabolites such as organic acids, histidine, phytochelatins, glutathione, metallothioneins and salicylic acid in several mechanisms, either directly as ligands or indirectly as mediators of stress response (Callahan *et al.* 2006; Haydon and Cobbett 2007; Horvath, Szalai, and Janda 2007). Upon exposure to metals, plants synthesize specific amino acids and peptides (Sharma and Dietz 2006),

and organic acid synthesis, accumulation, transport and exudation are increased by environmental stress (Lopez-Bucio *et al.* 2000). The chelation of Zn with organic acids is favoured by the pH of the vacuole and the xylem, indicating that these metabolites may be involved in sequestration in specific cell compartments and also in long-distance transport (Salt *et al.* 1999). Higher constitutive concentrations of organic acids were reported for *S. nigrum* compared to *Solanum torvum* plants, which are characterized as low Cd accumulators (Xu *et al.* 2012). Furthermore, the positive correlation found for Cd accumulation and acetic and citric acids in *S. nigrum* (Sun, Zhou, and Jin 2006) and the increase in organic acid exudation in response to Cd treatment (Bao, Sun, and Sun 2011), suggests a role for organic acids in the tolerance and accumulation of heavy metals in this plant.

The concentration of organic acids varies with plant age and tissues (Lopez-Bucio *et al.* 2000) which, as mentioned above, may have important effects on plant tolerance and accumulation of metals and consequently on phytoremediation efficiency. Most of the phytoremediation studies address tolerance and accumulation of contaminants in the early stages of plant development (i.e. plantlets), and surprisingly overlook the remediation behaviour at later stages of plant development, namely pre-flowering and post-flowering. However, the broad physiological changes that take place in plants at later stages of plant development are likely to affect their bioremediation fitness. In this context, the current study details the plant organ-dependent tolerance and accumulation of Zn in pre- and post-flowering *S. nigrum* plants, focusing on the changes of organic acids at these developmental stages.

4.2 Material and Methods

4.2.1 Plant material, culture conditions and biometric analysis

S. nigrum seeds, collected from the Porto district (Portugal) were supplied by the Department of Biology of the University of Porto. Seeds were disinfected and germinated on moist filter paper. Germinated seedlings were transferred to plastic containers, 4 seedlings per container, with 1.5 L Hoagland nutrient solution (Taiz and Zeiger 1998) and polypropylene granules (Battke, Schramel, and Ernst 2003). The nutrient solution was composed of 607 mg L^{-1} KNO_3; 945 mg L^{-1} $Ca(NO_3)_2.4H_2O$; 230 mg L^{-1} $NH_4H_2PO_4$; 246 mg L^{-1} $MgSO_4.7H_2O$; 3.73 mg L^{-1} KCl; 1.55 mg L^{-1} H_3BO_3; 0.338 mg L^{-1} $MnSO_4.5H_2O$; 0.576 mg L^{-1} $ZnSO_4.7H_2O$; 0.124 mg L^{-1} $CuSO_4.5H_2O$;

0.08 mg L^{-1} H_2MoO_4 and 23.5 mg L^{-1} NaFeEDTA (Taiz and Zeiger 1998). The nutrient solution was renewed frequently to avoid over-concentration due to evapotranspiration, or the deficiency of essential elements. Plants were grown in a chamber with 16 h day length, 19-22 ºC and light intensity of 70 µMol m^{-2} s^{-1}. Plants were divided in two groups according to their development. The first group, composed of plants cultivated in the aforementioned conditions for 50 days was designated as the pre-flowering stage. This group was characterized by fully developed plants undergoing vegetative growth. The plants in the second group, which corresponded to the post-flowering stage, were cultivated in the same conditions for 70 days, time at which these plants were flowering. Plants of both groups, i.e. after 50 and 70 days of growth, corresponding respectively to the pre-flowering and post-flowering stage, were challenged with Zn at 0.10 g L^{-1} (supplied as $ZnSO_4.7H_2O$). After 27 days of exposure to Zn, the plants belonging to the two groups were harvested for analysis.

At harvest, plants were carefully removed from the nutrient solution and the roots were washed with deionized water. Root and stem length were measured, the plants were separated into root, stem and leaves, which were frozen in liquid nitrogen and stored at -80 ºC until further analysis.

4.2.2 Zinc concentration in plant tissues

Zinc concentrations were determined in roots, stems and leaves. Individual plant organs were ground with a pestle and mortar in liquid nitrogen and Zn concentration was determined by the method described by Macnair and Smirnoff (1999). This method employs the colorimetric agent zincon (Merck) and is based on the formation of a coloured Zn-zincon complex which can be measured spectrophotometrically (Macnair and Smirnoff 1999). Absorbance at 606 nm was measured using a Shimadzu UVmini-1240 spectrophotometer.

4.2.3 Organic acids analysis

Plant samples of corresponding plant organs of the same treatment were pooled, lyophilized and powdered. For organic acids extraction, 0.4 g of powdered tissue was extracted by 30 min sonication and 60 min agitation at 200 rpm with 25 mL

of 0.01 N H_2SO_4. Extracts were filtered, concentrated to dryness and redissolved in 0.5 mL of 0.01 N H_2SO_4. The separation of organic acids was carried out on a system consisting of an analytical HPLC unit (Gilson) with an ion exclusion column, Nucleogel® Ion 300 OA (300x7.7mm; Macherey–Nagel, Düren, Germany), in conjunction with a column heating device set at 30 ºC, as before (Couto *et al.* 2011). Elution was carried out isocratically, at a solvent flow rate of 0.2 ml min^{-1} of 0.01 N H_2SO_4. The injection volume was 20 µL. Detection was performed with a UV detector set at 214 nm. Organic acids identification was performed by comparison of the retention times with those of authentic standards. Quantification was achieved by the absorbance recorded in the chromatograms relative to the external standards. The peaks in the chromatograms were integrated using a default baseline construction technique.

4.3.4 Statistics

Data of root and stem length, Zn and organic acid concentration were analysed for statistically significant differences (95% confidence interval) by one-way analysis of variance (ANOVA). Pairwise comparisons were performed using Tukey's Multiple Comparison Test. All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad software).

4.3 RESULTS AND DISCUSSION

4.3.1 Effect of zinc on S. nigrum

In a preliminary experiment aimed to determine the lethal Zn concentrations for *S. nigrum*, plantlets were cultivated in hydroponics with nutrient solution supplemented with Zn at 0.05 and 0.10 g L^{-1} over a period of 3 weeks (unpublished data). Plantlets subjected to a Zn concentration of 0.05 g L^{-1} showed a severe growth reduction and toxicity symptoms such as leaf necrosis resulting from Zn exposure (Fig. 4.1, A and B). Furthermore, it was shown that a Zn concentration of 0.10 g L^{-1} was lethal to plantlets (Fig. 4.1 C).

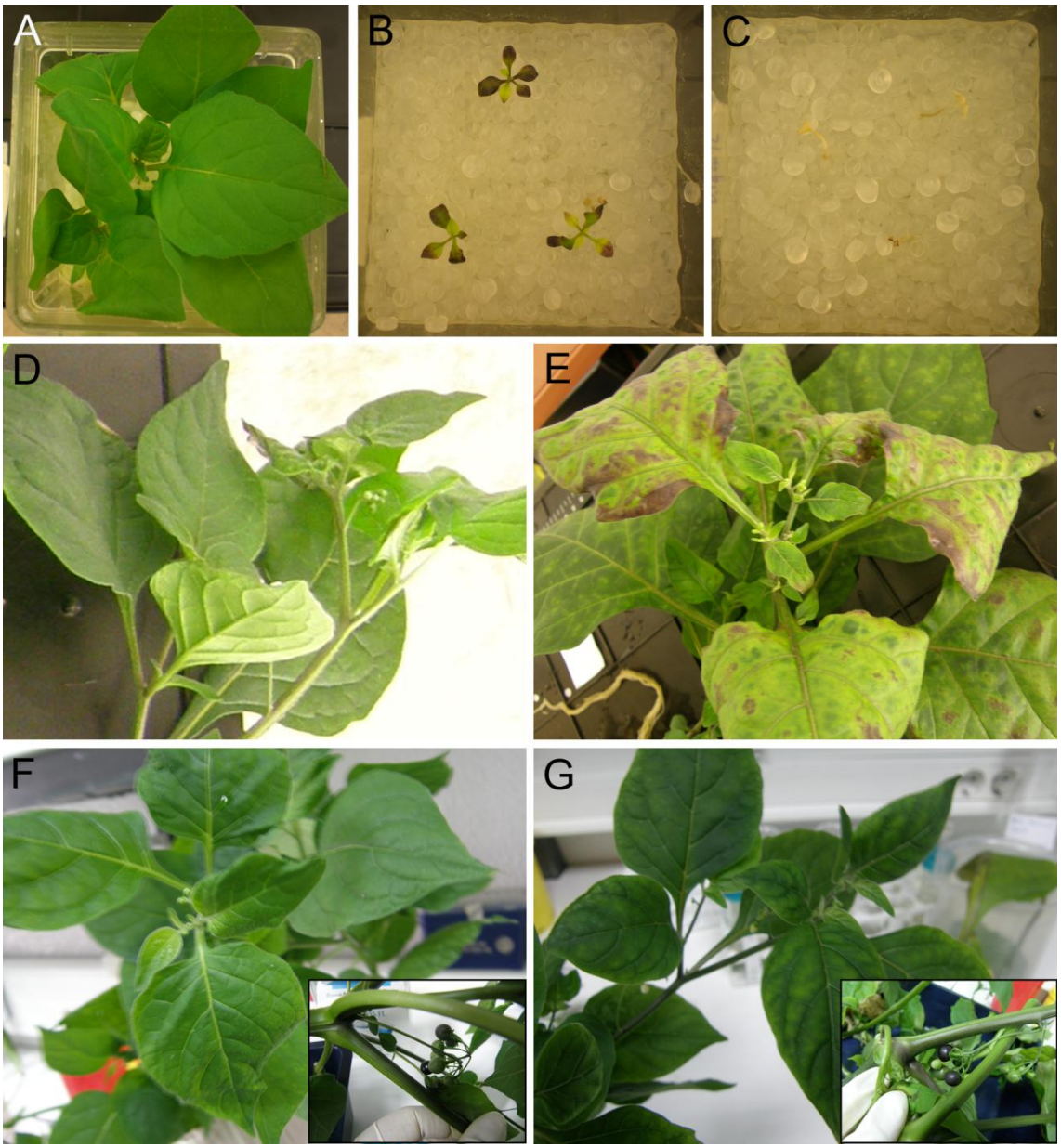

Fig. 4.1 A, B and C) *Solanum nigrum* plants cultivated during 20 days in A) Control nutrient solution. B) Nutrient solution supplemented with zinc at 0.05 g L^{-1}. C) Nutrient solution supplemented with zinc at 0.10 g L^{-1}. D-G) Plants cultivated with zinc at 0.10 g L^{-1} and control nutrient solution during vegetative and flowering growth stages. D) Pre-flowering control plant. E) Pre-flowering plant challenged with zinc at 0.10 g L^{-1}. F) Post-flowering control plants, bottom right showing a detail of fruits. G) Post-flowering plants challenged with zinc at 0.10 g L^{-1}, bottom right showing a detail of fruits.

In order to assess the behaviour of fully developed *S. nigrum* plants to this plantlet-lethal concentration, pre-flowering, i.e. mature plants undergoing vegetative growth, and post-flowering plants were challenged with nutrient solutions containing Zn at 0.10 g L^{-1}. Interestingly, it was observed that in both growth stages, exposure to this Zn concentration, over a period of 27 days, was not lethal, nevertheless, distinct effects

on plant development were observed (Fig. 4.1, D-G). Root and stem length were used as parameters to assess tolerance to Zn of the pre- and post-flowering *S. nigrum* plants (Fig. 4.2). Although Zn at a concentration of 0.10 g L^{-1} was not lethal to pre-flowering plants, shoot length reduction and necrotic lesions on the leaves were observed indicating that plant growth was affected (Fig. 4.1, D-E and 4.2, B). On the contrary, no reduction of root or stem length was observed in plants treated with Zn at the post-flowering stage (Fig. 4.2, B). These biometric data, together with the lack of toxicity symptoms showed that plants at this stage are tolerant to otherwise lethal Zn concentrations (Fig. 4.1, F-G). In addition, at the end of the 27 day period, both control and Zn treated post-flowering plants had developed fruits indicating that in these plants sexual reproduction was not impaired (Fig. 4.1, F-G).

