PHYTOEXTRACTION OF HEAVY METAL BY HYPERACCUMULATING AND NON HYPERACCUMULATING PLANTS: COMPARISON OF CADMIUM UPTAKE AND STORAGE MECHANISMS IN THE PLANTS

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Summary

Although phytoextraction using hyperaccumulating plants is seen as a promising technique, a lack of understanding of the basic physiological, biochemical, and molecular mechanisms involved in heavy metal hyperaccumulation prevents the optimization of the phytoextraction technique and its further commercial application. The best-long term strategy for improving phytoextraction is therefore to understand and exploit the biological processes involved in metal acquisition, transport and shoot accumulation in plants. In order to compare Cd allocation in leaves and the role of the leaf cells in hyperaccumulating and non hyperaccumulating plants, we used a wide range of techniques and approaches on contrasting ecotypes of *Thlaspi caerulescens, Arabidopsis halleri*, *Arabidopsis thaliana* and one high biomass crop *Salix viminalis*.

As a first step, the extent of hyperaccumulation and tolerance was studied in different plants. Five Swiss *T. caerulescens* populations were compared for their tolerance and Cd hyperaccumulation to two well studied populations: *T. caerulescens* Ganges (France) and *T. caerulescens* Prayon (Belgium). We showed that the behaviour of the populations in hydroponics was linked to the characteristics of their soil of origin. However, growing plants in hydroponics with 1 µM Cd seemed to be adequate to discriminate the various populations tested for hyperaccumulation. Cadmium effect on morphological parameters and Cd accumulation in *S. viminalis* leaves was also monitored. *Salix viminalis* was surprisingly tolerant to Cd and high Cd concentration in shoots.

Because it may give an indication of the tolerance mechanisms employed by the different plants, we studied the general Cd allocation in leaves of hyperaccumulating (Ganges) and non-hyperaccumulating plants (Prayon, *S. viminalis*). Surprinsingly, when grown in hydroponics the only differences found between Prayon and Ganges at the leaf level was concentration of Cd found in the storage sinks. Results also showed similarities between *T. caerulescens* and *S. viminalis* in Cd storage, although the Cd concentrations found in leaves differed greatly. Both point-like accumulation and accumulation at the edges of the leaves were observed. A link could be established with visible symptoms (necrosis). At last differences in Cd localization between young and mature leaves were observed. These differences were attributed to physiological differences between leaves. *Salix viminalis* and *T. caerulescens* were both able to reduce Cd toxicity by allocating Cd in less sensitive tissues. Cadmium was found inside the cells and in the cell walls of the leaves of *T. caerulescens*, but mainly in cell walls and to a lesser extent in the symplasm in *S. viminalis*. Metal allocation in both plants indicated that the plant general growth strategy governed metal accumulation.

We further investigated the role of the leaf cells in allocating Cd in leaves of Ganges, *A. halleri* and Prayon by characterizing Cd uptake in mesophyll protoplasts. Results indicated that differences in metal uptake could not be explained by different constitutive transport capacities at the leaf protoplast level. However, pre-exposure of the plants to Cd induced an increase in Cd accumulation in protoplasts of Ganges, whereas it decreased Cd accumulation in *A. halleri* protoplasts. The experiment with competitors eventually showed that probably more than one single transport system

are carrying Cd in parallel into the cell.

Metal allocation indicates that the principle of metal storage in metabolically less sensitive plant parts governs metal accumulation. Vacuolar compartmentalization and cell wall binding in leaves could therefore both play a role in accumulation of heavy metals. Based on these various results, we suggested that metal storage in plant demands the involvement of more than one compartment. Further work is however needed to assess many steps of the trafficking of metals that remain enigmatic.

Résumé

La phytoextraction basée sur l'utilisation de plantes hyperaccumulatrices semble être une technique prometteuse pour la réhabilitation des sols contaminés. Néanmoins un manque de compréhension et de connaissance de base des mécanismes d'hyperaccumulation de métaux lourds au niveau tant physiologique, biochimique, que moléculaire retarde le développement, notamment commercial, de cette technique. Afin de progresser dans la compréhension de ces mécanismes, l'élucidation des processus biologiques de l'absorption et de l'accumulation des métaux dans les parties aériennes des plantes est indispensable. Dans l'optique de comparer la localisation de Cd dans les feuilles de plantes hyperaccumulatrices et non-hyperaccumulatrices, des techniques et approches variées ont donc été mises en oeuvre avec différents écotypes de *Thlaspi caerulescens, Arabidopsis halleri*, *Arabidopsis thaliana* ainsi qu'une plante à forte biomasse, *Salix viminalis*.

Les capacités d'accumulation et de tolérance des différentes plantes ont tout d'abord été évaluées. Cinq populations de *T. caerulescens* provenant de sols suisses ont été comparées avec deux populations bien étudiées: Ganges et Prayon. Un lien entre les performances de ces populations en hydroponie et les caractéristiques de leur sol d'origine a été mis en évidence. La concentration de Cd semblant la plus adéquate pour évaluer le potentiel d'hyperaccumulation de ces plantes en hydroponie était 1 µM Cd. L'effet de Cd sur les caractères morphologiques et l'accumulation de Cd dans les feuilles de *S. viminalis* ont ensuite été évalués. *Salix viminalis* s'est montré étonnamment tolérant à Cd et aux hautes concentrations de Cd dans ses parties aériennes.

La distribution de Cd dans les feuilles donne des indications sur les mécanismes de tolérance mis en oeuvre par les plantes. Nous avons donc étudié la répartition de Cd dans les feuilles de plantes hyperaccumulatrices (Ganges) et non-hyperaccumulatrices (Prayon, *S. viminalis*) en hydroponie. Seule la concentration de Cd dans les différents sites de stockage changeait entre Prayon et Ganges. Il existait aussi des similarités de distribution de Cd entre *T. caerulescens* et *S. viminalis*, alors que les concentrations en Cd mesurées dans les feuilles différaient grandement. Chez les deux plantes, nous avons observé une localisation de Cd sur le bord des feuilles et sous forme de points répartis sur la surface des feuilles. Une relation entre ces points et les symptômes visibles observés (nécroses) a été établie. Enfin la différence de distribution entre les feuilles jeunes et plus âgées a été attribuée à des différences physiologiques. *Salix viminalis* et *T. caerulescens* étaient tous deux capables de réduire la toxicité du Cd en le séquestrant dans des compartiments moins sensibles. Le cadmium était localisé dans les cellules ainsi que dans les parois cellulaires de *T. caerulescens*. En revanche Cd était localisé principalement dans les parois cellulaires de *S. viminalis* et plus rarement dans le symplasme. La distribution du métal indiquait dans les deux cas que celle-ci découlait de la stratégie générale de croissance des plantes.

La contribution des cellules dans le stockage de Cd au niveau des feuilles a été évaluée par l'étude de l'absorption du métal par les protoplastes du mésophylle de Ganges, *A. halleri* et Prayon. Les résultats indiquent que les différences de localisation de Cd dans les feuilles de ces espèces ne peuvent être expliquées par

une différence constitutive de transport au niveau des protoplastes. Cependant le pré-traitement des plantes avec Cd augmentait l'accumulation dans les protoplastes de Ganges, et au contraire la diminuait dans les protoplastes d'*A. halleri*. Une expérience avec divers compétiteurs a établi que vraisemblablement plusieurs transporteurs participaient en parallèle à l'absorption de Cd dans les cellules.

La distribution du métal indiquait que son accumulation était basée sur le principe d'utilisation de compartiments métaboliquement moins actifs. Le stockage dans les vacuoles ainsi que dans les parois cellulaires dans les feuilles peuvent donc jouer un rôle en parallèle dans l'accumulation des métaux lourds. Sur la base de nos résultats, nous suggérons que la séquestration des métaux dans les plantes implique la contribution de manière associée ou simultanée de plusieurs compartiments. Des recherches complémentaires seront néanmoins nécessaires pour identifier plusieurs étapes du transport des métaux au sein de la plante qui restent inexpliquées.

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List of abbreviations

AAS ..... atomic absorption spectroscopy
ABC.....ATP-binding cassette transporters
ATP..... adenosine 5’-triphosphate
CAX..... calcium and other divalent cation exchange antiporters proteins
CDF..... cation diffusion facilitator proteins
cDNA..... complementary DNA
dCTP ..... deoxycytidine 5'-triphosphate
DNA..... deoxyribonucleic acid
EDTA..... Na ethylenediamine tetraacetic
EDXMA ..... energy dispersive X-ray micro-analysis
EELS..... electron energy loss spectroscopy
ESI ..... electron spectroscopic imaging
EST ..... expressed sequence tag
EXAFS ..... X-ray absorption fine structure
GF-AAS..... graphite furnace-atomic absorption spectroscopy
GSH ..... glutathione
HBED ..... N, N’-Di(2-hydroxybenzyl) ethylenediamine-N,N’-diacetic acid monohydrochloride hydrate
ICP-AES.....inductively coupled plasma-atomic emission spectroscopy
MES .....2-Morpholinoethanesulfonic acid monohydrate
mRNA.....messenger RNA
MTs.....methallothioneins
Nramp .....natural-resistance-associated macrophage protein
PCR.....polyremase chain reaction
PCs .....phytochelatines
P-type ATPase .....metal transporting ATPases
RNA.....ribonucleic acid
RT-PCR.....reverse transcriptase polymerase chain reaction
SEM .....scanning electron microscope
SIMS .....secondary ion mass spectroscopy
TEM.....transmission electron microscope
ZIP.....ZRT, IRT-like protein

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Aim of the work

Although phytoextraction using hyperaccumulating plants is seen as a promising technique, a lack of understanding of the basic physiological, biochemical, and molecular mechanisms involved in heavy metal hyperaccumulation prevents the optimization of the phytoextraction technique and its further commercial application. The best long-term strategy for improving phytoextraction is therefore to understand and exploit the biological processes involved in metal acquisition, transport and shoot accumulation in plants.

This study is a contribution to the understanding of the mechanisms involved in Cd storage at different levels from the whole plant physiology, to single cell analysis and gene expression in potential candidates for phytoextraction. In particular we wanted to assess the differences between the well-described Cd hyperaccumulator *Thlaspi caerulescens* ecotype Ganges and plants accumulating at lower degree or not at all Cd. To be able to find out the extent of a possible improvement of phytoextraction efficiency, it is necessary to understand the degree and type of differences occurring between these plants. More in detail, the questions to be answered were:

- to test the natural variability and potential of hyperaccumulation in new *T. caerulescens* populations, and the best way to screen them,
- to study the final location (storage) of Cd and how it differed between Ganges and the other plants tested,
- to determine if differences in Cd uptake and storage location could be explained by an enhanced transport at the cell level,
- to find if specific genes were induced in presence of Cd and how this differed between Ganges and the other plants tested.

Seven natural populations of *T. caerulescens* and one close relative, *Thlaspi perfoliatum,* were first assessed for their Cd tolerance and accumulation capability. Several plant species were further compared to Ganges. Four closely relative species of the Brassicaceae family: *Arabidopsis halleri* another hyperaccumulator of Cd, *T. caerulescens* Prayon an ecotype that does not hyperaccumulate Cd, *Arabidopsis thaliana* the best-studied genetic model in the plant kingdom, and one high biomass crop, *Salix viminalis*. To guarantee the homogeneity of the results the same set of samples were further studied.

In order to learn more about Cd allocation in leaves of *T. caerulescens,* we

performed macro-autoradiographs on plants grown in hydroponics and treated with increasing concentrations of Cd. Autoradiographs were confronted to visual symptoms, biomass production and metal concentration in shoots. Distribution of Cd at the macroscopic level was completed by observation in leaves at the cell level by optical and electronic microscopy. At last, separation of different plant compartments was performed to measure in detail the involvement of these compartments in Cd storage. Cadmium effect on morphological parameters and Cd accumulation in *S. viminalis* leaves was similarly monitored. In addition, the involvement of genes and proteins in Cd transport into the different Brassicaceae species was compared. We eventually investigated the role of the leaf cells in allocating metal in leaves of hyperaccumulating plants by characterizing Cd uptake in mesophyll protoplasts of Ganges, *A. halleri* and Prayon.

2

General introduction

As a consequence of the industrial revolution there is an enormous and increasing demand for heavy metals that leads to high anthropogenic emission of heavy metals in the biosphere (Vangronsveld and Cunningham, 1998). Nonradioactive As, Cd, Cu, Hg, Pb and Zn and radioactive Sr, Cs, and U are the most important metallic pollutants (Raskin *et al.*, 1997). These metals become an environmental concern when their concentrations begin to affect human health and the environment. A common characteristic of heavy metals, regardless of whether they are biologically essential or not, is that they may already exert toxic effects at low concentrations (Kabata-Pendias and Pendias, 2001). Unlike organic molecules, toxic metals can not be degraded but only be remediated. It requires consequently the intervention of mankind. The present work focused on Cd, one of the most ecotoxic metals (Adriano, 2001; Kabata-Pendias and Pendias, 2001).

2.1 Cadmium in the environment

2.1.1 Cadmium origin in the biosphere

Heavy metals are important environmental pollutants particularly in areas where there is a high anthropogenic pressure, but they also occur naturally (Alloway, 1995a; Sanità di Toppi and Gabbrielli, 1999). Anthropogenic Cd contamination often results from mining or smelting of metal ores, but Cd is also released into the environment by power stations, heating systems, waste incinerators, urban traffic, cement factories and as a by-product of phosphate fertilizers (Alloway, 1995a; Sanità di Toppi and Gabbrielli, 1999). Use of sewage sludges as fertilizers has further contributed to a significant contamination of agricultural soils (Alloway, 1995a; Sanità di Toppi and Gabbrielli, 1999). In areas with low anthropogenic pressure, natural high concentrations are observed for example in soils formed on metal rich rocks, such as serpentine soils that release Cd as a result of rock mineralization processes (Alloway, 1995a; Sanità di Toppi and Gabbrielli, 1999; Adriano, 2001).

2.1.2 Environmental and health hazards of Cd

Apart from some emission into the atmosphere in the form of dust particles or vapors, that may be controlled by the installation of adequate air filters, heavy metals stay largely in the aquatic and soil phases of the planet. Cadmium presence in the soil

can cause serious problems to all organisms. Although not essential for plant growth, when bioavailable, Cd is readily taken up by roots and translocated into aerial organs where it can accumulate to high levels. The most apparent visible symptoms of Cd toxicity in plants are retardation of plant growth, chlorosis and stunting (Das *et al.*, 1997; Sanità di Toppi and Gabbrielli, 1999; Kabata-Pendias and Pendias, 2001). Cadmium accumulation in plants represents the main source of contamination for animals and humans (i.e. food or tobacco smoke), although an additional risk exists for children by direct ingestion of contaminated soil (Wagner, 1993). In animals and humans, Cd may produce disorders in the metabolism of Ca, vitamin D, including bone degeneration and kidney damages (Wagner, 1993; Burgat-Sacaze *et al.*, 1996).

2.1.3 Cadmium in soils

Soils are the ultimate sink for heavy metals released in the environment, and Cd concentration tends to increase in soils over time. The risk arising from heavy metals depends largely on their mobility and availability to living organisms. In soils, both depend on metal concentration, speciation and complexation to soil components, modulated by the pH, redox potential, temperature and concentrations of other elements (competition).

Soils are composed of a wide variety of constituents: more or less weathered primary minerals, secondary constituents such as phyllosilicates, oxyhydroxydes of Al and Fe, as well as various organic and organo-mineral compounds. Such a heterogeneous medium acts as an effective sink for sequestering heavy metals released in the environment (Nriagu, 1979; Alloway, 1995b). In soils, endogenous and exogenous metallic elements are associated with various soil constituents, depending on their origin and their interaction with the reactive surface of soil components. During weathering it may also form several complex ions such as $CdCl^+$, $CdCl_3^-$, $CdCl_4^{2-}$, $CdOH^+$, $Cd(OH)_3^-$, $Cd(OH)_4^{2-}$ or organic chelates. Under conditions of strong oxidation, Cd is likely to form minerals such as CdO et $CdCO_3$ and is also likely to be accumulated in phosphate and biolith deposits (Kabata-Pendias and Pendias, 2001). Cadmium is known to mainly occur as Cd^{2+} in the natural environment and is taken up as such by plants.

