Chapter 1

General Introduction

Metal contamination
Some metals, such as copper (Cu), zinc (Zn), iron (Fe) etc., are essential nutrients required by plants, whereas others, such as lead (Pb), arsenic (As) and cadmium (Cd), among others, do not have known biological functions (Memon et al., 2001). Non-essential metals can be actively taken up by plants, due to incomplete ion selectivity of the transmembrane transporters responsible for the uptake of essential elements and they can be toxic even at very low concentrations (Clemens, 2001). However, also essential metals can become toxic when present in excess. Lead (Pb) is the most common heavy metal contaminant in the environment. Sources of Pb contamination in soils can be classified into three broad categories: industrial activities such as mining and smelting processes, agricultural activities such as application of insecticides and municipal sewage sludge, as well as urban activities such as the use of leaded gasoline, paints, and other materials (Shen et al., 2002).

**Pb uptake, transport and localization**

Pb is available to plants from soil and aerosol sources. Pb uptake studies in plants have demonstrated that roots have an ability to take up significant quantities of Pb whilst simultaneously greatly restricting its translocation to above ground parts. Uptake of Pb in plants is regulated by pH, particle size distribution, cation exchange capacity and other physico-chemical parameters of the soil, as well as by root exudation (Sharma and Dubey, 2005). At the root surface Pb binds to the carboxylic groups of mucilage uronic acids, and the galacturonic acid residues of pectin in the outer cell walls. Pb transported from the soil to the root cells has to cross the root-cell plasma membrane (PM). One possible transport pathway of Pb across the PM appears to be through PM cation channels, such as Ca-channels (Huang and Cunningham, 1996; Liu et al., 2009). In most, if not all plant species, the translocation of Pb from root to shoot is very limited (Kumar et al., 1995). The content of Pb in various plant organs tends to decrease in the following order: roots>leaves>stem>inflorescence>seeds. However this order can vary with plant species (Sharma and Dubey, 2005). Foliar Pb accumulation increases with leaf age (Godzik, 1993). Pb deposits of various sizes are
present mainly in the intercellular spaces, cell walls and vacuoles (Wierzbicka and Antosiewicz, 1993).

**Pb toxicity**

The visual non-specific symptoms of Pb toxicity are rapid inhibition of root growth, stunted growth of the plant and chlorosis (Burton et al., 1984). After entering the cell, Pb inhibits activities of many enzymes, disturbs mineral nutrition and water balance, changes the hormonal status and affects membrane structure and permeability. These disorders upset normal physiological activities of the plant (Seregin and Ivanov, 2001). Pb may also decrease the rate and percentage of germination (Lerda, 1992). At the cellular level Pb inhibits the activities of enzymes containing sulphhydryl (-SH) groups necessary for their activity (Van Assche and Clijsters, 1990). The activities of metalloenzymes may decline due to displacement of an essential metal by Pb from the enzymes’ active sites (Alloway, 1995). Pb decreases the photosynthetic rate by distorting chloroplast ultrastructure, diminishing chlorophyll synthesis, obstructing electron transport and inhibiting activities of Calvin cycle enzymes (Sharma and Dubey, 2005). At low concentrations Pb stimulates respiration and increases ATP content whereas higher concentrations are inhibitory to respiration and decrease ATP (Reese and Roberts, 1985). Pb causes nutritional imbalances of K, Ca, Mg, Mn, Zn, Cu and Fe within the tissues by physically blocking the access of these ions to the absorption sites of the roots (Godbold and Kettner, 1991). A decline in transpiration rate and water content in tissues occurs in plants growing under Pb exposure (Sharma and Dubey, 2005). One of the phytotoxic effects of Pb appears to be induction of oxidative stress in growing plant parts due to enhanced production of reactive oxygen species (ROS) resulting in an unbalanced cellular redox status (Verma and Dubey, 2003).