4.3.2 Variation of zinc accumulation and tolerance in pre- and post-flowering S. nigrum plants

Zinc accumulation was highest in the roots of both pre- and post-flowering Zn treated plants, followed by the stems and leaves (Fig. 4.3). The concentration found in the roots of pre- and post-flowering plants was similar, showing no significant differences, 1033±369 mg kg^{-1} and 910±273 mg kg^{-1} fresh weight (f.w.), respectively. However, significant differences between the two growth stages were observed in Zn accumulated in stems and leaves. In pre-flowering plants, Zn accumulation of 254±66.8 and 69.6±21.0 mg kg^{-1} f.w., observed in the stems and leaves respectively, was several fold higher than the Zn measured in these plant organs in post-flowering plants (58.4±24.1 and 10.9±7.81 mg kg^{-1} f.w. respectively) (Fig. 4.3). A previous study addressing Cd and Zn accumulation during specific stages of *Brassica juncea* plant growth and development did not find differences between the Zn accumulation in the root during vegetative growth, i.e. pre-flowering, and flowering (Sankaran and Ebbs 2008). Post-flowering *S. nigrum* plants accumulated significantly less Zn in the stems and leaves when compared to pre-flowering plants, indicating a reduction of Zn translocation during this growth period which may have a role in the increased tolerance of post-flowering plants. It has been shown that *S. nigrum* plants, cultivated with Cd from the plantlet stage, had accumulated in the shoots at flowering 87.5% of the total Cd accumulated by the plants at the seed-maturity stage, which suggests a post-flowering decrease in metal translocation (Wei, Zhou, and Koval 2006). Interestingly, *Brassica napus* plants when exposed to Zn up to flowering and maturity stages, i.e. post-flowering, were found to translocate less Zn to the shoots at post-

flowering than at flowering (Rossi, Figliolia, and Socciarelli 2004). In fact, while at flowering of *B. napus* the highest Zn concentrations were recorded in the shoot*;* at post-flowering the highest accumulation was detected in the root (Rossi *et al.* 2004). Distinct responses were also observed for the stems and leaves of *B. juncea,* in which the highest Zn and Cd concentrations in the stems were registered during seed set stage, while the highest leaf concentrations of these elements were obtained during vegetative growth (Sankaran and Ebbs 2008). In *Arabidopsis thaliana* leaf concentrations of Zn and other elements decreased with plant development (Waters and Grusak 2008).

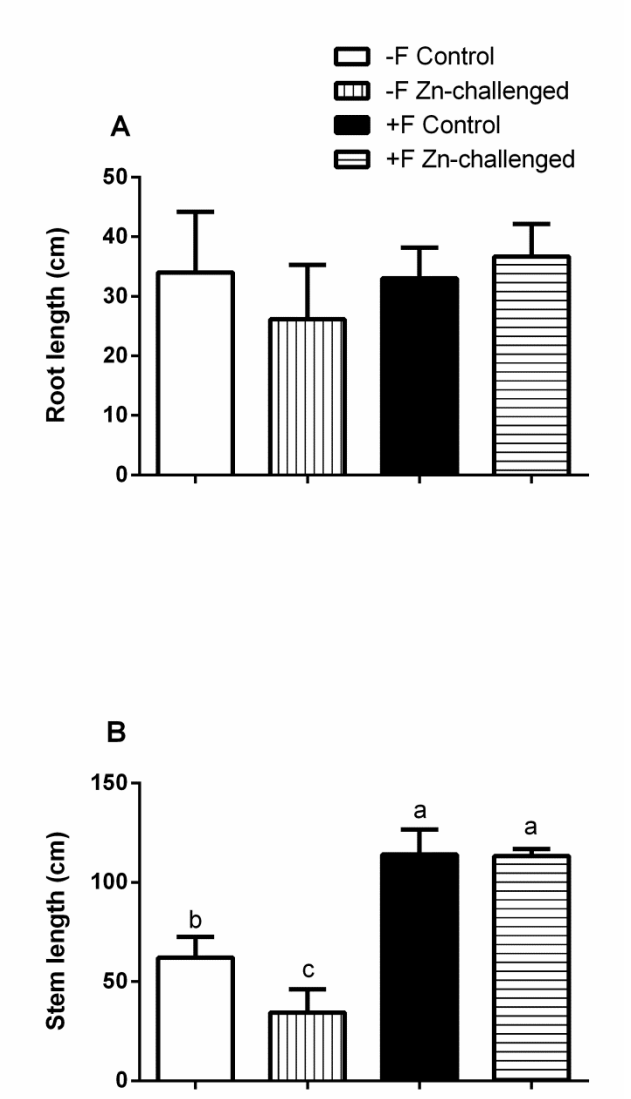

Fig. 4.2 - Biometric analysis of pre-flowering (-F) and post-flowering (+F) *S. nigrum* plants. A) Root length. B) Stem length. Significant differences are indicated by different letters at $P < 0.05$ level by Tukey's Multiple Comparisson Test. Bars represent Standard Deviation.

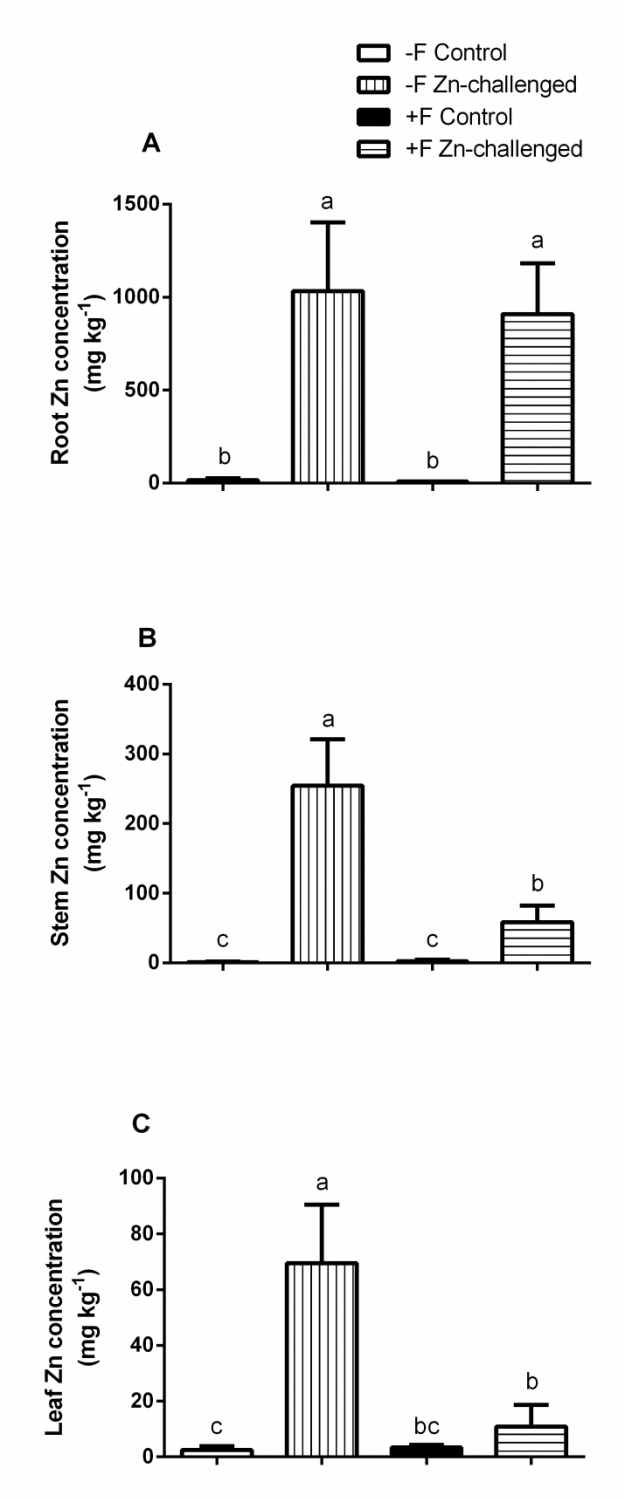

Fig. 4.3 - Zinc concentration in pre-flowering (-F) and post-flowering (+F) *S. nigrum* plants. A) Roots. B) Stems. C) Leaves. Significant differences are indicated by different letters at $P < 0.05$ level by Tukey's Multiple Comparisson Test. Bars represent Standard Deviation.

Important metabolic changes detected in *Cajanus cajan* plants, namely in photosynthetic rate, stomatal conductance, intercellular carbon dioxide and soluble protein content, were found to be down-regulated in flowering and post-flowering stages, although the effect of Zn on the metabolic pattern observed was not conclusive (Khudsar *et al.* 2008). Also, leaf accumulation of Cd in *B. juncea* was reduced upon treatment with abscisic acid, resulting in stomatal closure, indicating that Cd transport is driven by transpiration (Salt *et al.* 1995). This suggests that less Zn is transported to the leaves with reduced transpiration rates at the post-flowering stage. Taken together these data suggest that translocation and accumulation of Zn change during plant growth and development, with flowering revealing the most dramatic shifts which may explain the higher tolerance of post-flowering *S. nigrum* plants observed in the present study.

4.3.3 Organic acids response to zinc in pre- and post-flowering S. nigrum plants

We were able to identify citric, malic, shikimic and fumaric acids in *S. nigrum* in pre- and post-flowering plants where citric and malic acids, indicated in many studies to be involved in metal tolerance and accumulation (Tolra, Poschenrieder, and Barcelo 1996; Haydon and Cobbett 2007; Xu *et al.* 2012), were the most abundant. In general, the levels of all but citric acid, where slight increases were observed, were maintained or down-regulated in all organs of post-flowering control plants when compared to pre-flowering control plants.

Three of the identified compounds, namely citric, malic and fumaric acids, are intermediate metabolites of the tricarboxylic acid (TCA) cycle, which is a major hub of primary metabolism. The involvement of housekeeping metabolic pathways in tolerance and accumulation of metals has been detailed by several studies. For instance, Cd treatment in *Populus tremula* decreases the expression of proteins involved in primary metabolism, including glycolysis and the TCA cycle (Kieffer *et al.* 2009), while in the roots and leaves of *Lycopersicon esculentum* it elicits an increase in the activity of various enzymes involved in the TCA cycle (Lopez-Millan *et al.* 2009). An activation of the TCA cycle may provide energy and reducing power necessary in heavy metal containing cells (Hossain and Komatsu 2012). In fact, while photosynthetic rates were reported to decrease in Zn treated *Beta vulgaris,* respiration was increased

and it was suggested that root carboxylates would be exported to the leaves to be used as respiratory substrates (Sagardoy *et al.* 2010; Sagardoy *et al.* 2011).