2.1.4 Toxicity of heavy metals in plants

Uptake of metal ions is an essential part of plant nutrition. However, the existence of a complex metal homeostasis network in plants has only recently come into focus (Clemens *et al.*, 2002). Several heavy metals and metalloids, such as Fe, Mn, Zn, Cu and Mo play important roles in enzyme induction, reaction, and isozyme activity and therefore are essential micronutrients (Marschner, 1995). Other metals such as Al, Cd, As, U, Pb, Tl, Cr, Hg, Ag, and Au are not micronutrients for plants (Marschner, 1995). The response of plants to non-essential metals varies across a broad spectrum from tolerance to toxicity with increasing concentration (Figure 2.1; Baker and Brooks, 1989). In order to survive, plants must have developed, on one side, efficient and specific mechanisms by which heavy metals are taken up and transformed into physiologically tolerant form, providing the essential elements for metabolic functions. On the other side, excess of these essential elements or those

toxic ions that do not play a role in metabolism, have to be metabolically inactivated.

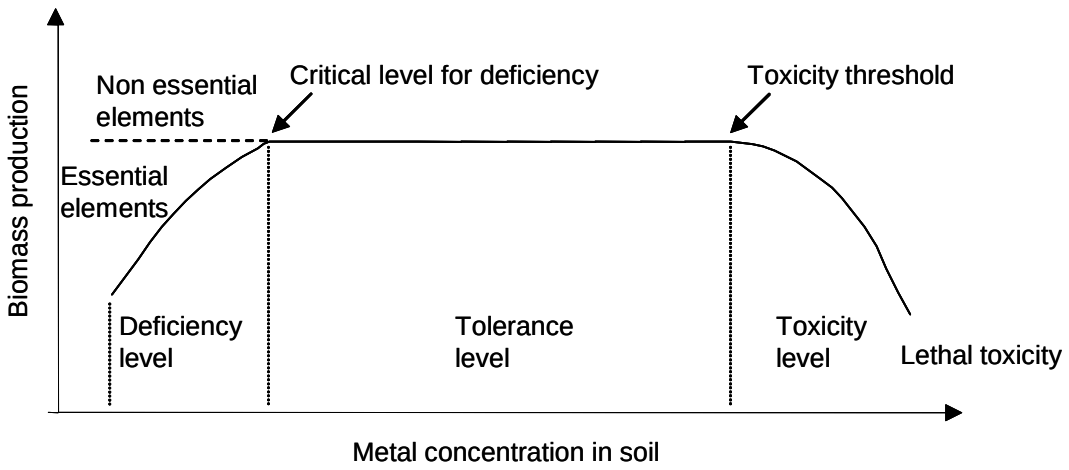

Figure 2.1: Effects of metal concentration in soils on plant biomass production (adapted from Baker and Brooks, 1989).

2.1.5 Heavy metal tolerance in plants

Three basic strategies have been proposed to explain tolerance of plants to toxic heavy metals (Figure 2.2): 1) exclusion, whereby plants avoid excessive uptake and transport of metals until the mechanism breaks down resulting in unrestricted transport, 2) indication, whereby plants poorly control their metal uptake so that the internal accumulation reflects the concentration found in soils, and 3) accumulation and sequestration, whereby plants take up large amounts of metal, and transfer the metal to the shoots where it is accumulated (Baker, 1981). An extreme strategy for metal tolerance, and in sharp contrast to metal exclusion, is hyperaccumulation (Adriano, 2001).

2.1.6 Phytoremediation

The only way of reducing Cd hazard is to reduce its mobility or bioavailability in soils. However, the remediation by conventional technologies (soil capping, soil washing, vitrification, *etc*.) previously developed for small, heavily contaminated sites would not be adequate for large volumes of soil containing low but significant levels of Cd (Vangronsveld and Cunningham, 1998). Phytoremediation has therefore emerged as an alternative to the engineering-based methods because of its technical and economic advantages (Flathman and Lanza, 1998; Vangronsveld and Cunningham, 1998).

Phytoremediation refers to a diverse collection of technologies based on the use of plants to remove or render harmless organic and inorganic pollutants (Cunningham *et al.*, 1997; Salt *et al.*, 1998; Vangronsveld and Cunningham, 1998). Phytoremediation processes applicable to heavy metal-contaminated soils include

phytostimulation, phytovolatilization, phytostabilization, and phytoextraction. Phytostabilisation is used where phytoextraction is not possible or desirable and seeks to stabilize polluted soils and to reduce the flow of the contaminant in the environment. Phytoextraction implies that the pollutant is removed from the medium (Susarla *et al.*, 2002).

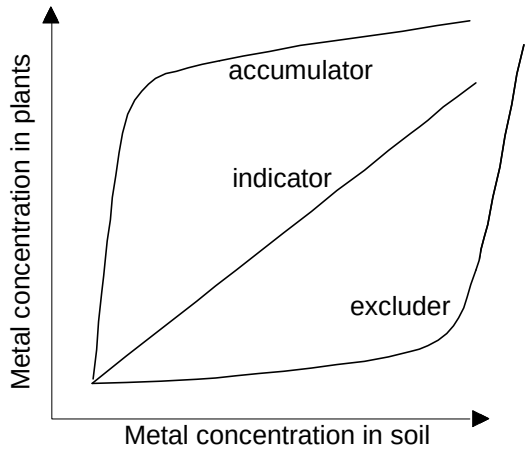

Figure 2.2: The three different strategies of metal uptake adopted by plants in relation to soil metal concentration.

Phytoextraction of heavy metals is recognized as one of the largest economic opportunity for phytoremediation (Raskin *et al.*, 1997; Vangronsveld and Cunningham, 1998; Angle *et al.*, 2001). This technique has a broad public acceptance as a “green” technique preserving soil fertility (Susarla *et al.*, 2002). It is indeed an *in situ* and solar driven technique that produces less waste because the biomass may be recycled for both energy and metals. Additionally, phytoextraction potentially resolves most of the legislative requirements that ask for metal removal down to a given threshold (Keller, 200.). The general drawbacks of phytoextraction are well known: phytoextraction is a slow process in comparison to the classical remediation methods (Susarla *et al.*, 2002). As a technology, it is still at its development stage and a lot of research is required for further optimization. Its application is limited because of the lack of established methods and successful completed remediation case studies, the lack of recognized economic performance and the risk of food chain contamination. Additionally it may not be able to remove 100% of the contaminants and its efficiency has been proved for some contaminants only. Feasibility and success of phytoextraction depends on the degree of metal contamination, the soil characteristics (fertility, metal bioavailability, etc.) and the capacity of the plant to withstand highly toxic concentrations of contaminants. Effective phytoextraction eventually requires that plants efficiently take up and translocate the metal to be extracted, sequester the metals in their tissues so that they can be tolerated, and have a high annual

production of above-ground biomass. Unfortunately to date, no plant has been described that fulfils all these criteria.

Two different approaches have been therefore promoted: 1) the use of so called hyperaccumulating plants with exceptional metal accumulating capacity but usually low biomass or 2) the use of selected conventional high biomass crops (which can also sometimes accumulate large quantities of heavy metals) usually with the help of additives to increase heavy metal bioavailability and thus uptake (Raskin *et al.*, 1997; Salt *et al.*, 1998). This second approach has however to date brought disillusion because uptake is not strictly proportional to bioavailability and the risk of groundwater contamination increases with increased metal mobility (Wenzel *et al.*, 2003). On the contrary, phytoextraction using hyperaccumulator plants is seen as a promising technique (Brown *et al.*, 1995; Hammer and Keller, 2003).

2.2 Phytoextraction of Cd

2.2.1 Hyperaccumulation and phytoextraction

Hyperaccumulating plants are often endemic to naturally metal-rich soils. Heavy metal contents in hyperaccumulators are at least 100 times those found in non hyperaccumulating plants grown in soil under the same conditions (Brooks *et al.*, 1998). The threshold applied for defining Cd hyperaccumulation is 100 mg Cd kg^{-1} shoot dry weight. This criterion is also applied to other metals including Ni (1000 mg kg^{-1}) and Zn (10’000 mg kg^{-1}). Some 400 taxa of terrestrial plants have been identified as hyperaccumulators, with about three-quarters of them being Ni hyperaccumulators (Baker and Brooks, 1989; Brooks *et al.*, 1998). Most probably, many more remain to be discovered. Only two Cd hyperaccumulators, both members of the Brassicaceae family have been reported to date, *Arabidopsis halleri* and *Thlaspi caerulescens* (Dahmani-Müller *et al.*, 2000; Lombi *et al.*, 2000).

Because they have overcome the physiological bottlenecks limiting metal accumulation in shoots and metal tolerance, metal hyperaccumulators are highly attractive model organisms (Salt and Krämer, 2000; Assunção *et al.*, 2003b). Several authors have proposed to exploit the unusual characteristic of these plants for the cleaning of metal-contaminated soils (Baker *et al.*, 1994a; Raskin *et al.*, 1997; McGrath *et al.*, 1998). However, the main constraint of this type of phytoextraction is the generally low biomass of metal hyperaccumulating plants.

Several researchers investigated ways of increasing the biomass production of hyperaccumulating plants. Brewer *et al.* (1999) reported the results of somatic hybridization of *T. caerulescens* with the high biomass crop *Brassica napus*. The hybrids obtained were taller and produced more biomass than *T. caerulescens*. They were able to accumulate Zn and Cd at levels that are toxic to *B. napus*, but below the levels commonly associated with hyperaccumulation (Brewer *et al.*, 1999). Another contemplated possibility is to use genetic engineering to confer the hyperaccumulation characteristics to high biomass plants (Krämer and Chardonnens, 2001; Pilon-Smits and Pilon, 2002). However, the basic mechanisms responsible for

hyperaccumulation are not fully understood yet and this lack of information is limiting the application of molecular engineering techniques. Besides, although genetic engineering might be the quickest way of developing efficient plants for phytoextraction purpose, the use and release of genetically engineered organisms into the environment is still restricted in many countries and is often criticized by the public. It would therefore be prudent to complement genetic engineering approaches with the search for “natural” accumulators. Presently there are only few species available for phytoextraction application (lack of nurseries and seed producers) and within these species very few cultivars or populations. It would be therefore valuable to screen new populations and develop conventional breeding programs that may help to select the more efficient plants as well as those able to hyperaccumulate multiple heavy metals.

2.2.2 High biomass species and phytoextraction

A critical parameter to evaluate phytoextraction efficiency is the total metal uptake per hectare. It is calculated from the concentration in the harvested parts and the biomass production (Ebbs *et al.*, 1997). Plants that employ other mechanisms than hyperaccumulation generally have lower shoot concentrations of metals but larger biomass (McGrath, 1998). Tobacco (*Nicotiana tabacum*), sunflower (*Helianthus annus*), Indian mustard (*Brassica juncea*) and in-bred lines of corn (*Zea mays*) produce high yields and are known to accumulate heavy metals but they have been investigated mainly in conjunction with additives (Kayser, 2000; Wenzel *et al.*, 2003). Indian mustard has been so far the most studied of those plants because of its positive response to heavy metals and additives like EDTA (Begonia *et al.*, 1998). Nevertheless tree species, especially pioneer plants (willow, poplar, …) seem also promising for phytoextraction (Pulford and Watson, 2003; Dickinson, 200.). Alternatively, these tolerant plants can be used on contaminated sites to phytostabilize contamination by reducing Cd transfer through leaching or land erosion (Vangronsveld and Cunningham, 1998; Rosselli *et al.*, 2003).

2.3 Mechanisms of tolerance and hyperaccumulation

2.3.1 The genetic basis

The understanding of metal tolerance and hyperaccumulation genetic basis is of great practical importance for phytoextraction. Tolerance and hyperaccumulation traits have been shown to be genetically determined and genetically independent in plants (Baker *et al.*, 1994b; Meerts and Isacker Van, 1997; MacNair *et al.*, 1999). Several studies on the inheritance of metal tolerance have further demonstrated that only one or two major genes might control metal tolerance in a completely or partially dominant way (Pollard *et al.*, 2002; Bert *et al.*, 2003). Concerning hyperaccumulation the results are not conclusive yet, it might have either a polygenic basis or on the contrary a major gene basis with multiple modifier genes (Pollard *et al.*, 2002; Bert *et al.*, 2003). While tolerance is correlated with soil concentration, the ability to accumulate does not show any positive correlation with soil concentration (Bert *et al.*, 2000; Assunção *et al.*, 2001; Bert *et al.*, 2002; MacNair, 2002; Assunção *et al.*,

2003a). A major conclusion from these results is that the present state of knowledge does not allow more than a superficial understanding of metal-resistance genetic basis in higher plants and particularly the exact and complex relationship between metal tolerance and metal hyperaccumulation has not been resolved yet.

2.3.2 Cadmium localization in plants

The distribution of metals within plant organs and tissues is an indirect indicator of detoxification and tolerance mechanisms employed by plant species. Indeed, the phytochemistry involved in metal transport and storage seems to vary considerably depending on the type of the metal treatment imposed on plants and plant species (Brooks *et al.*, 1998). For example, it has been reported that the youngest leaves of *T. caerulescens* (ecotype Vivier) and *B. juncea* exhibited higher Cd concentration than older leaves (Salt *et al.*, 1995; Perronnet *et al.*, 2003) but that Cd was found at higher concentration in the older leaves in *Silene vulgaris* (Chardonnens *et al.*, 1999), *Empetrum nigrum* (Uhlig *et al.*, 2001) and *Armeria maritima* ssp. *halleri* (Dahmani-Müller *et al.*, 2000). However, no such variation in concentration was reported for Cd in willow (Sander and Ericsson, 1998).

Cadmium localization was also assessed at the cellular level. In tobacco, *A. halleri*, *A. thaliana,* and *B. juncea* sequestration of metals in trichomes has been reported (Salt *et al.*, 1995; Küpper *et al.*, 2000; Zhao *et al.*, 2000; Choi *et al.*, 2001; Ager *et al.*, 2002). In *B. juncea* and *Silene vulgaris*, the leaf epidermis was a major site of Cd accumulation (Salt *et al.*, 1995; Chardonnens *et al.*, 1998), whereas in *A. halleri* and *B. napus* the highest concentration of Cd was found in the mesophyll (Küpper *et al.*, 2000; Carrier *et al.*, 2003). In tobacco Cd was localized predominantly in the vacuoles of leaf cells (Vögeli-Lange and Wagner, 1990). The cell walls were identified as another site of Cd storage in *Zea mays* shoots (Khan *et al.*, 1984; Lozano-Rodriguez *et al.*, 1997). In *B. napus* leaves, Cd was found in both vacuoles and cell walls (Carrier *et al.*, 2003). In roots of *T. caerulescens* Cd has been found in the apoplast as well as in the vacuole (Vázquez *et al.*, 1992a; Boominathan and Doran, 2003), but no data are available for Cd localization in leaf cells.

2.3.3 Plant mechanisms for Cd detoxification

From the early results, it seems that compartmentation in metabolically less active parts of plants could play a major role in heavy metal tolerance (Wang and Evangelou, 1995; Sanità di Toppi and Gabbrielli, 1999). At the subcellular level, these compartments are mainly extracellular cell walls and intracellular vacuoles.

Vacuolar compartmentation appears to play a major role in keeping heavy metals in the form of soluble complexes and/or insoluble precipitates away from the cytoplasm, where important metabolic functions are performed (Wang and Evangelou, 1995). Complexation of metal ions by specific high-affinity ligands reduces the concentration of free metal ions in cells, thereby reducing their phytotoxicity. Two major classes of chelating peptides have been investigated to date in plants: methallothioneins (MTs; Robinson *et al.*, 1993) and phytochelatins (PCs; Rauser, 1990; Zenk, 1996; Rea *et al.*, 1998). Methallothioneins-like proteins are implicated in metal homeostasis in cyanobacteria, yeasts, animals and plants (Hamer *et al.*, 1985;

Zhou and Goldsbrough, 1995; Huckle *et al.*, 1996; Murphy *et al.*, 1997). A number of researchers have introduced MTs from animal sources into plants in a transgenic approach (Yeargan *et al.*, 1992; Hasegawa *et al.*, 1997). These approaches increased plant tolerance, but not accumulation. On the other hand PCs synthesis seems to be the principal response of plants and many fungi to toxic metal exposure, and apparently also of certain animals (Cobbett, 2000; Clemens *et al.*, 2001; Vatamaniuk *et al.*, 2001). For example PCs are essential for Cd detoxification in *Arabidopsis thaliana* (Howden *et al.*, 1995). It is believed that PCs and their precursor glutathione could shuttle Cd ions from the cytosol to the vacuoles (Vögeli-Lange and Wagner, 1990; Sanità di Toppi and Gabbrielli, 1999; Heiss *et al.*, 2003). Therefore numerous attempts have been made to boost PCs formation in plants by overexpressing enzymes involved in the synthesis of the PCs precursor glutathione. In some cases this led to a slight enhancement of Cd tolerance and accumulation (Zhu *et al.*, 1999), whereas in others it did not (Xiang *et al.*, 2001; Lee *et al.*, 2003). Ligands participating in complexing heavy metals in vacuoles may be also metal chaperones, organic acids such as citric, malic and malonic acids or even histidine, nicotianamine and phytates (Krämer *et al.*, 1996; Stephan *et al.*, 1996; Rauser, 1999; Salt *et al.*, 1999).