**Lead tolerance**

Despite the adverse effects of toxic metal ions in most of the higher plants, certain plant species, called metallophtyes, have evolved the capacity to grow and reproduce in environments contaminated with high
concentrations of certain heavy metal ions (Antonovics et al., 1971; Baker, 1987). This ability is often called ‘metal tolerance’ or, more precisely, ‘metal hypertolerance’ (Clemens, 2006). Metal hypertolerance usually is a constitutive and heritable trait (Macnair, 1993). Baker (1981) proposed two basic strategies by which higher plants can tolerate the large amounts of metals in their environment: (1) exclusion, whereby transport of metals is restricted, and low, relatively constant metal concentrations are maintained in the shoot over a wide range of soil concentrations, and (2) accumulation, whereby metals are accumulated in the upper plant parts at both high and low soil concentrations.

Mechanisms responsible for metal tolerance in higher plants can be based either on the ability to prevent excessive uptake of metal ions (avoidance), or the ability to cope with high amounts of heavy metals in tissues (tolerance sensu stricto) (Levitt, 1980; Baker, 1987).

Avoidance can be achieved by immobilizing metals in the soil, for example through root exudates, changes in specific transporter activities in the plasma-membrane of the root cells, active efflux from the roots, or storing the metals in the apoplast. Once the metal passes the plasma membrane, tolerance may be effected in different ways, including cytosolic sequestration, enhanced efflux from the cytosolic compartment into a metabolically inactive compartment, either through tonoplast transport, efflux across the plasma-membrane, or via the secretory pathway (Clemens, 2001). In general, combinations of all these mechanisms do contribute to metal tolerance.

The Pb exclusion capacity of plants may be dependent on the capacity to immobilize Pb in the rhizosphere, e.g. through binding to carboxylic groups of mucilage uronic acids, external precipitation by oxalate exudation, etc. (Ye et al., 1997; Yang et al., 2000; Sharma and Dubey, 2005). Within the plant the major part of the Pb burden is found in the cell walls (Wierzbicka and Antosiewicz, 1993; Sharma and Dubey, 2005). A smaller part of the Pb burden is sequestered in the vacuole in the form of complexes. Plants exposed to Pb and certain other heavy metals like Cd, Zn and Cu synthesize metal binding cysteine-rich low molecular weight polypeptides, called phytochelatins, in the cytoplasm and transport the resulting metal-phytochelatin complexes into the vacuole.
(Cobbett, 2000). Several non-specific defense systems are also activated when plants are exposed to Pb. These include synthesis of osmolytes (like proline) and polyamines (putrescine), changes in the chemical composition of the cell wall (callose and suberin deposition), changes in hormonal balance (primarily that of ethylene and ABA), and activation of the antioxidant defense system (Seregin and Ivanov, 2001; Sharma and Dubey, 2005).

**Metal hyperaccumulation in plants**

A relatively small number of metallophytes (about 500 species worldwide) accumulate metals at extra-ordinarily high concentrations in their shoots, rather than their roots (Baker, 1981; Baker and Brooks, 1989; Verbruggen et al., 2009). Metal hyperaccumulating plants have been defined as plants that accumulate more than 1% Zn or Mn, 0.1% Ni, Cu, Co and Pb, or 0.01% Cd in their above ground parts (on a dry weight basis) when growing on their native soils (Baker and Brooks, 1989). These concentrations are lethal to normal plants (Marschner, 1995). Metal hyperaccumulators are found in a large number of plant families, but are particularly represented among the Brassicaceae family (Verbruggen et al., 2009; Krämer, 2010). The great majority of them are confined to Ni-enriched serpentine soil and hyperaccumulate Ni. A much smaller number of species hyperaccumulate other metals or metalloids, such as Mn, Zn, Cd, Pb, Co, or As (Verbruggen et al., 2009). Among them are a number of facultative metallophytes, including the model species *Arabidopsis halleri* and *Noccaea caerulescens*, both of which hyperaccumulate Zn, occasionally even from non-metalliferous soil (Reeves et al., 2001; Assunção et al., 2003b) and, though much less consistently, Cd, or in case of *N. caerulescens*, Ni or Pb. While Zn hyperaccumulation capacity is a species-wide property in both, although there is heritable variation in degree (Macnair, 2002; Assunção et al., 2003b,c; Deniau et al., 2006), the capacities to hyperaccumulate Cd or Ni seem to be rather population-specific (Reeves et al., 2001; Assunção et al., 2003b). In *A. halleri*, but possibly also in *N. caerulescens*, Zn hypertolerance is to a certain degree a species-wide trait, at least in comparison with non-metallophytes or non-hyperaccumulating
facultative metallophytes, although metallicolous populations are, on average, more Zn-tolerant than non-metallicolous ones (Assunção et al., 2003b; Pauwels et al., 2006). In *N. caerulescens*, this may also apply to Ni hypertolerance, but certainly not for Cd hypertolerance, which is clearly confined to metallicolous populations (Assunção et al., 2003b). To date 14 hyperaccumulators of Pb belonging to 7 different families have been reported (Verbruggen et al., 2009; Krämer, 2010).