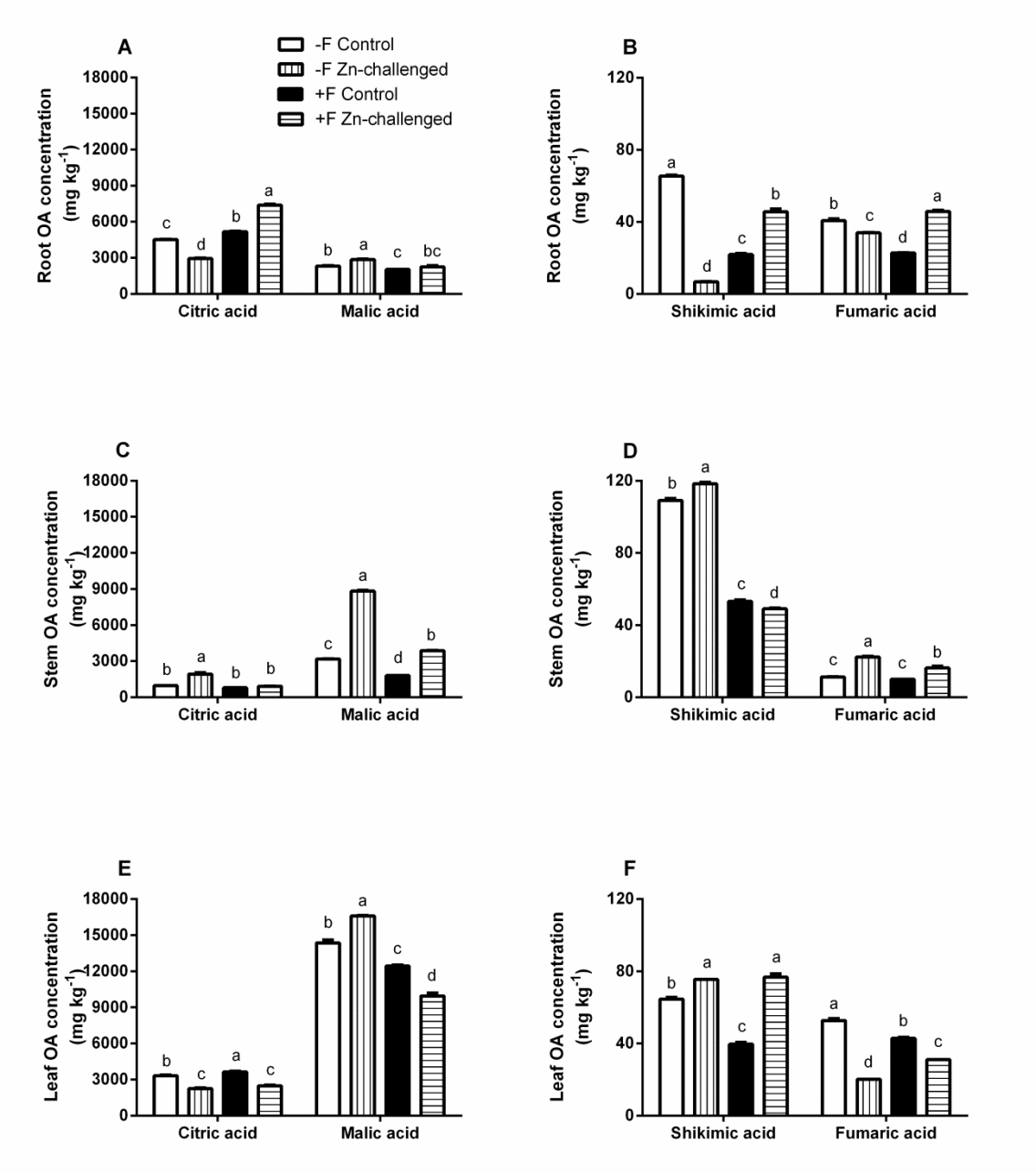

Fig. 4.4 - Organic acid (OA) concentration in pre-flowering (-F) and post-flowering (+F) *S. nigrum* plants. A and B) Roots. C and D) Stems. E and F) Leaves. Significant differences within the data for each organic acid and organ are indicated by different letters at $P < 0.05$ level by Tukey's Multiple Comparisson Test. Bars represent Standard Deviation.

The concentrations of the identified organic acids varied between plant organs, growth stage and in response to Zn treatment. In the roots, citric acid was the most abundant followed by malic, fumaric and shikimic acids (Fig. 4.4, A-B). In pre-flowering plants Zn treatment resulted in a decrease of all organic acids with the exception of malic acid where concentrations of 2853±74.2 and 2314±58.8 mg kg^{-1} dry weight (d.w.) were obtained for Zn treated and control plants, respectively (Fig. 4.4, A-B). Contrary to this pattern, in post-flowering plants the concentration of citric, shikimic and fumaric acids was increased in the roots of Zn treated plants, reaching 7385±135, 45.6±2.86 and 45.7±1.48 mg kg^{-1} d.w. respectively, in comparison with the controls where concentrations of 5177±54.3, 21.9±1.24 and 22.8±8.02x10^{-2} mg kg^{-1} d.w., were measured for these organic acids. Interestingly, although the Zn concentration in pre- and post-flowering plant roots were similar, citric and shikimic acids presented increases of 2.5 and 6.7 fold, respectively, in post-flowering plants as compared to pre-flowering plants.

Increases in citric acid upon Zn exposure have been observed in other plants, namely in the roots of *Beta vulgaris* (Sagardoy *et al.* 2011) and in the Zn hyperaccumulator *Arabidopsis halleri* (Zhao *et al.* 2000). In a comparative study of Cd accumulation of high and low Cd accumulator *Solanum* species, the hyperaccumulator *S. nigrum* was shown to possess higher constitutive concentrations of malic and citric acids and responded to Cd treatment with an increase in citric acid concentration while no significant differences were observed for *S. torvum* (Xu *et al.* 2012).

Shikimic acid, through the shikimate pathway, is a precursor of several important metabolites, namely flavonoids, stress induced phenylpropanoids, metal chelators such as protocatechuic acid, and salicylic acid, a relevant signal molecule involved in biotic and abiotic stress response known to be upregulated in Cd treated plants and lignin, a component of the cell wall (Dixon and Paiva 1995; Rice-Evans, Miller, and Paganga 1996; Diaz, Barcelo, and DeCaceres 1997; Rodriguez-Serrano *et al.* 2006; Horvath *et al.* 2007; Kovacik *et al.* 2009; Vogt 2010; Maeda and Dudareva 2012). The cell wall is indicated as one of the sinks for metals accumulated in plants and the involvement of shikimic acid as a precursor of cell wall constituents may explain its differential mobilization in Zn treated plants (Salt *et al.* 1999; Kramer *et al.* 2000; Callahan *et al.* 2006; Marques *et al.* 2007; Ahsan, Nakamura, and Komatsu 2012).

In the stems of pre-flowering plants, Zn treatment resulted in an increase of the identified organic acids: a two fold increase in citric (1930±208 mg kg^{-1} d.w.) and fumaric acids (22.4±0.691 mg kg^{-1} d.w.), 2.8 fold increase in malic acid (8834±115 mg kg^{-1} d.w.) and also a small, yet statistically significant increase in shikimic acid (118±1.77 mg kg^{-1} d.w.) (Fig. 4, C- D). The response in post-flowering plants was less pronounced for malic and fumaric acids, which experienced increases to 3879±32.6 and 16.2±1.94 mg kg^{-1} d.w. respectively, and there was no significant increase in citric acid (Fig. 4 C- D).This correlates with a lower Zn concentration in the stems of post-flowering plants.

Organic acids are known to be transported through the plant with the transpiration stream (Lopez-Bucio *et al.* 2000) and analyses of xylem sap have found Zn to be coordinated with organic acids (Salt *et al.* 1999; Lu *et al.* 2013). In *S. nigrum,* exogenous citrate addition to the roots lead to an increase of Cd in plant leaves and simultaneously to a decrease in root Cd concentration (Xu *et al.* 2012). Furthermore, it was shown that exogenous citric acid did not increase Cd influx into the root, suggesting that citric acid is involved in root-to-shoot Cd transport (Xu *et al.* 2012). Therefore, it might be hypothesised that the differences in citric acid content observed between pre- and post-flowering *S. nigrum* plant stems may be linked to the reduction of Zn translocation and that citrate, produced in the roots of pre-flowering plants, may be involved in the translocation of Zn to the stems where higher levels of citrate and malate correlate with higher Zn accumulation. It is noteworthy that our data show that malic acid consistently increased in all organs in the pre-flowering stage upon Zn treatment and the highest increases were observed in the stems. Malic acid was proposed to bind Zn in the cytoplasm and transport it across the tonoplast to the vacuole, where the complex would dissociate and Zn would be chelated by stronger ligands, while malate is transported back to the cytosol, which has been called the Zn-malate shuttle hypothesis (Broadley *et al.* 2007).

In the leaves, malic acid was the most abundant, followed by citric, shikimic and fumaric acids. An increase was observed for malic and shikimic acids in the leaves of pre-flowering Zn challenged plants. Malate was found to form complexes with Zn in the leaves of Zn hyperaccumulator *A. halleri* (Sarret *et al.* 2002; Sarret *et al.* 2009) and increases of malate were also observed in *B. vulgaris* when treated with 50 and 100µM Zn (Sagardoy *et al.* 2011). At flowering, only shikimic acid showed an increase in treated plants when compared to control, 76.8±3.28 and 39.6±1.75 mg kg^{-1} d.w. respectively (Fig. 4 E- F). The organic acids produced in the leaves may be exported to

the roots through the phloem (Lopez-Bucio *et al.* 2000) which may explain the decrease of organic acids observed in the leaves of Zn treated plants.

Shikimic acid was the only organic acid to increase significantly in the leaves of Zn treated post-flowering plants, which appears to correlate with the stress response to Zn and to the higher tolerance observed at this developmental stage. As referred to earlier, this organic acid is a precursor to several important metabolites which can act as metal chelators or alternatively mediate a stress-related response through salicylic acid (Horvath *et al.* 2007; Kovacik *et al.* 2009). Such hypotheses are supported by recent studies on other plant species (Diaz *et al.* 2001; Kovacik *et al.* 2009; Popova *et al.* 2009; Fuhrs *et al.* 2012).

Metal accumulation and tolerance are complex and clearly dependent on various mechanisms. We have shown that Zn tolerance and accumulation in *S. nigrum* vary during plant development, which is important for the improvement of phytoremediation strategies. In fact, this knowledge should allow to identify the plant growth stages more suitable to implement a broad range of phytoremediation technological solutions namely by increasing metal bioavailability as recently reviewed (Samardjieva *et al.* 2011). Post-flowering plants were more tolerant than pre-flowering plants to Zn concentrations previously shown to be lethal to *S. nigrum* plantlets. Coupled to this variation in tolerance, Zn accumulation, although similar in the roots of pre- and post-flowering plants, decreased in post-flowering stems and leaves. Furthermore, organic acids concentrations also varied between plant organs and developmental stages, indicating that they play a role and have different functions in *S. nigrum* tolerance and accumulation of Zn. Organic acids may be involved in these processes by participating in Zn root-to-shoot transport, subcellular sequestration and also in the mitigation of the toxic effects of Zn on plant metabolism, by providing metabolites for respiration. Further studies to detail Zn ultra-structural localization and also to characterize Zn effect on primary and secondary metabolism may help to further elucidate the mechanisms involved.

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CHAPTER V – ROOT PROTEOMIC PROFILE OF ZN-TREATED *Solanum nigrum* L.

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ABSTRACT

Plant fitness for phytoremediation is a result of the activation of several mechanisms that might be assessed by the differential expression of proteins. In this regard a proteomic analysis using 2-DE with MALDI-TOF/TOF mass spectrometry was carried out to determine differentially expressed proteins in the roots of Zn challenged *Solanum nigrum* plants. Pre- and post-flowering *S. nigrum* plants were exposed during 27 days to Zn at 0.10 g L^{-1}, a concentration lethal to plantlets but tolerated at these developmental stages. *Solanum nigrum* plants responded to Zn treatment with the induction and/or up-regulation of 19 protein spots in the root of which 11 were identified. The identified proteins were grouped into several categories according to their biological function and suggest that Zn response in *S. nigrum* roots integrates several metabolic pathways. Several of the up-regulated proteins were engaged in energy metabolism, namely enolase, malic enzyme and alcohol dehydrogenase, corroborating reports that metal tolerance and accumulation is an energy requiring process. Other proteins identified belong to stress response and proteolysis, suggesting a highly distressing plant response to Zn tolerance. Furthermore, the identification of an alpha-L-arabinofuranosidase, a protein involved in cell wall modification, highlights the involvement of the cell wall in tolerance and accumulation of metals in plants.

Altogether these data should contribute to further unveil the intricate network of plant physiological mechanisms primarily recruited in Zn treated *S. nigrum* plants.

5.1 INTRODUCTION

Phytoremediation, defined by Glick (2003) as the use of the natural capability of plants to remove, destroy or sequester hazardous substances from the environment, has emerged as a viable alternative to classical remediation methods. Regardless of the prospective of phytoremediation, much has still to be clarified about the mechanisms governing plant tolerance and accumulation of contaminants. Zinc is an important environmental contaminant and, although it is essential for plant growth and development, when in excess it is known to produce toxic effects (Jones 2003; Broadley *et al.* 2007).