Inherent characteristics regarding metal binding in cell walls is considered as one of the possible mechanisms in plant resistance and has been proposed by several authors as an explanation for the differences between species in Cd uptake and distribution (Florjin and Van Beusichem, 1993; Cakmak *et al.*, 2000). In the current literature the emphasis is mainly given to internal tolerance mechanisms, particularly vacuolar metal compartmentation and sequestration by PCs (Zenk, 1996). Little attention has so far been devoted to other tolerance mechanisms, although these mechanisms are likely to operate in combination (Baker, 1987; MacNair, 1997).

2.3.4 Putative Cd transporters for compartmentation

A number of genes and gene families with a putative role in intracellular compartmentation have been identified. These includes ZRT, IRT-like protein (ZIP; Guerinot, 2000), Cation Diffusion Facilitator proteins (CDF; Nies *et al.*, 1989), metal transporting ATPases (P-type ATPase; Williams *et al.*, 2000), natural-resistance-associated macrophage protein (Nramp; Thomine *et al.*, 2002), calcium and other divalent cation exchange antiporters proteins (CAX; Hirschi, 1999) and ATP-binding cassette transporters (ABC; Rea *et al.*, 1998). Although molecular genetics has greatly enhanced our understanding of metal homeostasis in recent years in bacteria and yeast, researchers are only starting to characterize the function of these transporters in plants.

Cadmium is suggested to be transported in the roots via divalent cation transporters for Fe, Zn and possibly Ca channels (Korshunova *et al.*, 1999; Pence *et al.*, 2000; Rogers *et al.*, 2000). Plants overexpressing ZAT1, encoding a putative zinc transporter in the CDF family of membrane transporters, accumulated higher Zn concentrations in their roots and were more tolerant to the metal than control plants (Van der Zaal *et al.*, 1999). Within the cell, it has been shown that the vacuolar Ca^{2+}/ H^{+} antiporter CAX2 also exchanged Cd with H^{+} and that the resulting transgenic plants accumulated more Cd (Hirschi *et al.*, 2000). On the contrary however another plant expressing the Mg^{2+}/H^{+} antiporter AtMHX1 appeared to be hypersensitive to Mg

and Zn (Shaul *et al.*, 1999). This highlights the limited knowledge about these transport mechanisms and also the need to better understand the role of these transporters. Mutagenesis of specific residues in these transporters might indeed be one target to develop interesting transgenic plants to use in phytoextraction (Rogers *et al.*, 2000). However, an improved understanding of metal homeostasis in plants is first needed.

2.3.5 The case of hyperaccumulating plants

So far the mechanisms of metal uptake by hyperaccumulating plants and the basis of their metal specificity are poorly understood. To date two mechanisms have been proposed to explain the high uptake of metals by hyperaccumulators: 1) enhanced absorption of metal into the roots (Lasat *et al.*, 1996) coupled with high rates of translocation of metal from roots to shoots (Shen *et al.*, 1997; Lasat *et al.*, 1998), and 2) foraging for metal by the roots, involving preferential allocation of root biomass into regions of metal enrichment (Schwartz *et al.*, 1999; Whiting *et al.*, 2000) and a large root system compared to shoot dry matter that might further favour soil prospecting as well as heavy metals uptake (Keller *et al.*, 2003).

A number of reports have suggested that in hyperaccumulators metals are detoxified by binding to organic acids, glucosinolates, histidine or phytochelatines (Krämer *et al.*, 1996; Salt *et al.*, 1999; Krämer *et al.*, 2000; Sarret *et al.*, 2002). However, complexation with such universal plant metabolites is considered by some researchers to be an insufficient explanation for the special properties of hyperaccumulating plants, including their metal specificity (Shen *et al.*, 1997; Küpper *et al.*, 1999; Frey *et al.*, 2000; Zhao *et al.*, 2003b). No putative high affinity Cd transporter has been identified in plants yet. It is generally believed that Cd uptake by plants represents opportunistic transport via cation channels or carrier for Zn or Fe (Pence *et al.*, 2000; Lombi *et al.*, 2002). It seems however to date that rapid removal of heavy metals from metabolically active cellular sites and sequestration within specific tissues (epidermis, trichomes) or inactive compartments (vacuoles) is the key to heavy metal hyperaccumulation (Vázquez *et al.*, 1992a; Vázquez *et al.*, 1994; Küpper *et al.*, 1999; Küpper *et al.*, 2000; Küpper *et al.*, 2001). These detoxification systems remain nevertheless to be characterized in order to explain the unusual behaviour of hyperaccumulating plants.

3

Testing criteria to compare Cd tolerance and hyperaccumulation in various *Thlaspi* populations

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3.1 Abstract

Few populations and species of hyperaccumulators have been studied so far. There is a need for 1) more choice in hyperaccumulating species or populations and 2) methods to screen these species:

- Five Swiss *Thlaspi caerulescens* populations accumulating Zn and hyperaccumulating Cd in the field were compared to Ganges and Prayon populations and a non-accumulating species, *T. perfoliatum* for 1) their tolerance and 2) Cd hyperaccumulation in hydroponics (0, 1, 5, 10, 20 and 50 µM Cd) and in a contaminated soil.

- In hydroponics, the shoot biomass was the best discriminating parameter for tolerance. At 20 and 50 µM Cd it was correlated with bioavailable Cd in the soil of origin. Root parameters did not discriminate populations except the total root length.

- 1 µM Cd was the adequate concentration to test hyperaccumulation: Cd concentration in shoots was correlated with the bioavailable metal concentration in the soils of origin and the plant efficiency to take up Cd.

- In contaminated soil the Swiss populations accumulated less than in their soil of origin and less Cd than Ganges , but more Zn than Ganges and Prayon.

3.2 Introduction

The extraction of heavy metals by plants has been proposed as a technique for remediation of contaminated soils. Hyperaccumulators that are specific to one or several metals are potential candidates, however the agronomic requirements of these species are often poorly known. Additionally they are often composed of

different populations restricted to one area and variously adapted to contaminated sites. The stock of seeds available is also limited, and to our knowledge, no systematic nursery programs have been launched so far on a commercial basis. The consequence is that today there exist a very limited number of species and/or populations that could be used in a large-scale remediation scheme. Additionally, the mechanisms underlying hyperaccumulation are still poorly understood and results obtained so far are based on only few populations and species of hyperaccumulators. There is thus a need for both more choice in hyperaccumulator species or populations and more seed reservoirs.

In the case of *Thlaspi caerulescens,* a Zn, Cd and Ni hyperaccumulator (depending on the population tested) that has been recognized as an interesting model for studying hyperaccumulation (Assunçao *et al.,* 2003b), approximately 20 populations have been studied, mostly originating from metalliferous or serpentine sites (Baker *et al*., 1994b; Meerts & van Isacker, 1997; Schat *et al.*, 2000; Roosens *et al*., 2003, Schwartz *et al.*, 2003). Few non-metallicolous populations have been investigated so far for their tolerance and metal uptake ability. All these populations presented such intra- and inter-populations variations in elements uptake (Cd, Zn, Ni, together or individually), in amounts accumulated (Zhao *et al*., 2003a) and site characteristics, that it seems probable that populations originating from other areas may present different properties (Schat *et al.*, 2000). This is especially true for Cd uptake that varies a lot between populations leading to 1) bioaccumulation factors either below or above 1 (Zhao *et al*., 2003a), ratio set as the limit to define hyperaccumulation (Baker, 1981), and 2) tolerance criteria discriminating metallicolous from non-metallicolous populations. The latter have been found to be less tolerant but able to accumulate more Zn but less Cd than the former (Ingrouille & Smirnoff, 1986; Meerts & van Isacker, 1997; Escarré *et al*., 2000; Schat *et al.*, 2000). It is however interesting to note that two of the most studied populations, Prayon and Ganges, are both clearly metallicolous populations growing on Zn and Cd contaminated soils (Roosens *et al*., 2003). They nevertheless differ in their Cd uptake ability both in soil and hydroponics, Ganges hyperaccumulating Cd whereas Prayon not (Lombi *et al.*, 2000). The criterion “metallicolous” or “non-metallicolous” might thus not explain solely the differences observed between populations. Additionally, it is based on soil characteristics but there is no consensus on metal concentrations setting the limit between the two. A continuum is therefore likely to exist between the two types of populations for both tolerance and hyperaccumulation (Pollard *et al.,* 2002).

Differences in Cd uptake by various *T. caerulescens* populations have been attributed to different Cd transporters: in the non-hyperaccumulating population Prayon, Cd could be transported via Ca channels or Zn or Mn transporters (Zhao *et al*., 2002), whereas in the hyperaccumulating Ganges populations this could be performed via a high-affinity system for Fe (Lombi *et a*l., 2002; Roosens *et al.*, 2003). However, it has also been shown that Cd uptake kinetics by protoplasts extracted from the leaf mesophyll is similar for both populations (Cosio *et al*., 2004).

Whereas hyperaccumulation is only measured by metal concentration in shoots, tolerance can be measured by different parameters and in different setups that are chosen according to the metal and the species to be tested (Köhl & Lösch, 1999). The methods differ if acute toxicity symptoms or chronic effects have to be studied. The root elongation rate (short-term elongation test) have been proved to be a relevant parameter to assess tolerance to toxic concentrations that may eventually inhibit root growth, whereas the shoot biomass production is the most widely used parameter for testing chronic effects of toxic metals (Köhl & Lösch, 1999). Other parameters have been used: the total root length is also interesting because it expresses the potential for absorption of nutrients or water from soil (Atkinson, 2000). It has been found to show more statistical differences between experimental treatments than total root weight in the case of wheat (Box & Ramseur, 1993) and it is more important for solute uptake than root weight (Nye & Tinker, 1977).

Tests in soil resemble most the natural conditions. However, scientists tend to use hydroponics systems because it is difficult to establish standardized and reproducible conditions in pots, as well as a sufficient number of replicates. The main drawback is the lack of some of the important soil-plant system components such as the mycorrhiza and the rhizospheric microorganisms, but hydroponics allow a reduction of the number of parameters to be tested.

In a contribution to investigate the extent of Cd tolerance and hyperaccumulation variability within the *T. caerulescens* species as well as to find relevant parameters to test them, five natural populations from the Swiss Jura and the Prealpes were collected on non-metalliferous soils with various degrees of Cd concentrations and low Zn concentrations. They were compared to two well studied populations growing on metalliferous soils: Ganges hyperaccumulating Cd and Zn and Prayon hyperaccumulating only Zn in the field, and to *Thlaspi perfoliatum* collected also in the Jura but not known to present hyperaccumulating traits. All populations were grown in hydroponics with a range of Cd concentrations and were compared for their tolerance and Cd hyperaccumulation using root and shoot biomass, root length, root elongation rate and concentrations in shoots as discriminative parameters. To validate the hydroponics results, the most successful populations were tested in a Cd, Cu and Zn-contaminated soil for survival and metal accumulation.

3.3 Materials and Methods

3.3.1 Origin of the Thlaspi *populations*

Seeds of *T. caerulescens* were collected from four natural populations growing on the Jura chain in Switzerland (le Sentier, les Avattes, Gurnigel 1 and Gurnigel 2). These populations were selected to cover a large range of Cd concentration in the soil of origin and were located far away from each other (Table 3.1). One population was collected in the Prealpes (Leysin) at approximately the same altitude. These five populations were selected from a larger panel of 25 populations originating from both the Jura and the Alps and Prealps (Basic et al., unpublished). They were compared to the Cd and Zn hyperaccumulating Ganges population (Les Avinières, St-Laurent-le-

Minier, France) and the Zn hyperaccumulating Prayon population (Prayon, Belgium). One population of *T. perfoliatum*, also from the Jura (Bern canton) was used as control, non-hyperaccumulating plant. It is a smaller plant than both *T. arvense* and *T. caerulescens* but unlike *T. arvense* its growth rate and the time needed to obtain mature plants are rather similar to *T. caerulescens*. Additionally its ecology and physiology have been studied in details (Baskin & Baskin, 1979; Koch & Hurka, 1999). Thus it would be a good alternative to *T. arvense* as control plant. All seeds were collected the year before the experiment.

Table 3.1: Selected sites and soil characteristics where *T. caerulescens* and *T. perfoliatum* populations were collected (SD in parentheses).

Populations	Localization	Altitude (m)	pH_{CaCl2}[3]	C_{org}[3]	CEC[3] cmol kg^{-1}	Pseudo-total[4] mg kg^{-1}		Soluble[4] mg kg^{-1}	
						Cd	Zn	Cd	Zn
T. c. Le Sentier	Jura (CH)	1100	6.1	10.2	69	2.1 (0.0)	67 (0)	0.01	0.88 (0.30)
T. c. Leysin	Prealpes (CH)	1480	7.1	4.7	43	1.1 (0.1)	158 (78)	0.00 (0.00)	0.28 (0.39)
T. c. Les Avattes	Jura (CH)	1450	5.8	12.8	55	4.0 (0.7)	126 (7)	0.08	3.00
T. c. Gurnigel 1	Jura, Mt d'Amin (CH)	1320	5.9	12.7	60	9.2 (4.5)	214 (74)	0.02 (0.01)	0.37 (0.52)
T. c. Gurnigel 2	Jura, Mt d'Amin (CH)	1320	5.6	9.2	39	2.3 (0.2)	92 (4)	0.02	0.25
T. c. Ganges[1]	St-Laurent-le-Minier (F)	1000	6.4	1.6	5.86	20 (10)	3045 (1473)	0.29 (0.14)	20 (13)
T. c. Prayon[2]	Prayon (B)	-	4.7-6.9	-	-	667 (85)	75700 (13500)	-	-
T. perfoliatum	Witzwilmoos (CH)	433	-	-	-	-	-	-	-

[1] Les Avinières population ; [2] Roosens *et al.*, 2003: pH in 1:1 (w:v) H_2O; Total metal: digestion in 4:1 (v:v) HCl/HNO_3; [3]FAL (1998); [4]Pseudo-total = 2 M HNO_3-extractable; [4]Soluble = 0.1 M $NaNO_3$-extractable.

All populations were collected on undisturbed soils originating from calcareous bedrock, whereas Ganges and Prayon were collected on metalliferous soils in industrial or mining areas. The overall vegetation of the five Swiss *T. caerulescens* sites was exploited as meadow. Mature plants from the five local *T. caerulescens* and *T. perfoliatum* populations and from the Ganges population were sampled (3 to 4 individuals) and analysed for their heavy metal content (see below for the method).

Selected soils characteristics are presented in Table 3.1. For the Jura and the Prealpes populations, soil samples were taken directly next to the plants sampled. Data for the Prayon and the Ganges population are given for comparison and were either taken from the literature (Prayon: Lloyd-Thomas (1995) in Roosens *et al.* (2003)) or from soil samples collected at les Avinières (Ganges) but not necessarily

related to the place were the seeds had been collected. The Swiss soils were distinctly different from the metalliferous ones as they had larger carbon content, a larger CEC and lower Zn and Cd total and soluble concentrations. Cadmium concentrations measured in the soils of the Jura populations were high and above the level set for background concentration according to the Swiss law (OIS, 1998). It is also a lot higher than the median value of 0.31 mg kg^{-1} Cd calculated for agricultural topsoils in Switzerland (Meyer, 1991). This is a common characteristic of the soils from the Swiss Jura (Atteia *et al.* 1994). The origin of Cd has been shown to be lithogenic (Dubois *et al*., 1998; Baize & Sterckeman, 2001) and this is probably also the case for the Prealpes site.