Hyperaccumulation of lead is particularly rare, due to the low solubility of most lead compounds, and the ready precipitation of lead by sulfate and phosphate within the root system (Rotkittikun et al., 2006). The degree of inter-specific and intra-specific variation in Pb tolerance and foliar Pb accumulation capacity among heavy metal hyperaccumulators has barely been explored to date. Evidence of Pb hyperaccumulation is largely based on analysis of samples collected from the field, which may have been prone to air-born contamination (Faucon et al., 2007). Experimental studies under controlled conditions are scarce and unambiguous evidence in favor of Pb hyperaccumulation has not been obtained thus far (e.g., Baker et al., 1994; Van der Ent et al., 2013). Regarding the question of why hyperaccumulators accumulate metals to concentrations that are toxic to most organisms, the different non-mutually exclusive hypotheses in literature are: tolerance or disposal of metal from the plant, protection against herbivores or pathogens, inadvertent uptake, drought resistance, and allelopathy (Boyd and Martens, 1992). The hypothesis of protection against herbivores and pathogens is by far the most popular one (Boyd and Martens, 1994; Ghaderian et al., 2000; Davis et al., 2001; Verbruggen et al., 2009). Heavy metal hyperaccumulation is not common among terrestrial higher plants. The metal hyperaccumulators identified thus far account for less than 0.2% of all angiosperms (Baker et al., 2000). Nevertheless, the phenomenon has attracted much attention for several reasons. First, there is an increasing interest in the exploitation of hyperaccumulating plants for phytoextraction and phytomining purposes. Second, it is a scientifically appealing phenomenon, raising a variety of fundamental questions with regard to plant metal homeostasis and trophic ecological interaction. For these reasons, heavy metal hyperaccumulation in plants is an ever growing and exciting field of research (Assunção et al., 2003a).
Phytoremediation

During recent years the concept of using plants to remediate heavy metal contaminated sites (phytoremediation) has received greater attention (Jarvis and Leung, 2002). Phytoextraction is the use of metal-accumulating plants that can transport and concentrate metals from the soil to the roots and aboveground shoots. As pointed out in the review by Salt et al. (1998), there are, at present, two strategies of phytoextraction: (i) continuous phytoextraction, which depends on the natural ability of some plants to accumulate, translocate and resist high amounts of metals over the complete growth cycle (e.g., hyperaccumulators), and (ii) chemically/chelate-enhanced, -assisted, or -induced phytoextraction, based on the application of chelating agents to the soil to enhance metal uptake by plants (Alkorta et al., 2004). A chelate is a chemical compound composed of a metal ion and chelating agent.

Chelating soil additives that form water-soluble metal complexes can readily desorb metals from the soil matrix, thus enhancing their concentrations in the soil solution, which in turn increases their mobility (Schmidt, 2003). In cases where diffusion or convection to the root surface is limiting the metal’s uptake into the root, such additives are expected to enhance the metal accumulation in the plant body, provided that chelate splitting at the root surface occurs readily, or that the metal can (also) effectively be taken up in the chelated form. In the latter case, chelating agents can also be expected to enhance plant metal accumulation when a metal’s uptake is limited by its concentration in the soil solution, rather than by its transport from the bulk soil to the root surface. Chelating agents, such as organic acids, amino acids and synthetic chelators, as well as biosurfactants have been previously studied for their potential to enhance metal accumulation in plants.