Currently, several plants such as *Thlaspi caerulescens* and *Arabidopsis halleri* are known to tolerate and accumulate high Zn concentrations (Assuncao, Schat, and Aarts 2003; Broadley *et al.* 2007). *Solanum nigrum* was also reported to accumulate Zn and also cadmium (Marques *et al.* 2006; Wei, Zhou, and Koval 2006). Recently we showed that Zn tolerance and accumulation in *S. nigrum* are growth dependent and this may be a valuable tool in the development of phytoremediation strategies (Samardjieva *et al.* 2011; Samardjieva *et al.* 2014a). Although tolerance was increased in post-flowering relative to pre-flowering plants, Zn accumulated to similar levels in the roots (Samardjieva *et al.* 2014a). Moreover, it was reported that several organic acids may be involved in this process (Samardjieva *et al.* 2014a). The production of carboxylic acids in response to metal treatment has been indicated by other authors, however, it is known that tolerance and accumulation of metals are a complex phenomenon, dependent on several different mechanisms which most likely vary between plants and metal treatments (Haydon and Cobbett 2007; Memon and Schroder 2009; Rascio and Navari-Izzo 2011). Mechanisms of acclimation to abiotic stress are reflected in changes in the proteome and reports of differential expression proteomics in response to several types of abiotic stress factors such as low and high temperature, excessive metal concentrations, among others, have been reviewed by Kosová *et al.* (2011). The response of the proteome to metals has been carefully reviewed by Hossain *et al.* (2012) and Visioli and Marmiroli (2013). Systematization of the reports on proteomic responses to metals have allowed to group differentially expressed proteins into several classes, namely, energy and carbohydrate metabolism; cellular metabolism; stress and antioxidant response; defense; regulation; metal chelators and transporters (Visioli and Marmiroli 2013). Proteomics, the systematic analysis of expressed proteins has emerged as a powerful tool for the understanding of metal tolerance and accumulation (Ahsan *et al.* 2007). However, it is also recognized

that the involvement of differentially expressed proteins metal tolerance and accumulation is still insufficient (Visioli and Marmiroli 2013).

The plant roots are the first organ to come into contact with the metal and the roots of Zn challenged *S. nigrum* plants were shown to be the organ with the highest Zn concentration (Roth, von Roepenack-Lahaye, and Clemens 2006; Samardjieva *et al.* 2014a). Previously we have shown that *S. nigrum* tolerance to Zn is growth dependent and have suggested the involvement of several organic acids in this response (Samardjieva *et al.* 2014a). In order to further elucidate *S. nigrum* response to Zn and gain insight into the metabolic changes elicited by Zn exposure, we addressed the changes in the root proteome at two plant developmental stages of *S. nigrum* plants. Differentially expressed proteins were determined by two-dimensional electrophoresis and MALDI-TOF/TOF-MS. To our knowledge, this is the first study to our knowledge, that addresses root proteome changes in *S. nigrum* under Zn treatment. These results contribute to elucidate the complex protein networks and metabolic pathways primarily involved in cellular detoxification and tolerance against heavy metal toxicity and provide data on plant proteome changes due to environmental stimuli.

5.2 Materials and Methods

5.2.1 Plant material, culture conditions and biometric analysis

S. nigrum seeds, collected from the Porto district (Portugal) were supplied by the Department of Biology of the University of Porto. Seeds were surface sterilized and germinated on moist filter paper. Seedlings with fully expanded cotyledonary leaves were transferred to plastic containers with Hoagland nutrient solution and polypropylene granules (Taiz and Zeiger 1998; Battke, Schramel, and Ernst 2003). Plant culture conditions were maintained as detailed previously (Samardjieva *et al.* 2014a). Plants were divided in two groups. One group was cultured in the aforementioned conditions during 50 days, corresponding to pre-flowering stage of development. At this time point the nutrient solution of half of the plants was replaced with a nutrient solution supplemented with Zn at 0.10 g L^{-1} (supplied as $ZnSO_4.7H_2O$). These plants were harvested after 27 days. The second group was cultivated in control conditions until 70 days, corresponding to the onset of flowering, i. e. the post-flowering stage, when the nutrient solution of half of the plants was replaced with a

nutrient solution supplemented with Zn at 0.10 g L^{-1}. The plants of group two were also harvested after 27 days. At harvest, plants were collected and the roots were washed with deionized water. Roots were frozen in liquid nitrogen and stored at -80 ºC until further analysis.

5.2.2 Two-dimensional electrophoresis

5.2.2.1 Protein extraction

Root samples of the same treatment were pooled and ground in liquid nitrogen. Protein extraction was performed essentially as described in Giavalisco *et al*. (2003). Frozen plant tissue was ground to a fine powder in liquid nitrogen and with 0.125 parts (v/w) of protease inhibitor mixture I (0.5 tablet/ml Complete™, 100 mM KCl, 20% glycerol v/v in 50 mM Tris pH 7.1) and 0.05 parts (v/w) of protease inhibitor mixture II (1mM pepstatin A and 1.4 µM phenylmethylsulfonylfluoride in ethanol). The homogenate was centrifuged for 90 min at 16100 x *g* at 4 ºC. The resulting supernatant was frozen in liquid nitrogen and stored at -80 ºC. The pellet resulting from the centrifugation was further ground in liquid nitrogen with 0.125 parts (v/w) of protease inhibitor mixture III (0.5 tablet/ml Complete™, 200 mM KCl, 20% v/v glycerol in 0.1 M K-phosphate buffer pH 7.1), 1 part (v/w) of 0.1 M K-phosphate buffer, pH 7.1 (containing 0.2 M KCl, 2 mM $MgSO_4$, 4% CHAPS w/v and 20% v/v glycerol) and 2% w/w of amidosulfobetaine 14 (ASB 14). After 0.025 parts (v/w) of DNase was added, the mixture was stirred for 45 min at 4 ºC. Following the addition of 23% v/w of a solution containing 7 M urea and 2 M thiourea the mixture was further stirred for 45 min at room temperature. The mixture was then centrifuged for 90 min at 16100 x *g* at 4 ºC. The resulting supernatant was pooled with the one resulting from the previous centrifugation, frozen in liquid nitrogen and stored at -80 ºC.

5.2.2.2 Protein preparation for two-dimensional electrophoresis

Protein extracts were treated with a 2-DE Clean-up kit (GE Healthcare) and ressuspended in rehydration buffer containing 7 M urea, 2 M thiourea, and 4 % w/v CHAPS. Protein content was determined with a protein quantification kit, 2-DE Quant kit (GE Healthcare), according to the manufacturers' instructions.

5.2.2.3 2-D gel electrophoresis

For isoelectric focusing, 200 µg of protein in rehydration buffer (7 M urea, 2 M thiourea, 4 % w/v CHAPS, 40 mM DL-dithiothreitol (DTT), 0.5 % v/v IPG buffer (pH 4-7) and 0.002 % w/v bromophenol blue) were applied for overnight in-gel rehydration of 13 cm, non-linear IPG strips, pH 4-7 (GE Healthcare). Isoelectric focusing was performed on a PROTEAN IEF Cell (Bio-Rad) with an initial voltage of 50 V for 1 h, gradient up to 500 V for 1 h, a step of 500 V for 3 h, gradient up to 8000 V during 2.5 h and a final step of 8000 V until 80000 vh. Current was limited to 50 µA/gel and temperature was maintained constant at 20 ºC. Following isoelectric focusing, the IEF strips were equilibrated by incubation with 1 % w/v DTT, followed by 2.5 % w/v iodoacetamide in equilibration buffer (75 mM Tris-HCl pH 8.8, 6 M urea, 30 % v/v glycerol, 2 % w/v SDS, 0.002 % w/v bromophenol blue) for 15 min each under gentle agitation. The strips were then fitted with a layer of low gelling agarose 1% w/v on top of vertical 12.5 % SDS-polyacrylamide gels for 2^{nd} dimension, electrophoresis performed at 350 V, 25 mA/gel, limited to 3 watt/gel during the first hour and the rest of the process at 9 watt/gel.

Gels were fixed in 7 % v/v acetic acid and 40 % v/v methanol and stained with Brilliant Blue G-Colloidal Concentrate (SIGMA) overnight, under gentle agitation. Destaining was performed with a solution of 10 % v/v acetic acid and 25 % v/v methanol, for 30 s, and gels were incubated in 25 % v/v methanol during 5 h in order to clear background. Image acquisition was performed on a Molecular Imager GS800 calibrated densitometer (Bio-Rad) and analysed in triplicate by the PDQuest 2-D analysis software (Bio-Rad).

5.2.2.4 MALDI-TOF/TOF mass spectrometry

Proteins were excised from the 2-DE gels and protein identification was carried out by MALDI-TOF/TOF mass spectrometry (4700 Proteomics Analyzer, AB SCIEX) as described by Gomes *et al.* (2013). MS and MS/MS spectra were searched against the UniProt (release 2014_04) protein sequence database using the Mascot search engine (Matrix Science, U.K.) with the taxonomic selection for Green plants.

5.2.2.5 Statistics

Differences in intensity of protein spots between treatments were analysed with the PDQuest software using the Student's *t* test (95% confidence interval).

5.1 RESULTS AND DISCUSSION

Solanum nigrum tolerance and accumulation of Zn vary with plant development and pre- and post-flowering plants show an increased tolerance to plantlet lethal Zn concentrations (Samardjieva *et al.* 2014a). The increase in tolerance by post-flowering plants was accompanied by a reduction of metal accumulation in the aerial plant parts while Zn concentration in the roots of pre- and post-flowering plant was similar (Samardjieva *et al.* 2014a). In order to discern specific mechanisms of tolerance in this plant, an analysis of differentially expressed proteins was carried out in the roots, which is the organ with highest accumulation of Zn.

Changes in the protein profile of pre- and post-flowering *S. nigrum* plant roots resulting from Zn exposure were analyzed by two-dimensional electrophoresis.. The analysis focalized on induced and 4 fold up-regulated proteins revealed 19 protein spots (Figs. 5.1 and 5.2) out of which 11 were identified (Table 5.1). The identified proteins belong to functional classes known to be affected in response to metal exposure, namely, energy metabolism, stress response, proteolysis and cell wall modification.

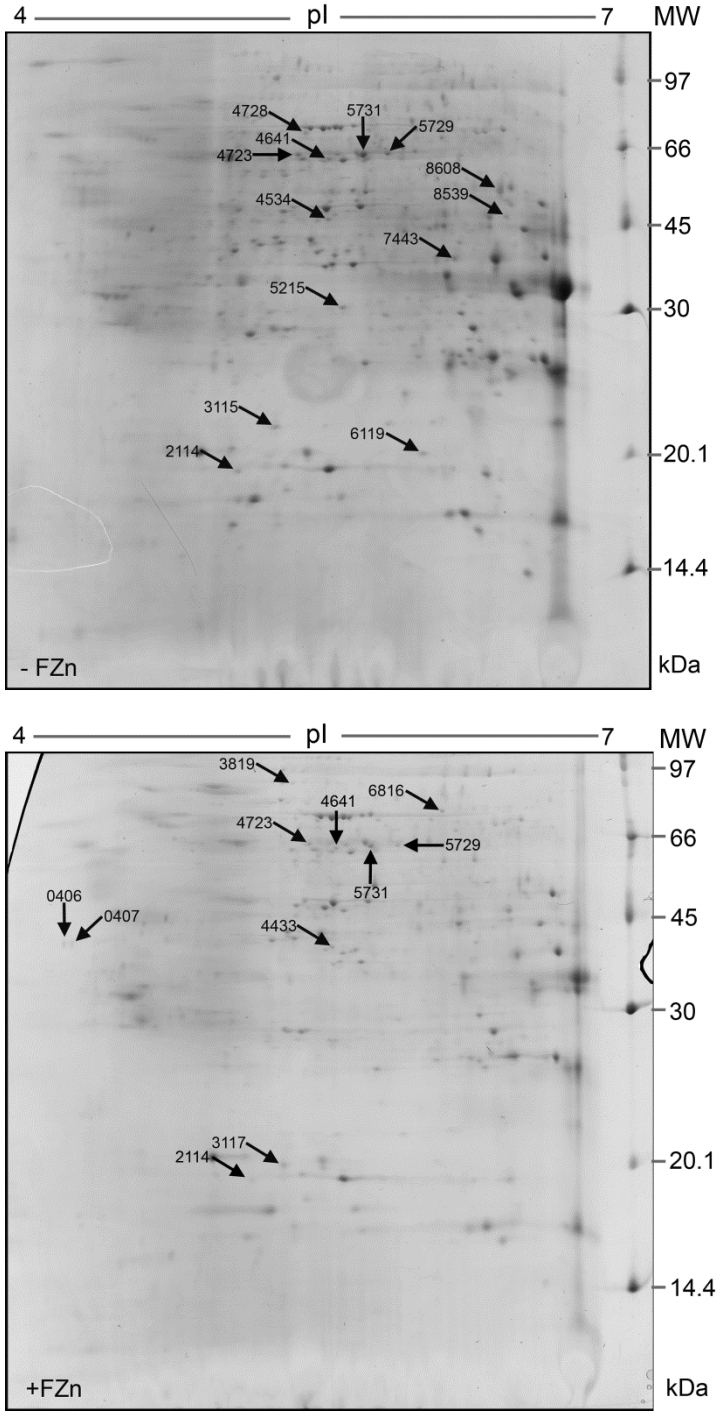

Fig. 5.1 – Representative images of the 2DE gels of Zn treated pre-flowering (-FZn) and post-flowering (+FZn) *S. nigrum* root protein extracts.