3.3.2 Plant cultivation in hydroponics

Seeds were germinated in the dark on filters moistened with deionized water. After two weeks, plants were exposed to light and water was replaced by the modified quarter-strength Hoagland's nutrient solution (Sigma, St Louis, USA) supplemented with 20 µM Fe-HBED (Strem chemical, Newburyport, USA). Three-week-old seedlings (with two pairs of leaves) were then transferred to 60-mL tubes (one plant per tube) filled with the same nutrient solution. The plants were allowed to grow two weeks (till four pairs of leaves were obtained) in hydroponics before treatment with Cd was started. Six different treatments in four replicates were then performed: control (0 µM), 1, 5, 10, 20 and 50 µM Cd added as [$Cd(NO_3)_2.4H_2O$]. Germination rate was quantified after one week (data not shown) and just before transfer in tubes.

Germination and plant culture were performed in a climate chamber (day/night period 16/8 h, day/night temperatures 20°C/16°C, and a light intensity of 500 lux). The nutrient solution was renewed every four days and Fe(III)-HBED was prepared as described by Chaney *et al.* (1998) in such a way that all HBED was saturated with Fe.

3.3.3 Tolerance to Cd

Tolerance to increasing Cd concentrations was quantified by three parameters: biomass production (shoots and roots), elongation of the longest root (calculated from the regular increase of the longest root or its average increase) and total root length.

3.3.3.1 Biomass production

Shoots and roots were harvested separately and quickly rinsed with deionized water. They were dried at 80°C and the individual dry weights were recorded.

3.3.3.2 Elongation rate

The elongation rate was calculated over a period of three-week-exposure to Cd using the technique described by De Koe *et al.* (1992) and Schat & Ten Bookum (1992). Before starting the Cd treatments the longest root was measured. The root system was then immersed in a suspension of charcoal (High grade powdered active charcoal, Fluka, Buchs, Switzerland) and rinsed before being put back into the

nutrient solution. The charcoal coloured the roots in black and the new root parts remained white. The measurement was performed at each nutrient solution renewal, which means five times within three weeks. The elongation was obtained by measuring the growth of the longest root defined by the length of the white part of the root. After six weeks the longest root was measured again.

Two types of data were obtained:

- the elongation rates calculated from the regular increase of the longest root growth of each plant (measurement five times within three weeks),
- the average elongation rate calculated from the difference in growth between the beginning and the end of the Cd treatment of the longest root.

The average elongation rate was calculated after Parker (1995) as follows:

ER (mm day^{-1})=[Average (final longest root length - initial longest root length)/(time of Cd exposure)].

The calculation of the Relative Net Elongation (RNE) as defined by Parker (1995) was also performed. It is calculated as the percentage of growth of the longest root of the treated plants related to the growth of the longest root of the control plants. It did not give consistent results and is not further detailed.

3.3.3.3 Total root length

It was measured using a desktop scanning device and is expressed as total root length per plant. Total surface areas as well as the distribution of roots within 16 diameter classes were also recorded. However, they did not give additional results and were thus not further detailed. Roots were spread in a glass tray in 2 to 3 mm of water. The software WinRHIZO (version Pro 5.0A, Régent Instruments, Quebec, Canada) was used to analyse images acquired using a desktop scanner STD1600 (Epson) provided by Régent Instruments. It is equipped with a positioning system (for trays receiving the roots) and two light sources preventing shadows and allowing for distinction between overlapping roots and forks. Limitations and accuracy of the technique have been tested by Bauhus & Messier (1999). All measurements were carried out at a resolution of 400 dpi.

3.3.3.4 Tolerance indexes

A tolerance index (Wilkins, 1978; Baker, 1987; Baker *et al*., 1994b) was calculated for the shoot and the root biomass as well as for the total root length as it follows:

TI_x (%)=Σ_1^4 [(x of the Cd treated plants)*100/ (average x of four control plants)]/4 with either,

x = dry matter weight of shoots for the tolerance index calculated for the shoot biomass (TI_s),

or, x = dry matter weight of roots for the tolerance index calculated for the root biomass (TI_r),

or, x = total root length for the tolerance index calculated for the root length (TI_l).

3.3.4 Characterisation of the hyperaccumulation

Element concentrations were measured after six weeks of plant exposure to Cd in the shoots of the different populations tested. Samples (previously dried and weighed for biomass) were ground in a mill made of titanium (ZM (type), Retsch, Haan, Germany) and hot-digested in HNO_3 65% sp. (Fluka, Buchs, Switzerland) and $HClO_4$ 70% p.a. (Fluka, Buchs, Switzerland). Cadmium concentration was measured in the digests by ICP-AES (Perkin Elmer Plasma 2000, Wellesley, USA).

3.3.5 Plant cultivation in pots

Seeds of les Avattes, le Sentier, Leysin, Gurnigel 1 and Ganges populations were germinated and grown for two months in a commercial autoclaved compost. They were regularly watered with deionised water till they had developed four pairs of leaves. They were then transferred into pots filled with 1 kg of air-dried soil homogenised and sieved to 1 cm. The soil was a heavy metal contaminated soil collected at Caslano (Southern part of Switzerland) that has already been described by Hammer & Keller (2003). Selected soil characteristics are presented in Table 3.2. The Ganges population had proved to grow well on this soil (Hammer & Keller, 2002). Six weeks before transferring the plants, a ground level fertilisation (130 mg kg^{-1} N (NH_4NO_3), 130 mg kg^{-1} P ($Ca(H_2PO_4).2H_2O$), 180 mg kg^{-1} K (50% as KCl and 50% as K_2SO_4), and 40 mg kg^{-1} Mg ($MgSO_4$)) was applied to all pots. Between eight and 14 replicates per population were harvested after eight weeks of growth in pots (the initial time span of 12 weeks was reduced because of plant mortality) for les Avattes, le Sentier, Leysin, and Ganges populations. Another set of the Ganges population (four replicates) and the Prayon population (three) were grown for 12 weeks and the Gurnigel 1 population (four) for one year. This latter population was initially rejected for the pot experiment because of the very small size of the indigenous population and thus the limited seed availability. Again as a control, *T. perfoliatum* was also tested in pot but was left to grow 14 weeks to reach maturity. Shoots were further processed as described for the hydroponics, and were analysed for Cd, Zn and Cu by ICP-AES.

Table 3.2: General characteristics of the contaminated soil used in the pot experiment to test the different *T. caerulescens* populations (SD in parentheses).

	Contaminated soil
pH_{CaCl2}	5.2 (0.3)
% C_{org}	6.3 (1.9)
% Clay	13 (5)
% Silt	19 (4)
% Sand	68 (8)
CEC_{pot}[1] in meq 100 g^{-1}	28 (4)
Cd_{tot}[2] in mg kg^{-1}	2.8 (0.7)
Zn_{tot} in mg kg^{-1}	1158 (216)
Cu_{tot} in mg kg^{-1}	264 (43)
Cd_{sol}[3] in µg kg^{-1}	13 (11)
Zn_{sol} in mg kg^{-1}	7.4 (5.9)
Cu_{sol} in mg kg^{-1}	0.4 (0.1)

[1]CEC cation exchange capacity (FAL, 1998); [2]Pseudo-total = 2 M HNO_3-extractable; [3]Soluble = 0.1 M $NaNO_3$-extractable . [2] and [3](FAC, 1989).

3.4 Results

3.4.1 Metal concentrations in the shoots of the plants collected in the field

The results are presented in Table 3.3. Except at Leysin, the local plants of *T. caerulescens* had Cd concentrations in their shoots higher than the hyperaccumulation level set at 0.01% Cd in DM (Reeves & Baker, 2000) whereas Zn concentrations, although very high, never exceeded the 1% threshold for hyperaccumulation (although Gurnigel 1 plants were relatively close to it). However metal transfer coefficient calculated as the ratio between metal concentration in *T. caerulescens* shoots and total metal concentration in the initial soils, was around 40 for Zn and the populations originating from the Jura, whereas for Cd it laid between 50 and 140. At Leysin, it was lower for both Cd and Zn (respectively 19 and 24). Cadmium and Zn concentrations measured in shoots of *T. perfoliatum* were low and in the range found for non-accumulating plants (Sauerbeck, 1989).

3.4.2 Germination ability

The percentage of germination gives information on the seed vigour and the ability to produce plants in the field. The results are presented in Figure 3.1. More than 100 seeds were put to germinate, except for the Gurnigel 1 population (25 seeds). In the same conditions and without Cd, *T. perfoliatum* gave the best results and, together with Gurnigel 1 had a germination rate above 50%. Seeds from Ganges and Prayon and the other populations gave similar results with a germination rate between 30 and 50%, whereas only 10% of the seeds from the Gurnigel 2 population were able to give

viable plants. This population was therefore further tested in hydroponics for only some of the Cd concentrations and not tested at all in pots.

Table 3.3: Selected element concentrations in shoots of *T. caerulescens* and *T. perfoliatum* collected in the field (n= 3 or 4 individual plants, SD in parentheses).

	Cd	Fe	Mg	P	Zn
Populations			mg kg^{-1} in DM		
Le Sentier	102 (26)	578 (325)	1597 (606)	2490 (932)	2881 (1651)
Leysin	24 (15)	581 (323)	1644 (303)	1736 (102)	3730 (2581)
Les Avattes	285 (191)	349 (126)	2562 (794)	4071 (3198)	4607 (2403)
Gurnigel 1	505 (91)	430 (198)	3132 (299)	2020 (729)	8794 (1173)
Gurnigel 2	327 (10)	1484 (725)	2111 (364)	2424 (243)	4156 (2285)
Ganges	1508 (364)	1822 (1729)	6080 (2799)	1982 (476)	17653 (8173)
T. perfoliatum[1]	0.6	1186	-	-	11

[1] pooled sample

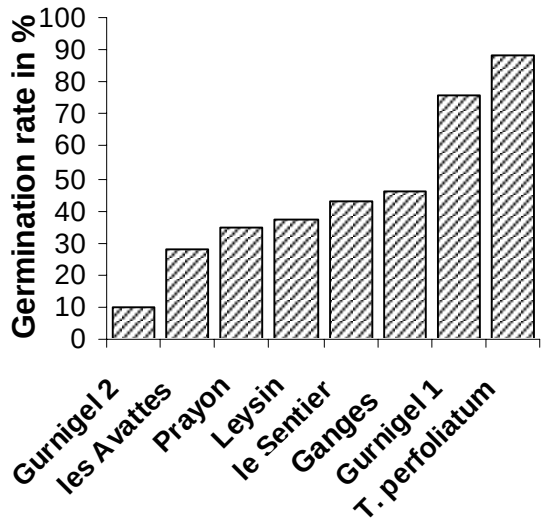

Figure 3.1: Germination rate of the different populations of *T. caerulescens* and *T. perfoliatum*.

3.4.3 Biomass and root parameters measured on plants grown in hydroponics

For all populations the shoot and root biomass as well as the total root length decreased with increasing Cd concentrations (Table 3.4 and Figure 3.2). *Thlaspi perfoliatum* was the most sensitive with no survival above 10 µM and a sharp decrease of both the shoot and the root biomasses at 1 µM Cd already. The shoot biomass production as well as the TI_S gave the same order for the populations:

Prayon was the most tolerant, followed by Ganges, les Avattes, Gurnigel 2, le Sentier and then Leysin and Gurnigel 1, although the differences were not always significant. It has to be noted that for les Avattes the shoot biomass presented a sharp decrease at 1 µM but then it did not decrease further till 50 µM. The total root length (Figure 3.2) gave a clear segregation between Prayon, les Avattes, le Sentier, Gurnigel 2 on one hand and Gurnigel 1, Leysin and *T. perfoliatum* on the other hand: the former did not show any change in total root length with increasing Cd concentration whereas the latter exhibited a decreased root length with increasing Cd concentration in the nutrient solution. When TI_l was calculated, Prayon appeared less tolerant than les Avattes and le Sentier. Observing the root biomass (Table 3.4), Prayon appeared to be again the most tolerant with an initial and subsequent higher biomass, whereas Ganges did not differ from the other populations tested giving intermediate results between les Avattes and le Sentier on one side and Leysin and Gurnigel 1 on the other side.

Table 3.4: Biomass production (mg per plant) of the different *T. caerulescens* and *T. perfoliatum* populations grown in hydroponics with increasing Cd concentrations. Values are average means (n=4 individual plants, SD in parentheses). Letters refer to the significance of the differences between treatments: for a given population, similar letters mean no difference between the means and different letters mean significant differences between means with *P*<0.05.

	Cd concentrations in nutrient solution (µM)					
Population	0	1	5	10	20	50
Shoot biomass						
Le Sentier	202 (42)a	201 (41)a	112 (45)b	79 (33)b	63 (5)b	70 (18)b
Leysin	228 (27)a	189 (18)a	161 (12)b	56 (11)c	37 (10)d	28 (7)d
Les Avattes	191 (46)a	83 (20)b	87 (38)b	121 (47)ab	77 (17)b	88 (29)b
Gurnigel 1	291 (104)a	232 (48)a	94 (39)bc	81 (10)b	54 (13)c	54 (23)cb
Gurnigel 2	nd[1]	200 (25)a	93 (52)b	121 (53)b	nd[1]	nd[1]
Ganges	205 (37)a	170 (16)a	138 (61)ab	131 (39)b	85 (52)b	101 (37)b
Prayon	235 (73)a	239 (47)ac	242 (5)a	287 (52)a	202 (20)bc	132 (58)b
T. perfoliatum	184 (25)a	23 (7)b	16 (7)b	20 (10)b	nd[1]	nd[1]
Root biomass						
Le Sentier	18.4 (2.6)a	18.8 (3.7)a	17.0 (5.1)ab	12.8 (4.2)ab	11.6 (2)b	11.6 (6.0)ab
Leysin	17.2 (4.5)a	17.1 (4.3)a	17.8 (3.8)a	10.5 (2.1)b	6.4 (0.8)c	5.7 (0.9)c
Les Avattes	14.4 (3.7)a	9.7 (2.5)ab	11.2 (4.7)ab	14.1 (2.6)b	11.2 (2.6)ab	10.7 (2.3)ab
Gurnigel 1	24.4 (6.8)a	16.0 (9.5)abc	11.6 (7.1)bc	10.2 (1.8)b	6.9 (0.8)c	7.6 (2.5)bc
Gurnigel 2	nd[1]	25.7 (3.9)a	13.5 (3.5)a	16.7 (6.6)a	nd[1]	nd[1]
Ganges	25.3 (3.7)a	24.3 (5.2)a	20.8 (9.3)ab	20.7 (6.8)ab	12.8 (6.4)b	14.0 (6.5)b
Prayon	21.4 (7.7)ab	23.9 (4.6)a	22.9 (5.0)ab	24.2 (5.0)a	19.1 (2.7)ab	15.5 (4.4)b
T. perfoliatum	13.1 (3.3)a	2.8 (1.2)b	1.9 (0.9)b	2.2 (1.3)b	nd[1]	nd[1]

[1] nd: no data because plant did not survive due either to poor initial fitness (Gurnigel 2) or Cd toxicity (*T. perfoliatum*).

Plotting the aboveground biomass versus the root biomass did not yield any additional information. The relations obtained between the tolerance indexes calculated for the shoot biomass (TI_s) and TI_r or between TI_s and TI_l did not differ between populations. However, for the populations from Leysin, les Avattes, le Sentier and Gurnigel 1 the shoot biomass response was more pronounced (larger decrease) than the root parameters to increased Cd concentrations in solution, whereas for Prayon and Ganges shoot biomass and root parameters decreased proportionally.

An average elongation rate (ER) was calculated from the difference between the lengths of the longest root at the end and at the beginning of the experiment. The results presented in Table 3.5 do not exhibit any clear trend, neither for a given population across the increasing Cd concentrations, nor between populations for a given Cd concentration in solution. Additionally, there was a large variability between replicates. Populations from les Avattes and le Sentier were the only ones showing no effect of the Cd treatment on the ER. Leysin, Ganges and Prayon gave similar uneven results. *Thlaspi perfoliatum* did not show any change in the longest root growth until the plant died. Observation of the regular increase of the longest root every three days did not show any change and thus did not yield additional information.