So far, most studies on chelate-induced phytoextraction have focused on Pb phytoextraction by means of using EDTA (ethylene diamine tetraacetic acid) (McGrath et al., 2002). But due to its low liability to biological degradation and, therefore, high environmental persistence, which may lead to secondary contamination (Tandy et al., 2006; Zhao et
al., 2010), it is now considered unsuitable for field use. Recently, the utilization of easily biodegradable chelating agents, such as EDDS (ethylene diamine disuccinic acid) and citric acid has been proposed. EDDS is a biodegradable structural isomer of EDTA (Vandevivere et al., 2001; Tandy et al., 2006), which has been shown to enhance the uptake of heavy metals such as Cu, Cd, Pb, Zn and Ni in various plant species (Luo et al., 2006). Citric acid is a low molecular weight organic acid present at high concentrations in the vacuoles of photosynthetic plant tissues (Gunawardana et al., 2010). It is also exuded from plant roots into the soil and, therefore, proposed as an alternative to synthetic chelators for use in chelator-assisted phytoextraction (Evangelou et al., 2006).

**Matthiola flava**

*Matthiola flava* Boiss is a perennial herb of the Brassicacea family. It is a densely tomentose, robust perennial hemicryptophyte found on dry rocky hills and sandy plains in Iran, Afghanistan, Pakistan, Kashmir, and Turcomenia (Sarwar, 2002). *M. flava* is a novel facultative metallophyte from Iran, which seems to be capable of considerable foliar lead accumulation in nature, and thus potentially suitable for the phytoremediation of Pb contaminated soils.

**Outline of the thesis**

In chapter 2 Pb tolerance and Pb accumulation are compared among calamine and non-calamine populations of the non-hyperaccumulator metallophyte *Silene vulgaris*, and the (facultative) Pb hyperaccumulators *Noccaea caerulescens* and *M. flava* growing in hydroponics. It is shown that 1) Pb hypertolerance is lacking in populations from calamine soil with high exchangeable soil Ca, 2) Ca counteracts Pb uptake and toxicity, but promotes root-to-shoot Pb transport in *M. flava*, 3) In *N. caerulescens* Pb hyperaccumulation is population-specific, and Pb hypertolerance is not consistently associated with Pb hyperaccumulation, 4) Natural Pb hyperaccumulation in metallicolous *M. flava* is not reproducible in hydroponics.
In chapter 3 Pb, Zn and Cd accumulation from two calamine soils with different pH and calcium contents are compared among metallicolous (M) and non-metallicolous (NM) populations of hyperaccumulator and non-hyperaccumulator metallophytes. In addition, a calamine and a non-metallicolous population of *M. flavida* are compared for Zn and Cd tolerance in hydroponics. It is shown that 1) Only the native M population of *S. vulgaris*, and no more than one out of four populations of *N. caerulescens* are able to grow in the low-pH/low-Ca soil, whereas both the N and NM populations of all the species survive and maintain normal growth in the high-pH/high-Ca soil, except for a serpentine, Cd-hypersensitive *N. caerulescens* population, 2) *N. caerulescens* hyperaccumulates Zn from the low-pH/low-Ca soil, but not from the high-pH/high-Ca one, 3) M and NM *M. flavida* are equally tolerant for both Cd and Zn.

In chapter 4 the possibilities for chelator-assisted Pb phytoextraction are explored, using the biodegradable chelators citrate and EDDS, and the Pb hyperaccumulator *M. flavida*. It is demonstrated that *M. flavida* has no regular uptake systems for undissociated Pb-citrate or Pb-EDDS complexes. At low Pb concentrations in the nutrient solution, complete chelation, either by citrate or EDDS, completely arrests Pb uptake. However, when Pb-EDDS is supplied at high concentration (>800 µM), *M. flavida* hyperaccumulates Pb, probably as Pb-EDDS, in its leaves. EDDS-assisted Pb hyperaccumulation is associated with heavy toxicity. Un-dissociated Pb-EDDS is toxic, albeit much less then free Pb, whereas free EDDS is non-toxic.

In chapter 5 the results of all the chapters are discussed from an evolutionary viewpoint and perspectives for future research are developed.

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