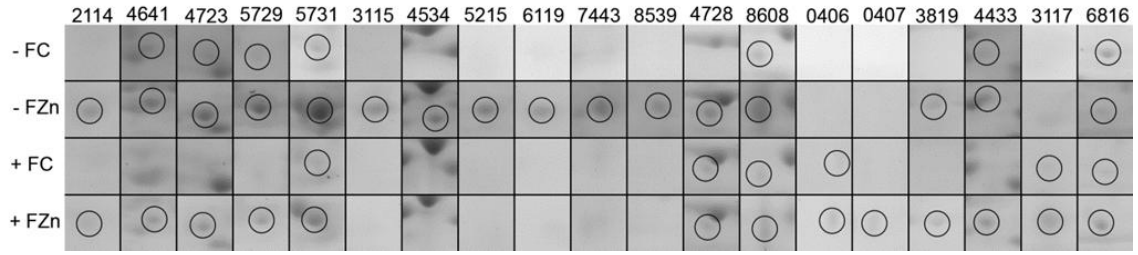

Fig. 5.2 – Details of the spots selected for identification showing an induction and/or 4 fold up-regulation between pre-flowering control (-FC) and pre-flowering Zn treated (-FZn) plants, and, post-flowering control (+FC) and post-flowering Zn treated (+FZn) *S. nigrum* plant roots.

5.1.1 Energy metabolism

A significant number of proteins found to be increased in abundance in response to metals are involved in energy and carbohydrate metabolism (Visioli and Marmiroli 2013). It was reported that important metabolic processes, such as photosynthesis and mitochondrial respiration may be modulated in order to meet the higher energy requirements of heavy metal stressed cells, and up-regulation of proteins involved in glycolysis and TCA cycle have been referred (Ahsan, Renaut, and Komatsu 2009; Hossain and Komatsu 2012; Visioli and Marmiroli 2013). In fact, in this study several spots, up-regulated or induced in Zn treated plant roots were identified as proteins involved in energy metabolism, namely enolase (spot nº5729 and 5731) (EC 4.2.1.11), malic enzyme (spot nº6816) and alcohol dehydrogenase 1 (spot nº8608) (EC 1.1.1.1).

Table 5.1 Protein identification by Peptide Mass Fingerprint and peptide sequencing by MS/MS. Response type in pre- and post-flowering *S. nigrum* roots, -FZn and +FZn respectively. Stars indicate an induction, arrows and circle indicate a 4-fold and 3-fold up-regulation relative to control, respectively.

Protein spot	Accession Id	MW / pI	Protein identification	Organism	Protein Score	Protein score C.I. %	Peptides	Response type -FZn	Response type +FZn
2114	P17642	17.4 / 5.7	Pathogenesis-related protein STH-2	*Solanum tuberosum*	70	99	9	★	★
4641	Q8GZD8	60.8 / 7.9	Leucine aminopeptidase 2, chloroplastic	*Solanum lycopersicum*	68	98	13	●	★
5729	P26300	48.0 / 5.7	Enolase	*Solanum lycopersicum*	139	100	18	⬆	★
5731	P26300	48.0 / 5.7	Enolase	*Solanum lycopersicum*	103	100	15	⬆	⬆
5215	Q3I5C3	27.5 /6.0	Cytosolic ascorbate peroxidase 2	*Solanum lycopersicum*	86	100	11	★	
7443	A2Q4Q3	69.1 / 7.6	Polyphenol oxidase	*Medicago truncatula*	74	93	4	★	
4728	Q76LU4	74.6 / 5.3	Alpha-L-arabinofuranosidase	*Solanum lycopersicum*	64	96	10	★	
8608	P14673	41.7 / 5.9	Alcohol dehydrogenase 1	*Solanum tuberosum*	99	100	14	⬆	
	P14674	41.8 / 5.9	Alcohol dehydrogenase 2	*Solanum tuberosum*	98	100	14		
3819	B9SMK4	88.1 / 5.7	Oligopeptidase A	*Ricinus communis*	102	100	8		★
4433	Q9LM08	36.6 / 5.3	Pepper esterase	*Capsicum annuum*	104	100	3		★
6816	K4C144	64.6 / 5.7	Malic enzyme	*Solanum lycopersicum*	72	99	14		⬆

Enolase catalyzes the conversion of 2-phosphoglycerate to phospho*enol*pyruvate, the penultimate step in glycolysis, and several reports have indicated an up-regulation of enolase and other glycolitic enzymes in response to metals (Van Der Straeten *et al.* 1991; Hossain and Komatsu 2012). An up-regulation of enolase was observed in suspension cell cultures of *Arabidopsis thaliana* and *Populus tremula* leaves in response to cadmium (Sarry *et al.* 2006; Kieffer *et al.* 2009). In tomato roots, cadmium at 10 µM elicited an increase in enolase, however, the enzyme was down-regulated at higher concentrations, indicating that the response is dose-dependent (Rodriguez-Celma *et al.* 2010). A variation of the response has also been reported between cultivars, for example a comparative study of a high and low cadmium accumulating cultivars of *Glycine max* showed up-regulation of enolase in the high cadmium accumulating cultivar (Hossain, Hajika, and Komatsu 2012a). Another study revealed an association of enolase and aldoase with the tonoplast and an interaction with V-ATPase subunits, suggesting an important role for enolase and aldolase in providing ATP, increasing proton-driven transport and facilitating Na^+ sequestration in the vacuole (Barkla *et al.* 2009). Phospho*enol*pyruvate is also a substrate the shikimate pathway (Herrmann and Weaver 1999). Organic acid analysis of post-flowering *S. nigrum* Zn treated roots showed an increase in the concentration of shikimic acid and a possible contribution of the increase in enolase to the shikimate pathway may also be relevant (Samardjieva *et al.* 2014a). Enolase was up-regulated

and induced (spot nº 5729) in pre- and post-flowering roots, respectively, and up-regulated (spot nº5731) in pre- and post-flowering roots, which correspond to the highest capacity of the plant to accumulate Zn, indicating that it is crucial in metal homeostasis in both growth phases.

The enzyme alcohol dehydrogenase (ADH) interconverts acetaldehyde and ethanol and in a recent review, Strommer (2011) indicates that in plants it participates in ethanol fermentation under oxygen limited conditions, aerobic fermentation and also in the production of volatile compounds that discourage predators. Furthermore, the expression of the *ADH* gene and the enzyme activity have been detected in plants cultivated with and without stress (Strommer 2011). Although the enzyme was detected in all Zn treatments in *S. nigrum*, it was up-regulated in pre-flowering plants. The activity of this enzyme in situations when normal respiration is impaired (Strommer 2011) is in line with the lower tolerance observed in *S. nigrum* Zn treated pre-flowering plants. There are also other studies suggesting that alcohol dehydrogenase 1 may respond to abiotic stress, as shown by the up-regulation of alcohol dehydrogenase 1 by low temperatures in *Zea mays* and *Oryza sativa* (Christie, Hahn, and Walbot 1991). Alcohol dehydrogenase was also shown to be up-regulated by wounding in *Zea mays* and *Lactuca sativa* (Kato-Noguchi 2001). This study further supports a role for alcohol dehydrogenase in metal stress related response.

Malic enzyme (ME) decarboxylates malate using NAD(P) yielding pyruvate, NAD(P)H and carbon dioxide, offering an alternative route for the synthesis of respiration substrates (Wedding 1989). This is advantageous as large reservoirs of carboxylic acids are available in plants and these can be mobilized for stress events characterized by a higher demand for energy (Wedding 1989). An up-regulation of NADP-ME was observed in response to cadmium in *Populus tremula,* and to arsenic in *Oryza sativa* (Kieffer *et al.* 2009; Ahsan *et al.* 2010). An induction of the expression of a gene encoding for a cytosolic NADP-ME in response to salt stress was observed in rice as well as an increase of the enzyme activity (Cheng and Long 2007). Moreover, *Arabidopsis* plants, expressing the rice *cytoNADP-ME* presented an increased tolerance to salt (Cheng and Long 2007). Although ME was found to be up-regulated in the present study, an analysis of organic acid content in Zn treated *S. nigrum* plants did not reveal an increase in malic acid in post-flowering plant roots (Samardjieva *et al.* 2014a). This might be explained by the fact that fumarate may act as an activator for ME (Wedding 1989) which is further sustained by an increase in fumaric acid observed in post-flowering *S. nigrum* plant roots (Samardjieva *et al.* 2014a). In the present study

ME was detected in all treatments, however, contrary to alcohol dehydrogenase, it was only found to be up-regulated in response to Zn in post-flowering plants.

5.1.2 Stress responsive proteins

Under abiotic stress, cells produce reactive oxygen species (ROS) such as hydrogen peroxide, which can cause oxidative stress and it is not surprising that an important group of proteins frequently up-regulated in response to metal stress are also involved in oxidative stress defense mechanisms (Ahsan *et al.* 2009; Hossain, Nouri, and Komatsu 2012b). Metals such as Zn, described as non-redox-active can displace other metals in the cell when present in excess (Pilon *et al.* 2009). Non-redox-active metals have been referred to promote indirectly the production of ROS and induce antioxidative response (Ahsan *et al.* 2009). Several enzymes may play a role in this response, for example cytosolic ascorbate peroxidase 2 (spot nº 5215), found to be induced in pre-flowering plants which were shown to be less tolerant to Zn than post-flowering plants. Ascorbate peroxidase (APX) reduces hydrogen peroxide to water and has been reported to play a role in antioxidative response to heavy metals, namely in response to cadmium treatment in *Arabis paniculata* and cadmium and copper treatment of *Matricaria chamomilla* (Ahsan *et al.* 2009; Kovacik *et al.* 2009; Zeng *et al.* 2011; Caverzan *et al.* 2012). An increase in several antioxidative enzymes, including APX, were reported for Cd treated *S. nigrum* plants (Deng *et al.* 2010; Liu *et al.* 2013). An increase in this enzyme was also observed in a high cadmium accumulating cultivar of *Glycine max* upon cadmium treatment (Hossain *et al.* 2012a). Rice *APX2* expression is also induced by other abiotic stimuli such as salt, drought and cold (Zhang *et al.* 2013).

Several spots were identified as responsive proteins to biotic and abiotic stress, namely, pathogenesis-related protein STH-2 (nº 2114), pepper esterase (nº 4433) and polyphenol oxidase (nº 7443). The pathogenesis-related (PR) protein STH-2, now PR-10a, is part of a large group of stress responsive proteins (van Loon *et al.* 1994; Ahsan *et al.* 2009; El-Banna *et al.* 2010). Group 10 are induced by biotic and abiotic stress, such as pathogens, cold, salinity, drought, heavy metals, oxidative stress or ultraviolet radiation, PR-10a protein, in particular was shown to be differentially expressed due to osmotic and salt stress in *Solanum tuberosum* suspension cultures and treatment of *Oryza sativa* with the glycoprotein elicitor CSB I (Liu and Ekramoddoullah 2006; Ahsan *et al.* 2009; Liao, Li, and Wang 2009; El-Banna *et al.* 2010). The PR-10a protein was

also found to be increased in abundance in response to copper treatment of *Oryza sativa* plants (Zhang, Lian, and Shen 2009). Contrary to other Zn up-regulated proteins mentioned before, this protein was found to be induced in response to Zn in both pre- and post-flowering plant roots.

A spot identified as a pepper esterase was induced in post-flowering plant roots. This enzyme caused a dose-dependent inhibition of appressorium formation by the fungus *Colletotrichum gloeosporioides* in *Caspicum anuum* (Kim *et al.* 2001). Esterases catalyze the formation or cleavage of ester bonds and have been reported to have activity toward xenobiotic compounds (Cummins, Burnet, and Edwards 2001; Radic and Pevalek-Kozlina 2010). These enzymes have been shown to be influenced by metals, with decreases observed in *Triticum aestivum* by treatment with cadmium, nickel and Zn and an increase by chromium (Karataglis, Symeonidis, and Moustakas 1988). On the other hand, increases of esterase activity were reported in response to treatment of *Lemna minor* with lead, cadmium, chromium, Zn, copper and mercury and in response to aluminium uptake in *Hordeum vulgare* (Mukherjee, Bhattacharyya, and Duttagupta 2004; Tamas *et al.* 2005). It may also be significant that esterases may participate in cell wall modification, namely pectin methylesterases, whose activity is enhanced by cations, among other factors (Cosgrove 2001; Micheli 2001; Krzeslowska 2011).