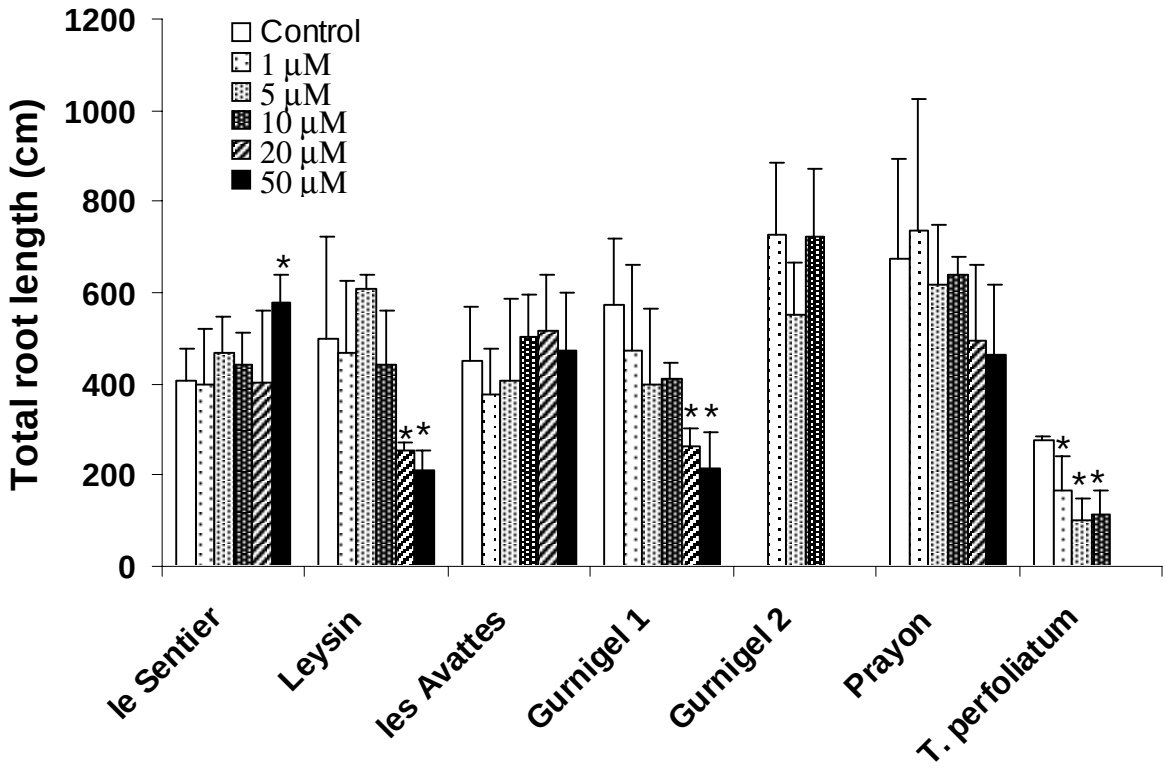

Figure 3.2: Total root length (cm) measured for the different *T. caerulescens* and *T. perfoliatum* populations grown in hydroponics with increasing Cd concentrations. Values are average means of 4 replicates with standard deviation. "*" refers to the significance (with $2P<0.05$) of the differences between a treatment and the control for a given population.

Table 3.5: Mean elongation rate (ER in mm d^{-1}) calculated from the final length of the longest root minus the initial length of the same root for the different *T. caerulescens* and *T. perfoliatum* populations grown in hydroponics with increasing Cd concentrations (SD in parentheses).

	Cd concentrations in nutrient solution in µM					
Population	0	1	5	10	20	50
Le Sentier	1.0 (0.6)	1.3 (0.5)	1.2 (0.6)	1.0 (0.2)	1.1 (0.4)	1.3 (0.3)
Leysin	0.2 (0.1)	0.2 (0.1)	0.1 (0.2)	0.9 (0.3)	0.4 (0.4)	0.3 (0.2)
Les Avattes	0.9 (0.8)	1.1 (0.7)	1.3 (1.3)	0.4 (0.4)	0.9 (0.4)	0.5 (0.3)
Gurnigel 1	2.3 (0.3)	0.5 (0.4)	1.1 (1.3)	0.3 (0.3)	0.3 (0.3)	0.3 (0.2)
Ganges	0.4 (0.3)	0.2 (0.2)	0.8 (0.4)	0.2 (0.3)	0.9 (0.4)	0.4 (0.1)
Prayon	0.6 (0.1)	1.0 (0.3)	0.7 (0.1)	0.3 (0.2)	0.4 (0.3)	0.3 (0.2)
T.perfoliatum	0.4 (0.1)	0.6 (0.4)	0.4 (0.7)	0.6 (0.6)	nd[1]	nd[1]

[1]nd: no data because plant did not survive due to Cd toxicity.

3.4.4 Cadmium concentrations measured on plants grown in hydroponics

The results are presented in Figure 3.3. The largest differences between the populations were observed at 1 µM Cd: all populations were able to accumulate 1% Cd or more in their aboveground biomass except Prayon and *T. perfoliatum*. Cadmium concentrations in shoots of *T. perfoliatum* were 0.4, 10, 62 and 129 mg kg^{-1} DM, for respectively 0, 1, 5, and 10 µM treatments and were thus clearly below Cd concentrations in Prayon plants for the same concentrations in solution. The five Swiss populations tested were not significantly different from Ganges. At higher concentrations in the nutrient solution Cd concentrations in plants increased except for Ganges at 50 µM Cd. When calculating ratios between the concentrations measured at 1 µM Cd and the concentrations at the other treatments, all populations presented the same increase except Prayon at 50 µM and les Avattes that gave the lowest ratios (2, 3, 6 and 15 for 5/1, 10/1, 20/1 and 50/1 resp.).

3.4.5 Validation in contaminated soil

The results are presented in Table 3.6. The populations chosen from the results of the hydroponics experiment (le Sentier, Leysin and les Avattes) did not grow well in the contaminated Caslano soil. They had to be harvested one month before the planned term because of reduced growth, chlorosis (although an Fe leaf treatment partially remediated the symptoms) and death of several plants. Ganges, Prayon, Gurnigel 1 and *T. perfoliatum* populations grew well and were harvested later. For comparison, a set of the Ganges population was harvested at the same time as the three non-tolerant populations. As expected Cd and Zn concentrations were low in the shoots of *T. perfoliatum.* Low Cd concentrations measured in the shoots of le Sentier, Leysin and les Avattes populations were more surprising compared to Ganges and Prayon. The time of exposure to the soil did not seem to be the reason of this low performance because Ganges gave similar Cd concentrations at eight and 12 weeks. On the contrary, Zn concentrations measured in these three populations were higher than in Ganges or Prayon, whereas Cu concentrations were similar in all plants.

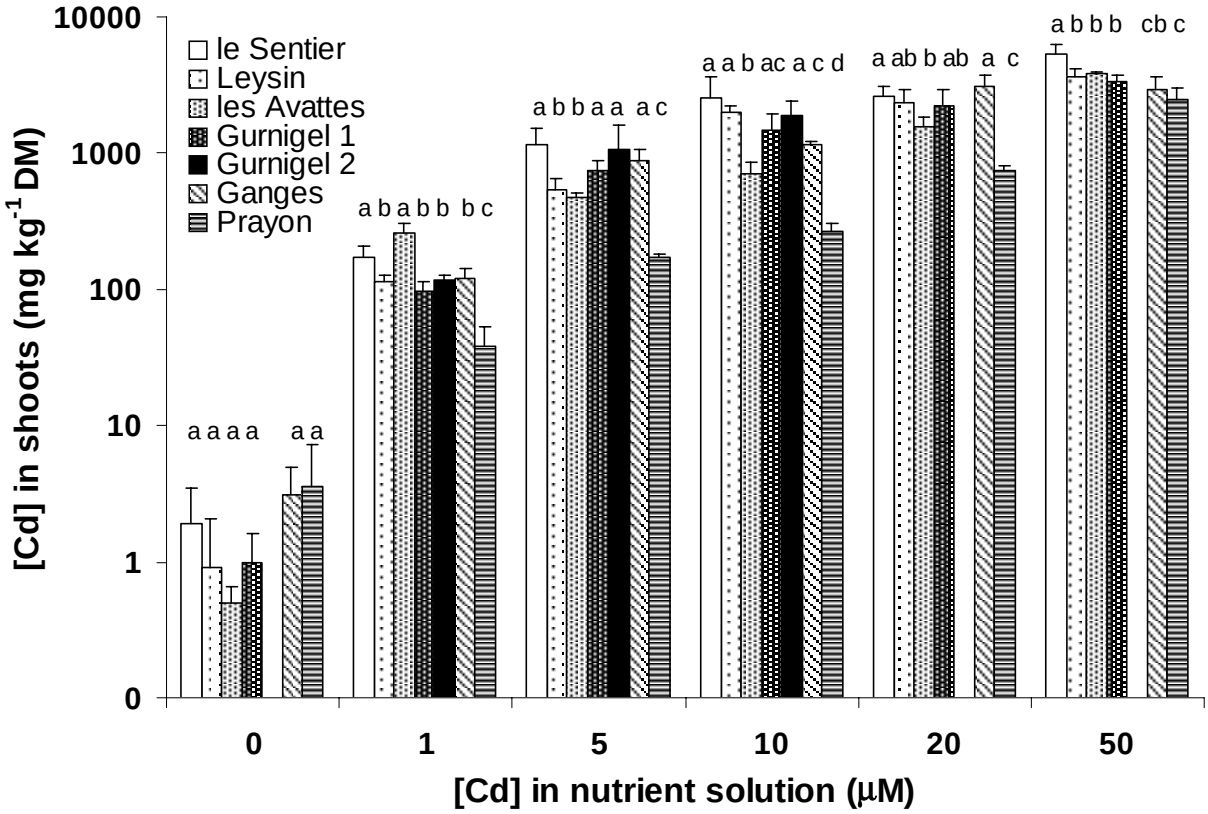

Figure 3.3: Cadmium concentrations (mg kg^{-1} DM) in shoots of the different *T. caerulescens* and *T. perfoliatum* populations grown in hydroponics with increasing Cd concentrations. Values are average means (n=4) with SD. Letters refer to the significance of the differences between populations: for a given treatment, similar letters mean no difference between the means and different letters mean significant differences between means with $P<0.05$.

Table 3.6: Biomass production (g per pot) and several metal concentrations (mg kg^{-1} DM) in the different *T. caerulescens* populations and *T. perfoliatum* tested in the contaminated soil from Caslano. See text for the explanation of the different times in pot (SD in parentheses).

Population	Time in pot	Biomass (g per pot)	Concentration in shoots (mg kg^{-1})		
			Cd	Zn	Cu
le Sentier[1]	8 weeks	0.06 (0.02)	5	6626	16
Leysin[1]	8 weeks	0.11 (0.08)	8	9693	23
les Avattes[1]	8 weeks	0.11 (0.06)	6	7125	16
Gurnigel 1	one year	8.10 (3.53)	7 (3)	7099 (2053)	22 (3)
Ganges[1]	8 weeks	0.49 (0.15)	64	5513	9
Ganges	12 weeks	1.19 (0.37)	58 (16)	5531 (524)	12 (3)
Prayon[2]	12 weeks	0.75 (0.45)	18 (1)	3826 (702)	17 (7)
T. perfoliatum	14 weeks	0.51 (0.27)	0.9 (0.2)	1089 (250)	nd[3]

[1]concentrations were measured on mixed samples of 4 replicates because plants were too small; [2] 3 replicates only; [3] not determined.

3.5 Discussion

3.5.1 Germination

The germination rate did not differ between the Swiss populations and Ganges and Prayon. Gurnigel 1 population is probably interesting because it seems easy to propagate by seeds, however, the population is very small and regular seeding remains uncertain. The poor results obtained by the Gurnigel 2 population could be due to the size of the population that was very small (although larger than Gurnigel 1). This characteristic is known to decrease the overall fitness of the population through an increase in inbreeding (Ellstrand & Elam, 1993).

3.5.2 Hyperaccumulation and tolerance in nutrient solution

In solution, populations differed in Cd accumulation. Growing plants in 1 µM Cd seemed to be an adequate medium to discriminate the various populations tested for hyperaccumulation because: 1) this is a concentration close to concentrations likely to be found in soil solutions, 2) it discriminated between Ganges and Prayon that are respectively hyperaccumulating and not hyperaccumulating in nature, 3) except for *T. perfoliatum*, this concentration did not induce any obvious toxicity effect and thus can be considered as below the NOEC (no observed effect concentration), which allows to distinguish between hyperaccumulation as a physiological trait from metal-accumulation as a toxicity effect (Köhl & Lösch, 1999) and 4) this was the only concentration for which it was possible to find a relationship between the level of Cd accumulation in hydroponics of the five Swiss populations tested and their behaviour in the field. Indeed, concentrations in plants grown at 1 µM Cd were negatively correlated with concentrations of $NaNO_3$-extractable Zn measured in the field, and less with $NaNO_3$-extractable Cd, probably because of Cd concentrations close to the detection limits. More importantly, Cd concentrations in plants (at 1 µM Cd) were negatively correlated with the ratio between Cd or Zn concentrations in the plants grown in the field and bioavailable Cd and Zn in the field (Figure 3.4a, b). When Ganges was included in the calculation the relationship was less obvious, but this may be explained by the fact that plants from Ganges were not collected exactly where the soil samples had been taken. This means that the more Cd is available in its original soil, the more the population is able to accumulate in solution (in concentrations close to soil solution concentrations). Although soil concentrations were not in the same range, Escarré *et al*. (2000) also observed a higher Cd uptake by metallicolous populations (populations growing on metalliferous soils) when compared to non-metallicolous ones. However, the reason why a population that was efficient in the field (large ratio $[Cd]_{\text{plant shoots in the field}}/[NaNO_3\text{-extractable Cd}]_{\text{soil}}$) took up less Cd in 1 µM than a less efficient one is unclear.

From our results we thus cannot say that *T. caerulescens* was more efficient when Cd in soil was lower, probably because the range of Cd concentration in the soils was limited. But it is also clear that the characteristics of the soil on which these populations have grown are of up most importance to understand and predict the behaviour of the *T. caerulescens* populations in hydroponics. It is not possible to know whether the hyperaccumulation capacity is a constitutive trait that may evolve

differently according to metal concentrations in the soil or if it was induced by pre-exposure to the soil. However, our results suggest that it might be important to keep in mind that pre-exposure of the plant (or the mother plant) may determine the final metal concentration in plant. This has also been observed at the cell level in experiments with mesophyll protoplasts extracted from *T. caerulescens* and *Arabidopsis halleri* leaves where pre-exposure of the plants to Cd led to changes in Cd uptake by protoplasts (Cosio *et al*., 2004).

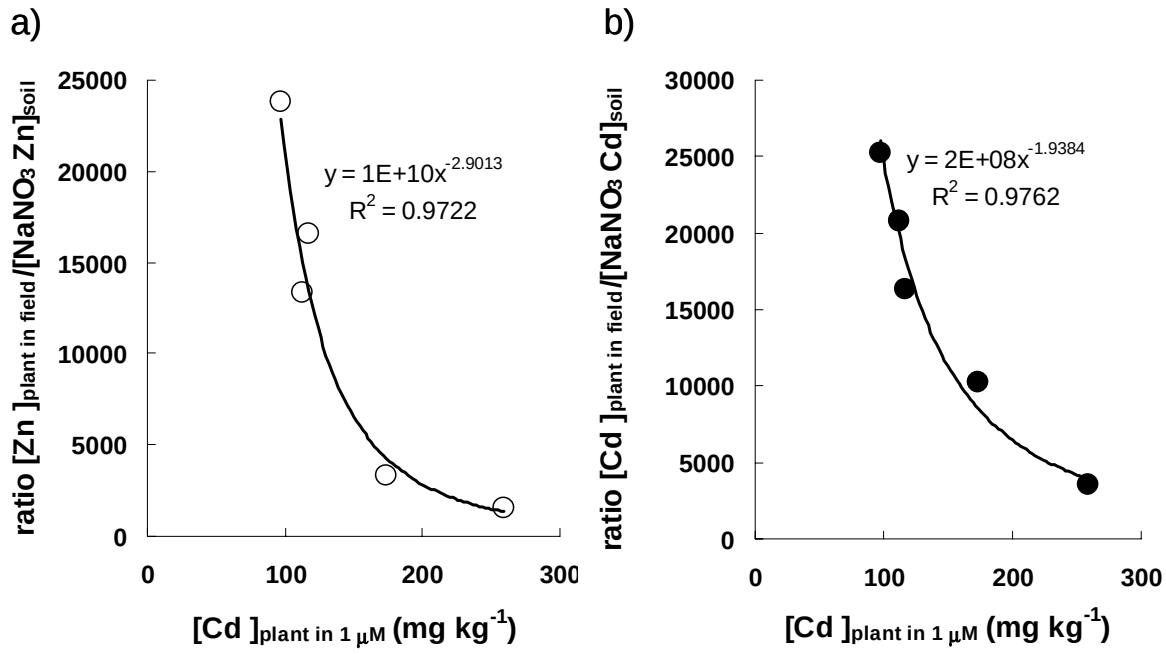

Figure 3.4: Relationship between Cd concentrations (mg kg^{-1} DM) in shoots of the different populations grown in hydroponics with 1 µM Cd in solution and the ratio [metal concentration in shoots of plants collected in the field] / [$NaNO_3$-extractable metal measured in the topsoil of the soils of origin]. a) Zn and b) Cd.

The different parameters tested to discriminate the different populations for their tolerance to Cd did not give consistent results when compared to each other. Moreover, there was a large variability between replicates leading to no significant differences between populations and treatments for a given parameter. In general, these differences could not be related to the initial soil characteristics too, except for the 20 and 50 µM treatments for which there was a positive exponential relationship (R^2=0.898) between the shoot biomass and the $NaNO_3$-extractable Cd in soil, indicating an increased tolerance to high Cd concentrations in populations originating from soils with larger bioavailable Cd. Escarré *et al.* (2000) found the same relationship while taking into account total instead of bioavailable Cd in soil.

The factor discriminating best between populations and also the most sensitive

to increasing Cd concentrations was the shoot biomass as also found by Cieslinski *et al.* (1996) for strawberry plants. This parameter is the most widely used for assessing metal tolerance in long-term growth tests (e.g. Ebbs & Kochian, 1997). Cadmium affected less the root system than the shoot biomass, but total root length was the most sensitive root parameter to metal treatment. Total root length has been indeed proposed as the best root parameter to quantify tolerance to Cd in long-term growth tests (Köhl & Lösch, 1999). The average elongation rate as well as the root dry weight and the daily elongation rate (measurement of the longest root growth 5 times within 3 weeks) were not the best parameters to discriminate between the different populations tested because little variation was measured. The ER is indeed more suitable for short-term growth tests (Schat & Ten Bookum, 1992). However the limited effect of Cd on root system is consistent with the idea that *T. caerulescens* roots forage for Cd and Zn (Schwartz *et al.,* 1999; Whiting *et al.,* 2000) and that consequently roots may develop well even at very high concentrations in the medium.

Several authors have discriminated between metallicolous and non-metallicolous populations, clearly placing Ganges and Prayon in the former category. The non-metallicolous populations have been found to tolerate and accumulate less Cd than the metallicolous ones (Escarré *et al*., 2000). From our results, it is clear that 1) Prayon is more tolerant than Ganges and 2) it accumulates less Cd than Ganges when confronted to close to natural Cd concentrations. The Ganges population has, however, evolved in a metalliferous environment as shown by our soil analyses as well as by other authors (Roosens *et al.*, 2003) and, according to this sole criterion, should be as tolerant and accumulating as Prayon. Our populations present a high capability of Cd hyperaccumulation in solution combined with a tolerance similar to either Prayon or Ganges: according to the results of the shoot biomass at 50 µM Cd, Prayon was the most tolerant, followed by Ganges, les Avattes, Gurnigel 2, le Sentier and then Leysin and Gurnigel 1, although differences were not always significant. On the contrary, at 1 µM Cd les Avattes was the least tolerant population while the other four natural populations behaved like Prayon and Ganges. However, for the five Swiss populations soil metal concentrations (Cd, Zn and other metals (data not shown)) were low according to Swiss standards: all metal concentrations in initial soils were below Swiss Guide values (OIS, 1998) except Cd that was above the threshold set at 0.8 mg kg^{-1} in 2M HNO_3 extracts. All values for $NaNO_3$-extractable (bioavailable) Cd were below the guide value set at 0.02 mg kg^{-1}. These Cd values were lower than those obtained for Prayon and Ganges soils. So, although the Swiss populations did not originate from a metalliferous environment, they behaved in solution like Ganges and Prayon. These results may be an indication that tolerance is not (only) related to initial soil characteristics but could be a constitutive trait (Schat *et al.,* 2000).

At last, from the results of the experiment in hydroponics, we could not find any clear relation between hyperaccumulation and tolerance as also observed by Schat *et al.* (2000) and Assunção *et al.* (2003a). Plotting the shoot biomass versus Cd concentrations did not discriminate the populations and more importantly, the general decrease of biomass observed with the increase in Cd concentration in shoots did not follow the same function. For a given concentration in plants, no difference could be found between different populations. As a consequence, it appeared that the origin of the populations did not seem to influence their tolerance to Cd in solution, unlike observed in soils by Escarré *et al.* (2000).

3.5.3 Validation in contaminated soil

In contaminated soil these five populations accumulated less Cd than Ganges, but more Zn than Ganges and Prayon. However they did not adapt well to the soil. It is difficult to conclude on a reduced tolerance to Cd or Zn contamination compared to Ganges and Prayon because other contaminants were present and also because soil characteristics were very different from their soils of origin. For example it has been shown that *T. caerulescens* is sensitive to Cu (McLaughlin & Henderson, 1999; Lombi *et al.,* 2001a). In fact, Schat *et al.* (2000) stated that hyperaccumulators were not inherently tolerant to high metal concentrations in soil and Assunção *et al.* (2003a) have shown that high level of tolerance is confined to metals that are at toxic levels in the soil of origin. However, the reasons why the five Swiss populations hyperaccumulated Cd when growing on their soils of origin and in hydroponics and did not hyperaccumulate on the Caslano soil are unclear. Indeed, in this soil Zn concentrations in plants were large, while Zn concentrations found in their initial soils were low. This is consistent with the idea that non-metallicolous populations accumulate more Zn than metallicolous ones (Meerts & van Isacker, 1997; Escarré *et al.*, 2000) as well as with the results obtained by Assunção *et al.* (2003a) who have found that concentrations in shoots of *T. caerulescens* plants growing on soils with less than 150 mg Zn kg^{-1}, were above 1000 mg Zn kg^{-1} in DM. But Escarré *et al.* (2000) also found that metallicolous populations accumulated more Cd than the non-metallicolous ones. Although it seemed to be the case for the five Swiss populations (if they are considered as non-metallicolous) when tested in contaminated soil, it is not the case at all when they had grown on their original soils. One explanation would be that Cd concentration in the Caslano soil is low in comparison with the other contaminants (which is not the case in their soils of origin) and that the latter may reduce Cd uptake through ion competition (Costa & Morel, 1993). Indeed, Zhao *et al*. (2002) and Roosens *et al.* (2003) found a reduction in Cd uptake by the Prayon population when Zn was added to the nutrient solution. Lombi *et al.* (2000) also suggested that competition between Zn and Cd could explain the reduced Cd uptake by different *T. caerulescens* (including Prayon and Ganges) populations grown in soils that were amended with 250 mg kg^{-1} Cd and 2000 mg kg^{-1} Zn compared to the same populations grown in 250 mg kg^{-1} Cd and 100 mg kg^{-1} Zn. Alternatively, it would also be possible that Cd uptake mechanisms are similar in the five Swiss populations and Prayon when they are grown in soil because these mechanisms may be related to similarities in uptake at the root level, and thus would have nothing to do with their environmental background.

Because of their geological history and their consistently high Cd concentrations (Atteia *et al.,* 1994), the soils of the Jura are unique. Except for *T. caerulescens*, Cd concentrations in shoots of plants growing on these soils are low (Cd remains in the roots) and in the topsoil the Cd available fraction is limited and originate mainly from the turnover of the vegetation (Benitez, 1999). Although it has been shown that *T. caerulescens* does not access a different Cd pool than non-accumulating plants (Hamon *et al.,* 1997; Gérard *et al.,* 2000), testing Prayon and Ganges on a range of these soils may provide information on Cd availability to *T. caerulescens* and clarify the specificity of the different *T. caerulescens* populations. Alternatively, the understanding of the mechanisms (and their origin) behind the difference observed between Ganges and Prayon would be the first step towards the

understanding of other *T. caerulescens* populations.

In general, and from an applied point of view, the first limiting parameter for the use of various populations in phytoextraction was the amount of available seeds and their viability. However, from the results in hydroponics and pots, it seems that limited differences in tolerance and accumulation between populations of *T. caerulescens* (as compared to other plants), the uncorrelated relationship between tolerance and hyperaccumulation, as well as the difficulty to define the best conditions for optimal growth and metal removal, may allow and even speak in favour of the use of a mix of seeds for an efficient phytoextraction.

3.5.4 Thlaspi perfoliatum

Thlaspi perfoliatum, although non-accumulating Cd, was found to have a limited tolerance to Cd in solution: the large Cd concentrations measured in its shoots can probably be attributed to a general breakdown of the root system. However it appeared surprisingly more tolerant in the pot experiment than the Swiss *T. caerulescens* populations. Also, it appeared to be more tolerant than the *T. arvense* population previously tested in the Caslano soil (Hammer & Keller, 2002) and commonly used in the literature as reference plant for non-hyperaccumulation (Lasat *et al*., 1996; Ozturk *et al.*, 2003). Cadmium and Zn concentrations measured in the shoots of *T. perfoliatum* were similar to those measured in the shoots of natural regrowth harvested in the field at Caslano (Hammer & Keller, 2003). These results confirmed that *T. perfoliatum* would be a good alternative to *T. arvense* as control plant.

3.6 Conclusion

The characteristics of the soil on which the populations had been collected were of up most importance to predict the behaviour of the *Thlaspi* populations in hydroponics. However, the criterion “metallicolous” or “non-metallicolous” did not seem to explain solely the differences observed between populations. Besides, it was not possible to know whether the hyperaccumulation capacity was a constitutive trait or if it was induced by pre-exposure of the mother plant to the soil. The understanding of the mechanisms (and their origin) behind the difference observed between Ganges and Prayon would be the first step towards the understanding of other *T. caerulescens* populations.

4

Localization of Cd in leaves of *Thlaspi caerulescens* and preliminary screening of Cd-responsive genes in Brassicaceae species

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Extended version of a paper in preparation for the Journal of Experimental Botany

4.1 Abstract

Cadmium effect on biomass production and Cd accumulation into two Cd hyperaccumulating species, *Thlaspi caerulescens* Ganges and *Arabidopsis halleri* and two non-hyperaccumulating species *T. caerulescens* Prayon and *A. thaliana* grown in nutrient medium containing varying concentrations of Cd (0, 5, 10, 50, and 100 µM) was monitored. A reduction in roots and shoots biomass and growth was observed with increasing Cd concentrations. On the contrary Cd accumulation increased with increasing Cd concentrations. Prayon was the most tolerant to Cd although it showed necrosis at high Cd concentrations, whereas *A. halleri* accumulated the highest Cd concentrations in leaves.

In leaves and cells of Ganges and Prayon, autoradiographs, SEM-EDXMA and tissue fractionation (apoplasm, cell walls, mesophyll protoplasts and lower epidermis) showed that Cd was distributed both inside the cells and in the cell walls, mainly in the large epidermal cells but also in small epidermal cells and sometimes in the mesophyll cells. Cadmium localization was clearly correlated with the necrotic spots observed on Prayon leaves. Cadmium-responsive genes were eventually screened through the use of DNA microarrays. Based on these results, we suggest that metal storage in the studied plants demands the involvement of more than one compartment.

4.2 Introduction

In view of the risk posed by Cd as environmental pollutant (Sanità di Toppi and Gabbrielli, 1999; Kabata-Pendias and Pendias, 2001), there has been interest in developing the use of hyperaccumulating plants to extract Cd from contaminated soils (Brown *et al.*, 1995; Hammer and Keller, 2003). However a lack of understanding of the mechanisms involved in heavy metal hyperaccumulation prevents the optimization

of the technique. A research priority is therefore to gain basic information on sink capacities and transport mechanisms of Cd into the cells of hyperaccumulating species.

Knowledge of Cd intracellular localization in leaves is necessary to further understand *Thlaspi caerulescens* specificities. Indeed earlier studies have shown that the phytochemistry involved in metal transport and storage seems to vary considerably with plant species (Brooks *et al.*, 1998). In tobacco, Cd was localized predominantly in the vacuoles of leaf cell (Vögeli-Lange and Wagner, 1990). The cell walls were identified as another site of Cd storage in *Zea mays* shoots (Khan *et al.*, 1984; Lozano-Rodriguez *et al.*, 1997). In *Brassica napus* leaves, Cd was found both in vacuoles and in cell walls (Carrier *et al.*, 2003). In *Brassica juncea* and *Silene vulgaris*, the leaf epidermis was a major site of Cd accumulation (Salt *et al.*, 1995b; Chardonnens *et al.*, 1998), whereas in *Arabidopsis halleri* and *B. napus* the highest concentration of Cd was found in the mesophyll (Küpper *et al.*, 2000; Carrier *et al.*, 2003). However, no data are available for Cd localization in leaves of *T. caerulescens*. The main efforts have indeed been put into root uptake assessment and Cd localization in roots because it is the primary step leading to hyperaccumulation. For example, kinetics studies of Cd uptake in *T. caerulescens* were only performed on roots (Lombi *et al.*, 2001b; Zhao *et al.*, 2002). In roots of *T. caerulescens* Cd has been found in the apoplast as well as in the vacuole (Vázquez *et al.*, 1992a; Boominathan and Doran, 2003). It should in addition be pointed out that in a number of studies the cellular distribution of Cd was only analyzed in cell fractions separated by biochemical or physical methods (Vázquez *et al.*, 1992a; Chardonnens *et al.*, 1998; Carrier *et al.*, 2003) with the possibility of artifacts and/or Cd losses during preparation as mentioned by Frey *et al.* (2000).

Although molecular genetics has greatly advanced our understanding of metal homeostasis in bacteria and yeast, researchers are only starting to characterize the function of transporter families in plants. Several data support the idea that different classes of transporters, maintaining metal homeostasis such as IRT1 or ZNT1 in the hyperaccumulating plant *T. caerulescens* can also transport Cd^{2+}, but no specific Cd transporter has been reported yet (Pence *et al.*, 2000; Lombi *et al.*, 2002). Recently, a direct correlation between Cd and the ATP-binding cassette (ABC) transporter transcripts suggested that in *Arabidopsis thaliana*, as in yeast (Ortiz *et al.*, 1995; Cobbett, 2000), ABC transporters were involved in heavy metal fluxes (Bovet *et al.*, 2003). With the completion of the *A. thaliana* genome sequence and because *Thlaspi* genes examined to date show 85% to 90% identity to those in *A. thaliana* (Guerinot and Salt, 2001), it should be possible through the use of DNA microarrays to determine if this mechanism also happens in *T. caerulescens*. Surprisingly to date no studies have combined different approaches in order 1) to validate the results obtained by a specific method and 2) to make the link between observation and measures performed at different scales.

In order to learn more about the general Cd allocation in leaves of *T. caerulescens* and because it may give an indication of the tolerance mechanisms employed by the plant, we performed macro-autoradiographies on plants grown in hydroponics and treated with increasing concentrations of Cd. Autoradiographs were confronted to visual symptoms, biomass production and metal concentration in shoots at various time of exposure for evaluation of the general process of Cd allocation.