Polyphenol oxidases, induced by Zn treatment in pre-flowering plants, use oxygen to oxidize phenolic compounds to their corresponding quinones, namely the *o*-hydroxylation of monophenols to *o*-diphenols and the dehydrogenation of *o*-dihydroxyphenols to *o*-diquinones, and are implicated to play a role in plant defense against stress, pathogens and herbivory (Mayer 2006; Thipyapong, Stout, and Attajarusit 2007; Constabel and Barbehenn 2008). The generated quinones are highly reactive and their interaction with proteins may cause the brown pigments in damaged plant tissues or extracts (Constabel and Barbehenn 2008). Interestingly, as reviewed by Thipyapong *et al.* (2007), it has been shown with transgenic *Lycopersicon esculentum* plants with suppressed polyphenol oxidase activity (SP) and plants overexpressing polyphenol oxidase (OP), that the OP plants were more resistant to pathogens such as *Pseudomonas syringae* and also insects. Moreover, there are also reports indicating an involvement of this enzyme in response to metal stress. For example, an increase of polyphenol oxidase activity was observed in *Vallisneria natans* in response to lead treatments, in *Panax ginseng* in response to copper and in

Matricaria chamomilla in response to copper and cadmium (Ali *et al.* 2006; Kovacik *et al.* 2009; Wang *et al.* 2011).

5.1.3 Proteolysis

Two spots were identified as proteins involved in proteolysis, an oligopeptidase A (nº 3819) (EC 3.4.24.70), induced in post-flowering roots and a leucine aminopeptidase 2 (nº 4641) (EC 3.4.11.1), up-regulated and induced in pre- and post-flowering roots, respectively. Although plant proteases have been viewed chiefly as part of the housekeeping machinery of amino acid turnover in cells, important roles in defense response, as pathogens recognition have been suggested (van der Hoorn and Jones 2004). An oligopeptidase A-like protein was found to be up-regulated in *Catharathus roseus* in response to lead (Kumar, Varman, and Kumari 2011). Leucine aminopeptidases (LAPs) are exopeptidases which catalyze the hydrolysis of leucine residues from the N-terminal of proteins and peptides (Matsui, Fowler, and Walling 2006). Leucine aminopeptidases have recently been shown to have molecular chaperone activity (Scranton *et al.* 2012). Certain conditions, such as environmental stress and reactive oxygen species can result in protein aggregation. This can be prevented by molecular chaperones which facilitate the folding or refolding of misfolded proteins (Tyedmers, Mogk, and Bukau 2010). Plant LAPs are classified in two groups according to their isoelectric point, the neutral (LAP-N) and acidic (LAP-A) which possess 77% amino acid similarity (Matsui *et al.* 2006). Evidence was presented that LAP-A from *Lycopersicon esculentum* is involved in defense against herbivores and LAP-A mRNA levels were increased in *S. nigrum* after mechanical wounding (Chao *et al.* 2000; Fowler *et al.* 2009). Importantly, *S. nigrum* plants silvenced for *SnLAP-N* were more susceptible to *Manduca sexta* caterpillars than control plants, emphasizing the defensive functions of LAP in *S. nigrum* (Hartl *et al.* 2008). The LAP identified in this study belongs to the neutral group of leucine aminopeptidases and is suggested to play a role in protein turnover of vegetative and reproductive organs (Tu, Park, and Walling 2003). Under metal stress, the urgency of this turnover is expectably higher, possibly due to protein damage induced by oxidative stress. It was recently shown that loss of function of LAP2 in *Arabidopsis thaliana* resulted in reduced vegetative growth and higher sensitivity to stress, further strengthening their importance in stress response (Waditee-Sirisattha *et al.* 2011).

5.1.4 Cell wall modification

The plant cell wall, a cellular structure continuously remodeled during plant growth, is one of the main sinks for metal sequestration in plant tissues (Fulton and Cobbett 2003; Krzeslowska 2011). Besides the structural polysaccharides, the cell wall contains many proteins, implicated in cell wall turnover and biosynthesis (Kaczkowski 2003). In this study, spot nº4728, induced in pre-flowering *S. nigrum* roots was identified as an alpha-L-arabinofuranosidase. This enzyme, known to play a role in fruit softening and textural changes, hydrolyses arabinofuranosyl residues found in a number of pectic and hemicellulosic polysaccharides of the cell wall, as reviewed by Tateishi (2008). Two genes from *A. thaliana* encoding for putative alpha-L-arabinofuranosidases, *AtASD1* and *AtASD2,* showed differential tissue and developmental expression (Fulton and Cobbett 2003). While the expression of *AtASD1* coincides with developmental processes such as cell proliferation, vascular development, morphogenesis, senescence and abscission of floral organs, *AtASD2* expression was limited to the vasculature of older root tissues in seedlings, floral organs and abscission zones (Fulton and Cobbett 2003). Other reports have also suggested a role for this enzyme in vascular development, in particular in the modification of the structure of xylan during xylem vessel formation (Ichinose *et al.* 2010). The cell wall of *S. nigrum* has been observed to be a preferential site of Zn sequestration (Samardjieva, Tavares, and Pissarra 2014b) and the induction of an enzyme involved in cell wall modification, such as alpha-L-arabinofuranosidase, in Zn treated plants, further suggests that this sequestration of Zn in the cell wall is the result of a dedicated plant response.

Solanum nigrum pre- and post-flowering plant roots responded to Zn treatment with the induction and up-regulation of several proteins in the root tissues. Plants in these growth stages differed in their tolerance to Zn, higher tolerance in post-flowering plants, and in their translocation of Zn to the aerial parts, which was reduced in post-flowering plants (Samardjieva *et al.* 2014a). However, Zn concentration in the roots of these plants was similar in both growth stages which may explain the lack of a specific pattern in the differential proteomic analysis of pre- and post-flowering plants. Zinc treatment induced and up-regulated the expression of proteins involved several important cellular processes however, as has been argued by others, it is not possible to discern whether this constitutes a direct response to Zn treatment or a secondary,

generalized response (Roth *et al.* 2006). The main group of Zn responsive proteins in *S. nigrum* was involved in energy metabolism confirming reports by other authors of a higher demand for energy production in metal treated plants. A significant number of the identified proteins were involved in defense response to biotic and abiotic stress. Other proteins involved in Zn response were involved in proteolysis. Also significant was the identification of an alpha-L-arabinofuranosidase, involved in cell wall modification, highlighting the importance of the cell wall as a tolerance and accumulation mechanism to metals in plants. Overall, these results present evidence for a complex metabolic network involved in Zn response in *S. nigrum*.

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CHAPTER VI - GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Anthropogenic activities, associated with economic and population growth, are continuously depositing in the environment organic and inorganic contaminants. Although the awareness for the need and urgency to solve this problem exists, the means available are in many cases expensive and invasive, rendering the contaminated sites unavailable for further use (Prasad and Freitas 1999; Arthur *et al.* 2005; Marques, Rangel, and Castro 2009). Cost effective methods, such as phytoremediation, may become a valuable tool for the solution of this problem, however, further knowledge into the biology of plant tolerance and accumulation of contaminants is needed. The main objective of this thesis was to gain insight into the mechanisms of tolerance and accumulation of zinc in *Solanum nigrum* plants. With this goal, key areas of interest and development of phytotechnologies were identified and the response to Zn accumulation by *S. nigrum* plants was analyzed at the structural, biochemical and molecular levels. Detailed microscopy studies using autometallography, allowed a comprehensive description of the cellular compartments envolved in Zn sequestration and flux through the plant. Zinc tolerance and accumulation in *S. nigrum* showed a clear trend with plant development stages, what may prove valuable in the development of phytoremediation strategies. Moreover, biochemical analysis revealed the involvement of several organic acids in this response, and proteomic analysis revealed the up-regulation of several groups of Zn responsive proteins.

Further than providing a detailed discussion of the main scientific questions tackled throughout this doctoral project, the current chapter is intended to offer an overview of how all these findings, of which the main highlights are schematically summarized in Fig. 6.1, may converge to offer further insights into tolerance and accumulation of Zn in *S. nigrum* plants.

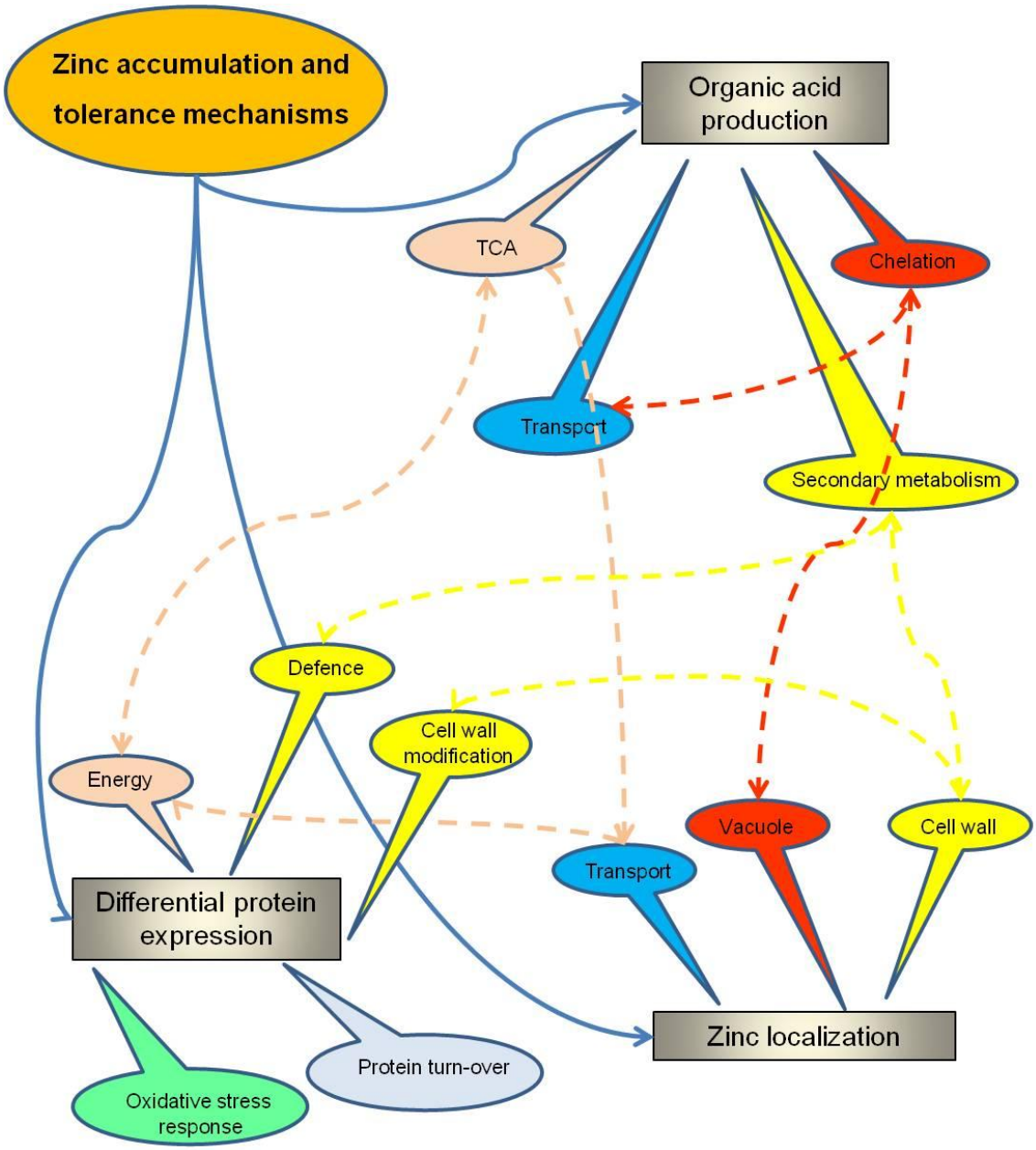

Fig. 6.1 – Tentative integrative model emphasizing the main metabolic features engaged in *S. nigrum* plant response to Zn.

A review of patents registered in the area of phytoremediation identified important subjects in which research and innovation might be instrumental for the development of new phytotechnologies (Samardjieva *et al.* 2011) (Chapter II). Clear-cut plant characteristics, such as high biomass and fast growth, are evidently essential for phytoremediation. However, these traits are inconsequential if the plants are not tolerant to the contaminants, and this is expected to be dependent on several mechanisms that are only now being uncovered. Therefore, it is not surprising that a

considerable amount of research has focused into the comprehension of tolerance and transport mechanisms. Essential metals such as Zn have detrimental effects when in excess, and their cytosolic concentrations must be tightly regulated (Martinoia *et al.* 2012). A number of mechanisms, such as sequestration in specific cellular compartments and the production of metal ligands have been shown to promote metal tolerance in plants and have been the subject of reviews (Cobbett and Goldsbrough 2002; Hall 2002; Callahan *et al.* 2006; Krzeslowska 2011; Rascio and Navari-Izzo 2011). Among these, the synthesis of phytochelatins, amino acids and organic acids have been the subject of patented claims (Samardjieva *et al.* 2011) (CHAPTER II). This is also the case for metal transporter proteins, essential for subcelular sequestration of metals as well as long distance transport, which have been the focus of literature revisions and also several patents (Colangelo and Guerinot 2006; Kramer, Talke, and Hanikenne 2007; Samardjieva *et al.* 2011) (CHAPTER II). Contributing to the numerous studies pinpointing the mechanisms of tolerance and accumulation, our results have shown that these constitute an network of responses at various levels, metabolic, physiological, morphological and molecular, and furthermore vary with plant development of *S. nigrum* (Samardjieva *et al.* 2014a; Samardjieva, Tavares, and Pissarra 2014b) (Chapters III, IV and V).

6.1 TOLERANCE AND ACCUMULATION OF ZINC IN *S. NIGRUM* ARE GROWTH DEPENDENT

Earlier reports of Zn accumulation in *S. nigrum* plants have highlighted the tolerance and accumulation potential of this plant. For example, a previous study showed that the Zn measured in the leaves, stems and roots of *S. nigrum* plants inoculated with arbuscular mycorrhyzal fungi, reached values of 1450, 3240 and 3810 mg kg^{-1} (d.w.), respectively, without toxicity symptoms (Marques *et al.* 2006). More studies, carried out in contaminated soil matrix, showed that the accumulation capacity of *S. nigrum* exposed to various Zn concentrations, changed with mycorrhyzal inoculation, manure and compost and with addition of chelating agents (Marques *et al.* 2007; Marques *et al.* 2008a; Marques *et al.* 2008b). Preliminary studies carried out by us in hydroponics to keep tight experimental settings, showed that Zn at the concentration of 0.10 g L^{-1} was lethal to *S. nigrum* plantlets, that is, Zn supplied at this concentration in the early stages of plant development caused death (Samardjieva *et*

al. 2014a) (Chapter IV). Plantlets challenged with 0.025 g L^{-1} Zn at this early developmental stages, for a period of 35 days showed a reduction in root and stem length and in biomass (Samardjieva *et al.* 2014b) (Chapter III). Extensive physiological changes occur along plant development and it has been indicated that the concentration of organic acids, indicated to play a role in metal tolerance, also varies (Lopez-Bucio *et al.* 2000). It follows that phytoremediation fitness, namely tolerance and accumulation of Zn, is likely to vary with plant development. In order to assess a possible link between *S. nigrum* plant development and Zn tolerance and accumulation, plants were challenged with Zn at two distinct development stages (Samardjieva *et al.* 2014a) (Chapter IV). The first stage contained plants undergoing vegetative development and the second contained plants at flowering, the two groups of plants referred to as pre- and post-flowering, respectively. These plants were subjected to Zn at 0.10 g L^{-1}, a concentration previously found to be lethal to plantlets. Zinc at this concentration, supplied at pre- and post flowering stages, was not lethal to the plants; moreover, post-flowering plants were more tolerant to this Zn treatment than pre-flowering plants. Regardless of the Zn concentration or the growth phase of the treatment, Zn concentration was always higher in the roots, followed by the stems and leaves (Samardjieva *et al.* 2014a; Samardjieva *et al.* 2014b) (Chapter III and IV). As would be anticipated, root Zn concentrations were higher in the 0.10 g L^{-1} treatment of pre- and post-flowering plants than in the 0.025 g L^{-1} treatment of plantlets (Samardjieva *et al.* 2014a; Samardjieva *et al.* 2014b) (Chapter III and IV). Surprisingly, higher stem and leaf concentrations were observed in plantlets subjected to 0.025 g L^{-1} of Zn than more developed plants subjected to a 4 fold higher Zn concentration (Samardjieva *et al.* 2014a; Samardjieva *et al.* 2014b) (Chapter III and IV). Moreover, lower Zn concentrations were detected in the aerial organs of post-flowering plants than in pre-flowering (Samardjieva *et al.* 2014a) (Chapter IV). This growth dependent decrease in Zn concentration in the aerial parts of the plants correlates with an increase in tolerance verified in the more developed plants. Additionally, an ascorbate peroxidase, an enzyme involved in antioxidative response by reducing hydrogen peroxide to water and referred to participate in metal stress response (Caverzan *et al.* 2012), was differentially expressed in pre-flowering plant roots. The induction of this antioxidative defense enzyme corroborates the observation of the lower tolerance of pre-flowering plants when compared to post-flowering plants. These data suggests that Zn tolerance in *S. nigrum* plants is dependent on the Zn treatment concentration, as expected, but also on Zn accumulation in the aerial parts which decreases with plant development while tolerance increases.

6.2 SEQUESTRATION IN THE CELL VACUOLE AS A MECHANISM FOR TOLERANCE AND ACCUMULATION

As mentioned beforehand, metal concentration in the cytoplasm must be tightly controlled in order to avoid toxic build-up and the vacuole, a cellular compartment which may occupy up to 90% of the cell volume, is known for its role in sequestering toxic compounds (Taiz 1992; Martinoia *et al.* 2012). A detailed histological and ultra-structural analysis of *S. nigrum* challenged at the plantlet stage with Zn at 0.025 g L^{-1} pointed to the vacuole of several cell types from different tissues as a sink for Zn (Samardjieva *et al.* 2014b) (Chapter III). Zinc deposits were detected in the vacuole or at the cytoplasm – vacuole interface, possibly associated to the tonoplast, in cells generally characterized by large vacuoles and intercellular spaces such as the root cortical parenchyma, stem cortical parenchyma, starch sheath and leaf mesophyll (Samardjieva *et al.* 2014b) (Chapter III). Particularly conspicuous Zn deposits were observed in the starch sheath which is the innermost layer of the stem cortex, characterized by numerous amiloplasts and that may develop Casparian strips, functioning as a barrier to apoplastic transport and therefore obstructing the flux of Zn through the cell wall and intercellular spaces (Kraehmer and Baur 2013; Samardjieva *et al.* 2014b)) (Chapter III). The vacuole, besides its crucial role in cell growth, is a storage compartment for many metabolites such as organic acids, sugars, proteins and secondary compounds (Taiz 1992; Martinoia *et al.* 2012). Most compounds are transported into the vacuole using the electrochemical gradient created by proton pumps (Martinoia *et al.* 2012). These pumps acidify this cell compartment relatively to the cytosol, and this characteristic of the vacuole has been indicated to favor the chelation of Zn with organic acids (Taiz 1992; Salt *et al.* 1999; Martinoia *et al.* 2012). In fact, the vacuole, where high concentrations of organic acids such as malic and citric acids are stored, has been indicated to be a sequestration site for Zn in the leaves of hyperaccumulators *Thlaspi caerulescens, Sedum alfredii* and *Potentilla griffithii* (Kupper, Zhao, and McGrath 1999; Salt *et al.* 1999; Frey *et al.* 2000; Lopez-Bucio *et al.* 2000; Li *et al.* 2006; Hu *et al.* 2009; Leitenmaier and Küpper 2013). In this work variations in citric and malic acids were observed in Zn challenged pre- and post-flowering *S. nigrum* (Samardjieva *et al.* 2014a) (Chapter IV). Moreover, malic acid, proposed to shuttle Zn into the vacuole, was observed to increase consistently in response to Zn treatment in pre-flowering plants (Broadley *et al.* 2007; Samardjieva *et al.* 2014a) (Chapter IV).

Several transporters have been implicated in the transport of Zn into the vacuole (Martinoia *et al.* 2012). The tonoplast has an inner positive membrane potential, therefore import of positive ions such as Zn^{2+}, requires active transport while their export is energetically favored (Olsen and Palmgren 2014). Therefore, the import of solutes into the vacuole may be an energy requiring process and recent reviews concerning the differential expression of proteins in response to metal treatment have highlighted the modulation of energy producing pathways (Taiz 1992; Hossain and Komatsu 2012; Martinoia *et al.* 2012; Visioli and Marmiroli 2013). In this study the analysis of differentially expressed proteins in pre- and post-flowering *S. nigrum* plant roots allowed the identification of several proteins with roles in energy metabolism, such as enolase, that were induced or up-regulated in response to Zn (Chapter V). An association of enolase and aldoase with the tonoplast and with V-ATPase subunits has been shown by Barkla *et al.* (2009), and the authors provide evidence suggesting an important role of enolase and aldolase in providing ATP, increasing proton-driven transport and facilitating Na^+ sequestration in the vacuole. All together, this indicates that the sequestration of Zn in the vacuoles of *S. nigrum* plants is likely a tolerance mechanism.

6.3 THE APOPLAST IS AN IMPORTANT SINK FOR ZINC IN *S. NIGRUM* PLANTS

The detailed analysis of Zn localization in *S. nigrum* plants showed that the apoplast is an important sequestration site for Zn, particularly in tissues characterized by intercellular spaces and large vacuoles, namely the root cortical cells, the stem medullary and cortical cells and the leaf mesophyll cells (Samardjieva *et al.* 2014b) (Chapter III). A previous study of Zn accumulation and about the contribution of mycorrhiza in *S. nigrum* plants, also showed Zn deposits in the cell walls of root cells (Marques *et al.* 2007). As was mentioned beforehand, the involvement of the cell wall in metal sequestration has been recently reviewed by Krzesloweska (2011) who indicates that several components of the cell wall posses an affinity for metals. It has been put forth that that metal binding to a group of cell wall components, the homogalacturonans (HGAs), may result in stiffening of the cell wall and an inhibition of cellular elongation, one of the main processes of plant growth (Eticha, Stass, and Horst 2005; Yang *et al.* 2008; Krzeslowska 2011). This may be a contributing factor to the stunted growth observed in Zn challenged plants. The constituent of the middle lamella, pectin, contains a HGA domain and we have observed Zn deposits in this apoplastic

compartment in *S. nigrum* (Krzeslowska 2011; Samardjieva *et al.* 2014b) (Chapter III). Another important component of the cell wall is lignin, a phenolic molecule, common in the secondary cell walls, but also found in the primary cell walls and in the middle lamella, whose components are synthesized from phenylalanine, which in turn is synthesized through the shikimate pathway which correlates with the increase in shikimic acid observed in post-flowering roots and in pre- and post-flowering leaves of Zn treated *S. nigrum* plants (Taiz and Zeiger 1998; Herrmann and Weaver 1999; Maeda and Dudareva 2012; Samardjieva *et al.* 2014a) (Chapter IV).

Cell wall lignification is indicated to be one of the responses to infection, wounding and metal treatment (Taiz and Zeiger 1998; Yang, Cheng, and Liu 2007; Ahsan, Nakamura, and Komatsu 2012). For example, an increase in lignin content and an up-regulation of proteins associated with lignin biosynthesis were observed in *Glycine max* plants upon cadmium treatment (Yang *et al.* 2007; Ahsan *et al.* 2012) The cell wall is described as a dynamic structure that undergoes remodeling during cell growth and also in response to metals (Krzeslowska 2011). Therefore, the observed induction of alpha-L-arabinofuranosidase in Zn challenged *S. nigrum* plants (Chapter V), involved in the modification of cell wall constituents, further supports the importance of the cell wall in the mechanisms of tolerance and accumulation of metals.