Distribution of Cd at the macroscopic level was completed by observation at the cell level in leaves by electronic microscopy of leaf cuttings. Chemical and physical separation of leaves was further performed to measure in detail the involvement of different plant compartments (cell wall, apoplasm and mesophyll protoplasts) in Cd storage. Two different populations of *T. caerulescens* were compared: Ganges Cd hyperaccumulating ecotype, and Prayon Cd non-hyperaccumulating ecotype (Lombi *et al.*, 2000). At last the two *T. caerulescens* populations were compared to another Cd hyperaccumulating species, *A. halleri* (Dahmani-Müller *et al.*, 2000), and a non-hyperaccumulating species *A. thaliana* for Cd-responsive genes and also more specifically for the involvement of genes and proteins related to the ABC transporters in Cd transport.

4.3 Materials and Methods

4.3.1 Plant material and culture

The plants studied were *T. caerulescens* ecotype Ganges (southern France), *A. halleri* (northern France) both known for Zn and Cd hyperaccumulation (Robinson *et al.*, 1998; Bert *et al.*, 2000; Dahmani-Müller *et al.*, 2001), and *T. caerulescens* ecotype Prayon (Belgium) known for Zn hyperaccumulation, and accumulating Cd at a lower degree (Lombi *et al.*, 2000). *Arabidopsis thaliana* (var. Columbia) was also studied for comparison purposes on microarrays.

Seeds were germinated in the dark on filters moistened with deionized water. Two-week-old seedlings were transferred to 1-L pots (four plants per pot) filled with modified quarter-strength Hoagland's nutrient solution (Sigma, St Louis, USA) supplemented with 20 µM Fe-HBED (Strem chemical, Newburyport, USA). Fe(III)-HBED was prepared as described by Chaney *et al.* (1998) in such a way that all HBED was saturated with Fe. Plants were allowed to grow 2 weeks in hydroponics before treatment with Cd was started.

Germination and plant culture were performed in a climate chamber (day/night period 16/8 h, day/night temperatures 20°C/16°C, and a light intensity of 500 lux). The nutrient solution was renewed every week and aerated continuously.

Concentrations tested were chosen according to 1) the plant species tolerance (1st experiment) and 2) the sensitivity of the method employed. When necessary and depending on the method chosen (see below) and the device used, labelled Cd (^{109}Cd) was used alone or in addition to the different Cd treatments. Radiotracers are very convenient and sensitive for determination of low concentrations in aqueous extract, but they can not be measured by β-counting in acidic samples or in intact leaves. When Cd had to be measured in such samples, unlabelled Cd was used in higher concentrations to allow detection by ICP-AES.

4.3.2 Tolerance and accumulation of Cd in plants

We measured the total concentration of Cd in leaves and roots of *T.*

caerulescens Ganges, and Prayon treated 8 weeks with non-labelled Cd (0, 5, 10, 50 and 100 µM Cd), *A. halleri* treated 8 weeks with non-labelled Cd (0, 5, 10, and 50 µM Cd), *A. thaliana* treated 3 weeks with non-labelled Cd (0, 0.2, 1, and 5 µM Cd). Four different mature plants grown in hydroponics were harvested, shoots and roots were separated and dried at 80°C for one week. Organs were individually weighted and hot-digested in HNO_3 65% sp. (Fluka, Buchs, Switzerland) and $HClO_4$ 70% p.a. (Fluka, Buchs, Switzerland). The concentration of Cd in digests was then measured by ICP-AES (Plasma 2000; Perkin Elmer, Wellesley, USA). Roots of *A. thaliana* and *A. halleri* and shoots of *A. thaliana* grown with 5 µM Cd were pooled before the metal concentration analysis because their biomass was too small, therefore the value obtained is a mean of the four plants without SD.

4.3.3 Visualization of heavy metals in plants

Both ecotypes of *T. caerulescens* were studied for a wide range of solutions with labelled Cd. Four different treatments (0, 5, 10, and 50 µM) and three times of exposure to the metal (3, 15, and 30 days) were used for the macro-autoradiographs. An additional treatment, 100 µM Cd was done for 15 days only. All nutrient solutions were labelled from the beginning of the Cd treatments with 0.1 kBq mL^{-1} (= 2.2 10^{-3} µM) of $^{109}CdCl_2$ (NEN Life Science Products, Boston, USA). Shoots and roots were collected separately, quickly rinsed with deionised water and then carefully wiped up. The samples were arranged as flat as possible and wrapped in a single layer of thin PE film. To obtain autoradiographs of adaxial and abaxial sides, samples were disposed between 2 X-OMAT AR-5 autoradiography films (Kodak, Rochester, USA) at room temperature. Time of exposure varied from 2 hours to 10 days depending on the ^{109}Cd activity present in the samples. Autoradiographs were developed with an automatic film-processor SRX-101A (Konica, Tokyo, Japan) and subsequently numerised on a Linotype-Hell Tango digitalisator (Heidelberg, Kiel, Germany). Control plants grown without ^{109}Cd were processed in parallel to radioactive samples to detect possible artefacts on the autoradiographs. Localization of Cd within leaf was determined by examining the exposed film. Leaves of plants grown in 50 µM Cd were then individually dried at 80°C, weighted, hot-digested one hour at 95°C in HNO_3 65% sp, and analyzed for Cd concentration.

4.3.4 Energy dispersive X-ray micro-analysis

The microanalysis of leaves was performed on both Ganges and Prayon populations grown 12 weeks in nutrient solution complemented with 100 µM Cd, because previous observation had shown that Cd could not be detected in plants treated with lower Cd concentrations. We used old leaves because preliminary analyses had shown that Cd concentrations in the whole leaves were 10 times larger in old leaves than in young ones and thus allowed Cd detection, which was not possible in young leaves. Fresh leaves were cut into small pieces (approximately 4 mm x 4 mm). The samples were mounted vertically on SEM stub using a Tissue-Tek and rapidly frozen by plunging into liquid propane. Microanalysis of freeze-fractured leaves was performed in a Philips SEM 515 equipped with an SEM cryo unit (SCU 020, Bal-Tec, Balzers, Liechtenstein) and a Tracor Northern energy dispersive X-ray analysis system interfaced with a Noran System Six Version 1.3 software package.

Electron-induced X-rays were detected by a Si(Li) spectrometer detector (Tracor Northern 30 mm^2 Microtrace) with an ultra-thin beryllium window. The microscope was operated at an acceleration voltage of 18 kV with a beam current of 80 nA and the stage-tilt was adjusted to obtain a take-off angle of 44 degrees. Working distance was 12 mm. The temperature on the SEM cold stage was kept below –120 °C. After preliminary measurements, we decided to carry out single spot (50 nm spot size) analyses at the edge and in the middle of the leaf and on mesophyll and epidermal cells as well as in the vein, the cuticle, the cell walls and intercellular space. Measurements of Cd are given in net counts obtained during 100 secondes at $Cd_{L\alpha}$ 3.13 keV. Elemental maps were also carried out to determine the distribution of S, Ca, K, P, C and Cd at the edge of the leaf in the Ganges population but did not give additional results. In particular, it was not possible to obtain a discriminative distribution of Cd within the leaf.

4.3.5 Leaf tissue fractionation

To measure the involvement of different plant compartments (cell wall, apoplasm and protoplasts) in Cd storage, mesophyll protoplasts were prepared from leaves of 8 different 12-week-old *T. caerulescens* grown with or without Cd (0, 5, 10, and 50 µM Cd) spiked with 0.1 kBq mL^{-1} (= 2.2 10^{-3} µM) of ^{109}Cd for better accuracy and because of the small volumes and low concentrations involved. The method used for protoplasts extraction has been described in detail elsewhere (Cosio *et al.*, 2004; Chapter 5). Briefly, the abaxial side of leaves, consisting of the lower epidermis and the cuticle, was pealed using a pair of tweezers, weighted and placed in a counting vial. Pealed leaves were weighted and processed for mesophyll protoplasts extraction. The first supernatant corresponding to the apoplasm/cell wall fraction was removed, filtered at 0.45 µm to remove all the debris and 10 to 20 aliquots were placed in counting vials. Protoplasts per mL obtained in the concentrated preparation were counted with a hemocytometer. Depending on the plant sample 10 to 20 aliquots of 200 µL concentrated protoplasts solution were placed in counting vials. For technical reasons the upper epidermis was not recovered. Four mL of scintillation solution (Ultima Gold LSC-cocktail, Packard Bioscience, Meriden, USA) were added in the counting vials to all the aliquots (lower epidermis, cell walls/apoplasm and protoplasts). The ^{109}Cd was quantified via scintillation counting (Packard, Tri-Carb, liquid scintillation analyser, Meriden, USA).

4.3.6 Preparation of the apoplast wash fluid and cell walls

To further differentiate Cd distribution between apoplasm and cell walls, two experiments were performed:

4.3.6.1 Extraction of apoplast wash fluid

For the apoplast wash fluid extraction that resulted in very small volumes, we used the treatment giving the largest signal and plants were grown in presence of the radiotracer only (= 2.2 10^{-3} µM ^{109}Cd) to avoid dilution of the activity. Leaves of *T. caerulescens* Ganges and Prayon were cut at the base with a razor blade. Leaves were then infiltrated with deionized water under vacuum 3 times for 4 minutes to

ensure complete infiltration. Fully infiltrated leaves sank, and were darker than the non-infiltrated ones. Leaves were carefully wiped out from water on paper towels, weighted and packed tip down into a 5 mL syringe that was centrifuged at 4°C for 5 minutes at 200g, 500g or 4500g. The volume of collected apoplastic solution was measured. The ^{109}Cd was quantified as previously described via scintillation counting. Two g of infiltrated leaves were then recovered from the syringe and hot-digested 1 hour at 95°C in HNO_3 65% sp. for Cd concentration determination. All samples were also tested for cytoplasmic contamination: since K^+ occurs in the cytosol at concentrations at least one order of magnitude higher than those in the apoplastic solute (Lohaus *et al.*, 2001), concentration of K^+ was analyzed in the apoplastic wash fluid and in the digest by Flame-AAS (AAanalyst 300; Perkin Elmer, Wellesley, USA).

4.3.6.2 Cell wall extraction

The distribution of Cd between the cell walls and symplast of leaves and roots was further estimated after isolating the cell walls using the method developed by Hart *et al.* (1992). This treatment isolates the cell walls without destroying their morphology (Hart *et al.*, 1992). Plants were grown 8 weeks without Cd prior being grown 4 weeks in 100 µM non-labelled Cd in order to reach high biomass production and concentrations above the detection limit of Cd by ICP-AES, and to avoid the problem of Cd phytotoxicity. Roots and shoots were harvested separately and quickly rinsed. Abaxial sides of leaves were pealed using a pair of tweezers to remove the cuticle. Pealed leaves and roots were weighted, soaked in 2:1 (v:v) methanol:chloroform for 3 days and then washed thoroughly in water. Tissues were processed as for the tolerance experiment and Cd was measured. The purity of the isolated cell walls was checked by an analysis of the fatty acids composition of the extracts performed by the Lipid Analysis Unit of the Scottish Crop Research Institute (Dundee, UK). It confirmed that all cellular components had been removed by the solvents.

Calculation of Cd content (µg) of the cell walls and of the whole leaves or roots was based on the fresh biomass weighted before the solvent extraction. Based on previous observation, dry weight was estimated from fresh weight (20% of fresh weight for shoots, 8% of fresh weight for roots). The dry weight of cell walls was measured after extraction. Roots were pooled before the metal concentration analysis, therefore the value obtained is a mean without SD.

4.3.7 Screening of Cd-responsive genes

At last, based on the results of the tolerance experiment, 12-week-old *T. caerulescens* Ganges, Prayon and *A. halleri* that had grown with 10 µM unlabelled Cd or 8-week-old *A. thaliana* that had grown with 0.2 µM unlabelled Cd were screened for Cd-responsive genes. *Arabidopsis thaliana* is the best-studied genetic model in the plant kingdom, and *A. halleri* is a close relative and also a Cd hyperaccumulator. These plants were therefore studied on microarrays for comparison to *T. caerulescens* but also as controls.

The plants were harvested, quickly rinsed with deionized water and separated in leaves and roots. Both organs were rapidly immerged in liquid nitrogen. When RNA extraction was not carried out immediately, plant materials were stored at –80°C to

prevent any RNA degradation. Isolation of RNA, preparation of fluorescent probes, hybridization reaction and microarray analysis were performed as described by Reymond *et al.* (2000). For our purpose, a fluorescent labeled cDNA probe was prepared from mRNA isolated from control tissues by reverse transcription in presence of Cy3-dCTP (Amersham Biosciences, Dübendorf, Switzerland). A second probe, labeled with Cy5-dCTP, was prepared from the Cd-treated tissues. Both probes were first hybridized on cDNA-microarray spotted with *A. thaliana* ABC-transporters genes as well as miscellaneous other genes (Bovet *et al.*, 2003). The fluorescence was analysed using the confocal laser ScanArray 4000 (GSI Lumonics, Ottawa, Canada), and the image was processed using the included software.

A study with a second set of microarray slides was then performed. Probes were hybridized on slides spotted in duplicate with 9'216 expressed sequence tags (ESTs) per slides (*Arabidopsis* 9.2K). These slides were available at HHMI Biopolymer/Keck Foundation Biotechnology Resource Laboratory, at the Yale University School of Medecine (http://info.med.yale.edu/wmkeck/dna_arrays.html). For this assay annotation for spotted ESTs was provided by the slide purchaser. From the duplicate showing comparative patterns (data not shown), one array was selected according to the level of background signals and hybridization intensities. The data were analyzed as described above and eventually expressed in terms of percentage of up-regulated genes using a cut-off of 2.5 and sub-classified in 12 broad families of gene products.

4.4 Results

4.4.1 Biomass production and metal uptake

To assess tolerance to Cd and the accumulation potential of the different species or population tested, Cd concentrations in shoots and roots and biomass produced were measured (Table 4.1). A reduction in roots and shoot biomasses was observed with increasing Cd concentrations in all populations and species. Prayon was the most tolerant with no significant shoot biomass reduction at 10 μM Cd and a 43% shoot biomass reduction at 50 μM. Ganges was more affected by low and medium Cd concentrations (36% shoot biomass decrease at 10 μM Cd) but showed a similar decrease in shoot biomass (51%) at 50 μM. On the other hand Prayon exhibited necrosis spots on leaves at 50 and 100 μM Cd (Figure 4.1a), whereas Ganges seemed perfectly healthy. *Arabidopsis halleri* was significantly less tolerant than *T. caerulescens* with a sharp decrease in shoot biomass even at low Cd concentration (64% at 10 μM) and showed an 89% decrease in shoot biomass at 50 μM Cd. *Arabidopsis thaliana* was highly sensitive to Cd with a 92% shoot biomass decrease at 5 μM Cd.

Arabidopsis halleri showed the largest Cd concentration in shoots for all the treatments performed. At 50 μM, Cd concentration in *A. halleri* shoots was 3-fold higher (8244±3228 mg Cd kg^{-1}) than in shoots of both *T. caerulescens* populations. Cadmium concentrations were always larger in Ganges than in Prayon (Table 4.1). For example at 10 μM Cd in the nutrient solution Ganges accumulated 4-fold more Cd

in the shoots (1091±160 mg Cd kg^{-1}) than Prayon (264±44 mg Cd kg^{-1}). However, this factor diminished with increasing concentrations of Cd and both plants exhibited similar large Cd concentrations in shoots at 50 µM Cd, reaching 2960±635 mg Cd kg^{-1} for Ganges and 2457±519 mg Cd kg^{-1} for Prayon.

Cadmium concentration found in shoots of *A. thaliana* grown with 5 µM Cd was higher (950 mg Cd kg^{-1}) than in Prayon and in the same order than Cd concentration found in Ganges (751±299 mg Cd kg^{-1}).

Table 4.1: Effect of Cd on biomass and accumulation: average biomass production (mg) and Cd concentration (mg kg^{-1}) per plant of 12-week-old *T. caerulescens* Ganges, Prayon, *A. halleri* grown 8 weeks and 7-week-old *A. thaliana* grown 3 weeks in hydroponics with or without Cd (n= 4, SD in parentheses).