6.4 INSIGHTS INTO THE INVOLVEMENT OF SECONDARY METABOLISM

The shikimic acid pathway is one of the main routes for the synthesis of plant phenolics, through it carbohydrates are converted into the intermediate aromatic amino acids phenylalanine, tyrosine and tryptophan (Taiz and Zeiger 1998; Herrmann and Weaver 1999). In plants, these amino acids are used in protein synthesis but also as precursors for secondary metabolites (Herrmann 1995). In this way, shikimic acid is the precursor of important secondary metabolites such as flavonoids, stress induced phenylpropanoids and salicylic acid, among others (Dixon and Paiva 1995; Taiz and Zeiger 1998; Rodriguez-Serrano *et al.* 2006; Horvath, Szalai, and Janda 2007; Kovacik and Klejdus 2008; Kovacik *et al.* 2009a; Popova *et al.* 2009; Maeda and Dudareva 2012). Secondary metabolites, although considered waste products in the past, are now known to have high importance in defense against herbivores and pathogens and may also act as attractants for pollinators (Taiz and Zeiger 1998). Moreover, certain phenolic acids and flavonoids are indicated to possess chelator potential (Kovacik and Klejdus 2008; Kovacik *et al.* 2009a; Symonowicz and Kolanek 2012). An increase in

soluble and wall bound phenolics, as well as thickened cell walls, were observed in response to several metal treatments of vetiver grass (Melato *et al.* 2012). This further indicates the relevance of the cell wall and secondary metabolites in plant metal response. Salicylic acid, a relevant signaling molecule in biotic and abiotic stress, is also a metabolite produced through the shikimate-phenylpropanoid pathway (Sticher, Mauch-Mani, and Metraux 1997; Horvath *et al.* 2007). For example, Popova *et al.* (2009) reported an increase of endogenous salicylic acid levels in *Pisum sativum* upon cadmium treatment, moreover, pre-treatment of the seeds with salicylic acid was shown to reduce the negative effects of cadmium. It is proposed that salicylic acid acts in the attenuation of abiotic stress by influencing the activity of specific enzymes or inducing genes responsible for protective mechanisms (Horvath *et al.* 2007). Analysis of differentially expressed proteins in pre- and post-flowering *S. nigrum* roots allowed the identification of several induced or up-regulated proteins involved in plant response to biotic and abiotic stress such as the pathogenesis-related protein STH-2, pepper esterase and polyphenol oxidase (Chapter V). The pathogenesis-related (PR) protein STH-2, now PR-10a, was induced upon osmotic and salt challenge, and over-expression of the protein increased tolerance to these factors (van Loon *et al.* 1994; El-Banna *et al.* 2010). The PR-10a protein belongs to a larger group of proteins which have been found to be responsive to stress conditions such as metals (Ahsan, Renaut, and Komatsu 2009). Additionally, the expression of *PR-10* genes is regulated by jasmonic acid, abscisic acid and salicylic acid (McGee, Hamer, and Hodges 2001; Liu and Ekramoddoullah 2006), further strengthening a metabolic network response to Zn in *S. nigrum* plants. Polyphenol oxidase (PPO), induced in pre-flowering *S. nigrum* roots upon Zn treatment, is also indicated to play a role in response to metal stress (Ali *et al.* 2006; Kovacik *et al.* 2009b; Wang *et al.* 2011). Polyphenol oxidases use oxygen to oxidize phenolic compounds to their corresponding quinones, namely the *o*-hydroxylation of monophenols to *o*-diphenols and the dehydrogenation of *o*-dihydroxyphenols to *o*-diquinones, and are implicated to play a role in plant defense against stress, pathogens and herbivory (Mayer 2006; Thipyapong, Stout, and Attajarusit 2007; Constabel and Barbehenn 2008). The physiological function of PPO was studied in walnut (*Juglans regia*) by Araji *et al.* (2014) by silencing the *jrPPO1* gene and the authors show that the activity of PPO is fundamental for secondary metabolism in walnut playing a role in the metabolism of phenolic compounds, the expression of phenylpropanoid pathway genes as well as acting as an indirect regulator of cell death.

6.5 ZINC TOLERANCE IN *S. NIGRUM* PLANTS AS AN ENERGY REQUIRING PROCESS

The analysis of differentially expressed proteins in metal treated plants can be a powerful approach in discerning metal tolerance mechanisms, however, knowledge in this particular field is still insufficient (Visioli and Marmiroli 2013). Heavy metal treated plants show an increased demand for energy and this is evidenced by effects on proteins involved in energy metabolism, as extensively reviewed by Ahsan *et al.* (2009), Visioli and Marmiroli (2013) and Hossain and Komatsu (2012). For example, the reported association of aldolase and enolase with the plant tonoplast (Barkla *et al.* 2009) points to a concentration of glycolytic complexes in regions of high demand for ATP or pyruvate, forming functionally compartmentalized energy networks. In order to meet this higher demand for energy, the up-regulation of enzymes involved in energy and carbohydrate metabolism is to be expected (Ahsan *et al.* 2009). Several organic acids of the tricarboxylic cycle, namely citric, malic and fumaric acids, were affected by Zn treatment in pre- and post-flowering *S. nigrum* plants and an analysis of differentially expressed proteins allowed the identification of up-regulated or induced proteins involved in energy metabolism in the root tissues, namely enolase, malic enzyme and alcohol dehydrogenase (Samardjieva *et al.* 2014a) (Chapter IV and V). Enolase is an important player in glycolysis where it catalyzes the conversion of 2-phosphoglycerate to phospho*enol*pyruvate and this enzyme has been reported to be affected by metal treatment by several authors (Van Der Straeten *et al.* 1991; Sarry *et al.* 2006; Kieffer *et al.* 2009; Hossain and Komatsu 2012). Malic enzymes, decarboxylate malate using NAD(P) yielding pyruvate, NAD(P)H and carbon dioxide, in this manner offering an alternative route for the synthesis of respiration substrates, which is advantageous as large reservoirs of carboxylic acids are available in plants (Wedding 1989). It has been observed that photosynthetic rates may be decreased as a result of metal treatment and Sagardoy *et al.* (2010) reported that in *Beta vulgaris* this was also accompanied by increases in respiratory rates. In a later report, Sagardoy *et al.* (2011), having observed an effect of Zn on intermediates of the TCA cycle, suggest that carboxylates produced in the root are transported to the leaf to compensate for the decreases in photosynthetic rates. On the other hand, a recent review of the proteomics of heavy metal hyperaccumulators presents a number of examples of up-regulation of proteins involved in photosynthesis in these plants in response to metal treatment and indicates that this up-regulation would itself enhance energy demand (Visioli and Marmiroli 2013).

6.6 A MODEL FOR ZINC FLUX IN *S. NIGRUM* PLANTS

The detailed ultrastructural study of Zn localization allowed for the identification of cells and cell compartments which are sinks for Zn and also shed light on the flux of the metal through the plant (Samardjieva *et al.* 2014b) (Chapter III). Interestingly, in the vascular tissues, Zn was often observed in association with the plasma membrane – cell wall (PM-CW) complex of vascular parenchyma cells (Samardjieva *et al.* 2014b) (Chapter III). In fact, plasma membrane exclusion and complexation at the PM-CW interface has been pointed out as a potential tolerance mechanism (Hossain *et al.* 2012). Therefore, zinc accumulation in the PM-CV complex may constitute a protection mechanism functioning as a barrier to Zn entrance into the cytoplasm. It has been put forward that the uptake of Zn into cells does not require active transport due to the existence of a cell membrane potential, negative on the inside, favoring the influx of Zn, however, active transport is necessary for the efflux of Zn from the cytoplasm, for example in xylem loading (Olsen and Palmgren 2014).

The coordination of Zn with organic acids, such as citrate, is favored by the low pH of the xylem (pH ~ 5.5) (Salt *et al.* 1999). Organic acids may participate in the long distance transport of metals in plants, for example, data presented by Xu *et al.* (2012) indicates that citric acid is involved in Cd root-to-shoot transport rather than transport into the root. In the xylem of Zn hyperaccumulators *Sedum alfredii* and *Thlaspi caerulescens* a portion of the metal was transported in the xylem in association with citric acid (Salt *et al.* 1999; Lu *et al.* 2013). Citric acid concentrations in the stem of *S. nigrum* were higher in Zn treated in pre-flowering plants when compared to control plants, on the other hand, in post-flowering plants, where Zn accumulation in the stems was several times lower, this increase was not observed (Samardjieva *et al.* 2014a) (Chapter IV). Moreover, an increase in citric acid was detected in the roots of post-flowering plants (Samardjieva *et al.* 2014a) (Chapter IV). The differences in citric acid content between pre- and post-flowering *S. nigrum* stems observed in this study may, therefore, be due to the concurrent reduction of Zn transport from the root in post-flowering plants (Samardjieva *et al.* 2014a) (Chapter IV). The Zn deposits detected in the phloem and associated parenchyma of roots, stems and leaves of Zn treated *S. nigrum* show that this tissue is highly relevant in Zn flux. The observation of Zn in the phloem of *S. nigrum* stems, by light microscopy, has been reported in previous studies (Marques *et al.* 2008b), and further contributes to strengthen this hypothesis. The

relevance of Zn transport in the phloem has also been reported recently for hyperaccumulator *Sedum alfredii* where Zn redistribution through the phloem was detected by the determination of the transport of ^{68}Zn from mature to growing leaves (Lu *et al.* 2013). In *Arabidopsis thaliana*, high accumulation of cadmium in the root phloem tissues is proposed to be an avoidance mechanism protecting the shoot tissues through metal redistribution (Van Belleghem *et al.* 2007). Together with these reports, our results suggest that the phloem transport is highly relevant plant response to metals. The detection of Zn in the xylem, phloem and their associated parenchyma, as well as the cambium tissue, suggests that the metal is transported from the root to the shoot tissues and also laterally from xylem to phloem and surrounding parenchyma cells.

CONCLUSIONS AND FUTURE PERSPECTIVES

A comprehensive understanding of the mechanisms of plant metal tolerance and accumulation requires an integrative approach and it is noteworthy that a number of mechanisms are involved in plant response to Zn in *S. nigrum*. This phenomenon is highly dependent on plant development, where an evident relationship exists between tolerance, plant growth and Zn accumulation in the aerial organs. In this plant, Zn flux occurs through the xylem and phloem vascular tissues and is ultimately sequestered in the vacuoles and apoplast of parenchyma cells. The process is accompanied by an up-regulation of proteins involved in energy metabolism, most likely to compensate for a higher energy requirement in Zn challenged *S. nigrum*. This is further supported by the effects of Zn challenge on organic acids involved in the tricarboxylic cycle, which may also participate in the process of tolerance by acting as Zn ligands in compartmentalization or in long-distance transport. Finally, an important role is also likely played by secondary metabolites, as suggested by the increases of shikimic acid and of defense proteins activated by these metabolites or involved in secondary metabolism.

The results presented in this thesis offer insight into the mechanisms of Zn tolerance and accumulation in *S. nigrum* plants and further contribute to the notion of a complex network of mechanisms involved in metal response in plants.

Further work is needed to fully unveil the tolerance and accumulation capabilities of *S. nigrum* plants, and in the line of future perspectives, insight into the phytoremediation fitness of *S. nigrum* plants, continues to pass through an integrative approach:

- Extended analysis of the differentially expressed proteins in response to Zn, especially in the stem and leaves of pre- and post-flowering *S. nigrum* plants may allow to clarify the mechanisms governing the switch in tolerance occurring between these stages.
- Characterization of the tissue dependent expression of Zn responsive proteins.

- The involvement of secondary metabolism, suggested by some of the data, should be explored. Namely, the activation of key pathways such as the shikimate and the phenylpropanoid pathways could be assessed by the measurement of enzymatic activities of enzymes involved, such as shikimate dehydrogenase and phenylalanine ammonia-lyase. In addition, phenolic content could further elucidate the engagement of secondary metabolism.
- A cDNA microarray analysis, offering the possibility to examine the expression of a high number of genes simultaneously, would allow the identification of relevant genes in Zn response. The data obtained would complement and add to previously obtained knowledge by the analysis of differentially expressed proteins.
- Attending to the importance of microorganisms in the rhizosphere, the involvement of plant growth promoting rhizobacteria (PGPR) cannot be neglected.
- Studies carried out under laboratory controlled conditions, although unavoidable to understand the biology of *S. nigrum* tolerance and accumulation of Zn, cannot substitute the need to study the phytoremediation fitness of *S. nigrum* under field conditions which is the ultimate goal of these studies.

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