Cd in nutrient solution (µM)	Biomass (mg)		Cd (mg kg^{-1})	
	Shoots	Roots	Shoots	Roots
Ganges				
0	205 (37)	25 (4)	3 (2)	104†
5	138 (61)	21 (9)	751 (299)	1938†
10	131 (39)	21 (7)	1091 (160)	3444†
50	101 (37)	14 (6)	2960 (635)	-
100	22 (9)	-	8163 (1058)	-
Prayon				
0	235 (73)	214 (77)	4 (4)	18 (19)
5	242 (5)	229 (50)	170 (11)	790 (94)
10	287 (52)	242 (50)	264 (44)	1801 (232)
50	135 (58)	155 (44)	2457 (519)	4977 (467)
100	43 (31)	-	5670 (558)	-
A. halleri				
0	334 (235)	43†	3 (2)	7†
5	124 (36)	24†	1927 (623)	1370†
10	119 (24)	26†	1167 (913)	2188†
50	37 (27)	12†	8244 (3228)	3067†
A. thaliana				
0	113 (82)	16†	1 (1)	1†
0.2	81 (45)	12†	89 (16)	109†
1	30 (15)	5†	325 (105)	948†
5	9 (9)	2†	950†	1120†

† pooled before analysis.

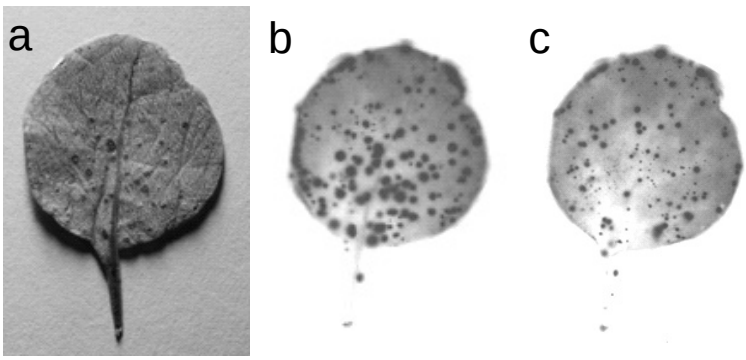

Figure 4.1: Leaf of *T. caerulescens* Prayon grown in hydroponics with 100 µM Cd spiked with 2.2 10^{-3} µM ^{109}Cd a) photograph of the adaxial side, b) autoradiograph of the adaxial side and c) autoradiograph of the abaxial side.

4.4.2 Visualization of heavy metals in plants

To assess if the concentration of Cd in the nutrient solution or the time of exposure to Cd had an influence on Cd localization in the plants, we performed autoradiographs. The technique did not give satisfactory results for discriminating between metal adsorbed onto the roots, and into the roots (data not shown), but it was very efficient at visualizing ^{109}Cd distribution in leaves.

The darkness of the autoradiographs depended on the ratio ^{109}Cd/total Cd. Concentration of ^{109}Cd remained constant (2.2 10^{-3} µM ^{109}Cd) in all solutions whereas total Cd concentration increased from 2.2 10^{-3} µM to 100 µM, the ^{109}Cd activity present in the samples proportionally decreased and autoradiographs became lighter with increasing total Cd concentration (Figure 4.2). Thus the intensity of the darkening did not reflect the total concentration of Cd found in the leaves and intensity of darkening could only be compared when plants were grown with the same ^{109}Cd/total Cd ratio.

In Ganges leaves we observed a change in Cd distribution pattern with increasing Cd concentrations (Figure 4.2). At low Cd concentration, younger leaves exhibited higher Cd concentrations than older ones as shown by the darker prints on the autoradiographs, but the reverse was true at high Cd concentrations. Cadmium was localized mainly at the edge of the leaf, but also in point-like accumulations spread on the whole limb surface. The number of the point-like accumulations increased with increasing Cd concentration. In some leaves Cd was found in the central vein, but on the contrary in other leaves the vein appeared white on the autoradiographs. The autoradiographs of the adaxial and abaxial sides of the leaves were always different (data not shown).

For Prayon a similar overall Cd distribution pattern was observed (Figure 4.2).

Both point-like accumulation and accumulation at the margin of the leaves were visible. Nevertheless, younger leaves exhibited lower Cd concentration than older leaves, as shown by the lighter prints on the autoradiographs observed for all the treatments. Besides, concentration in older leaves seemed more variable, some of the leaves appearing dark and others being very slightly colored. In this ecotype, the localization of the spots observed on the autoradiographs at high Cd concentrations (50 and 100 μM) could be directly related to the necrotic spots also observed at these concentrations (Figure 4.1). As for Ganges, the autoradiographs of the adaxial and abaxial sides of the leaves were different (Figure 4.1b and c).

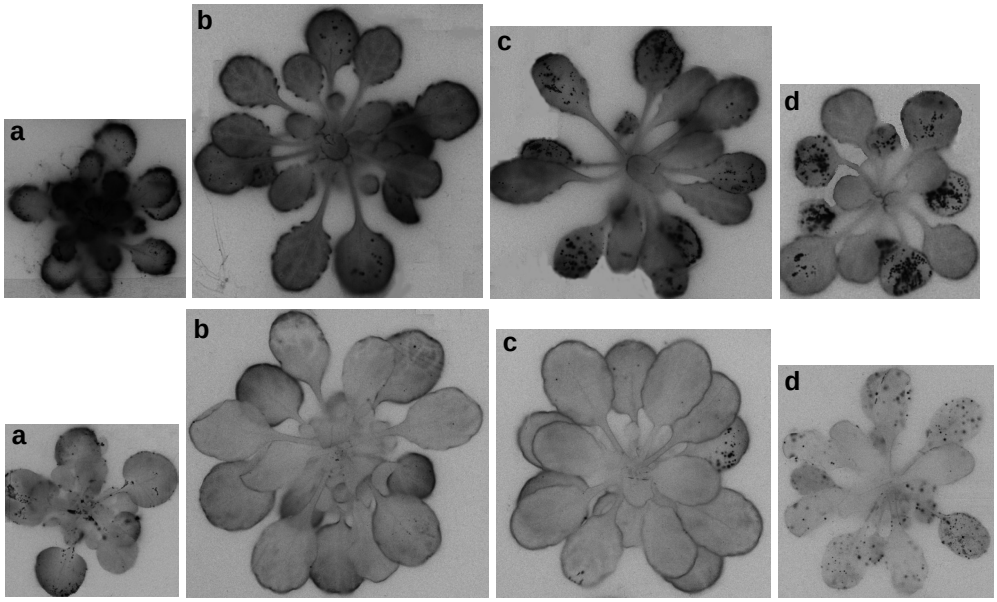

Figure 4.2: Autoradiographs of *T. caerulescens* Ganges (top) and Prayon (bottom) grown in hydroponics 30 days with 2.2 10^{-3} μM ^{109}Cd and a) 0 μM, b) 5 μM, c) 10 μM Cd, and d) 50 μM Cd.

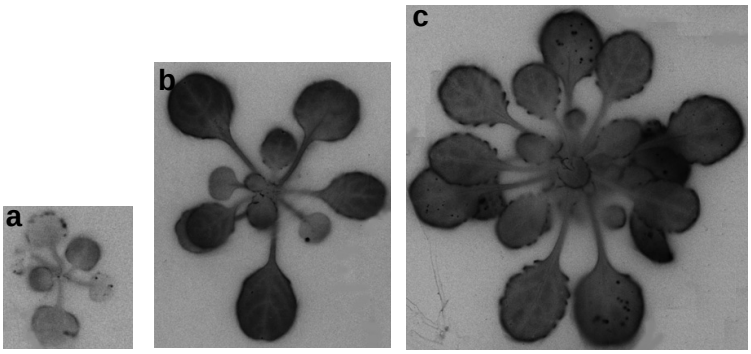

Figure 4.3: Autoradiographs of leaves of *T. caerulescens* Ganges grown in hydroponics with 5 μM Cd spiked with 2.2 10^{-3} μM ^{109}Cd at a) 3 days, b) 15 days and c) 30 days.

For both ecotypes the duration of exposure to the metal had no influence on the Cd distribution pattern: after 3, 15 or 30 days the allocation of Cd remained similar in the leaves, although Cd concentration increased in leaves as reflected by the increasing darkness of the autoradiographs (Figure 4.3).

4.4.3 Cadmium concentration in individual leaves of T. caerulescens

To confirm autoradiographs, we weighted individual leaves of Prayon and Ganges grown in 50 µM Cd and measured Cd concentration (Figure 4.4). Darker leaves on the autoradiographs of a plant corresponded to leaves with higher Cd concentrations.

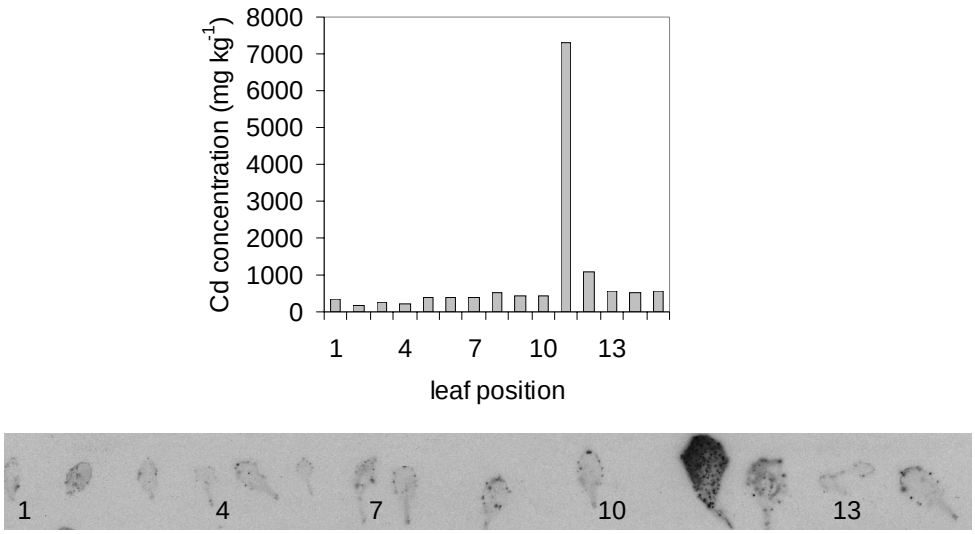

Figure 4.4: Concentration of Cd (mg kg^{-1}) and corresponding autoradiographs of leaves of *T. caerulescens* Ganges grown in hydroponics 30 days with 2.2 10^{-3} µM ^{109}Cd and 50 µM Cd. Leaves are classified according to their position in the rosette. The youngest leaf (n° 1) is on the left and the oldest on the right.

Weight and concentration varied with leaf position in the rosette for both Prayon and Ganges. For Prayon Cd concentrations found in leaves were proportional to leaf dry weight with a correlation coefficient r= 0.648 (1 plant, n= 80; sign. $2\alpha<0.001$). Bigger leaves had higher Cd concentration. On the opposite, the same comparison for Ganges resulted in r= 0.118 (3 plants, n= 37). When studying independently the three Ganges plants tested, we found r= 0.694 (n= 8; not sign.), 0.254 (n= 15; not sign.; Figure 4.4) and -0.750 (n= 14; sign. $2\alpha<0.05$) respectively. Surprisingly, average concentrations of leaves in the three plants were similar with 1142±180 mg Cd kg^{-1} for the first, 1153±171 mg Cd kg^{-1} for the second and 1257±30 mg Cd kg^{-1} for the last

one. There was no obvious relationship between the amount stored and either Cd concentration or the position of the leaf in the rosette.

4.4.4 Localization of Cd in leaves by SEM-EDXMA

We further studied the localization of Cd particularly at the edges of old leaves with SEM-EDXMA. Typical scanning electron micrographs and spots analyzed are presented in Figure 4.5. The pattern of Cd distribution was similar for both Ganges and Prayon (Figure 4.6). The main difference between Ganges and Prayon resulted in the size of the signals probably resulting from the 1.5-fold Cd concentration difference found between leaves of both populations when grown at 100 µM Cd (Table 4.1). Cadmium was found mainly at the edge of the leaves in the larger epidermal cells. A considerable amount of Cd was also found in cell walls of the epidermal cells and in smaller epidermal cells. Cadmium was eventually detected in the mesophyll cells at the edge of the leaf and in epidermal and mesophyll cells in the middle of the leaves, but in significantly lower amounts than at the edges. Cadmium was almost absent from the lateral veins. No central vein was analyzed.

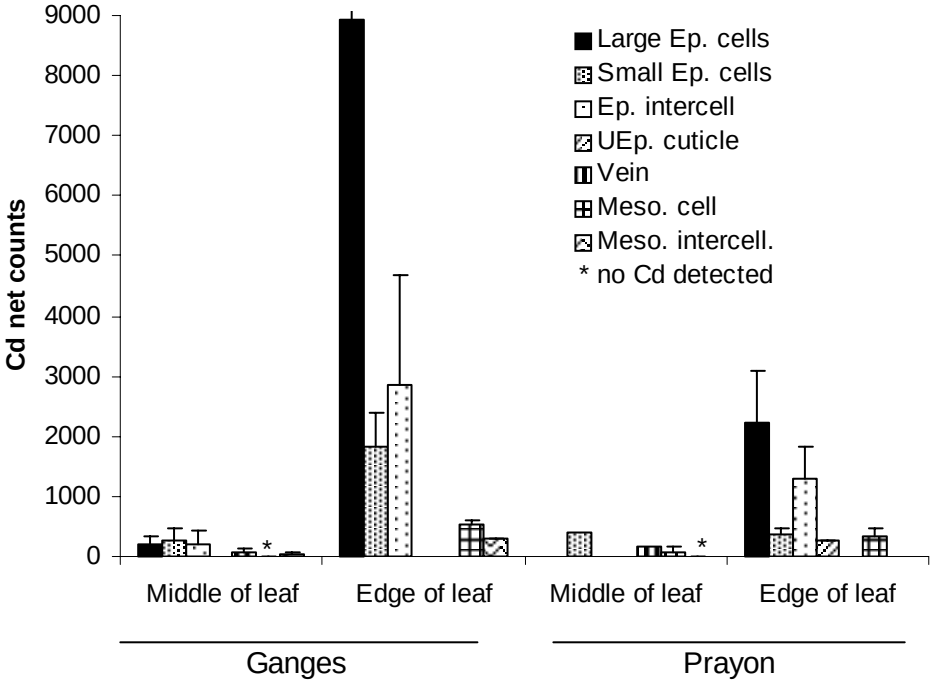

Figure 4.5: Cadmium net counts in different leaf compartment of Ganges and Prayon grown with 100 µM Cd determined by SEM-EDXMA (SD of Ganges large epidermal cells= 4516 Cd net counts; Ep.= epidermis; UEp.= upper epidermis)

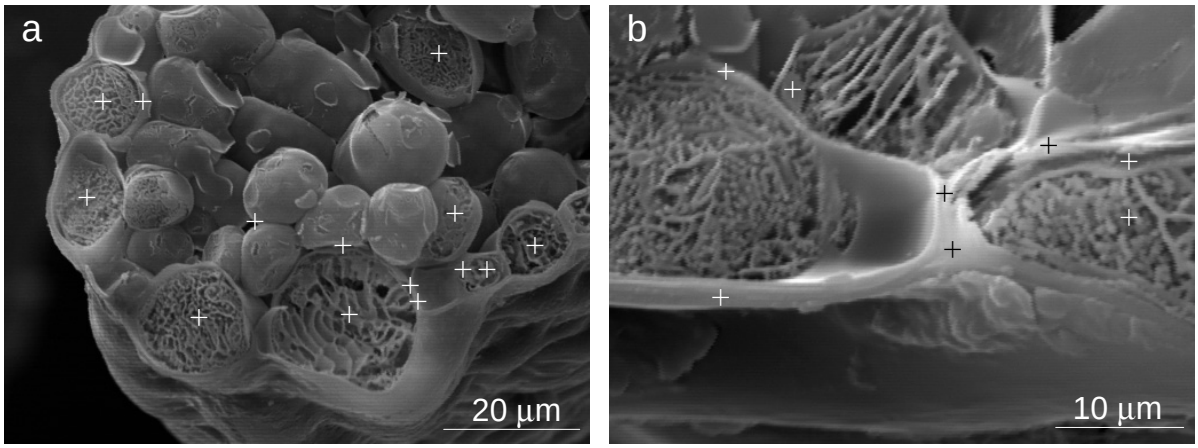

Figure 4.6: Scanning electron micrographs of a) the leaf edge of Ganges, and b) epidermal cells of the leaf edge of Prayon. The crosses represent the single spot analyses performed on the samples.

4.4.5 Subcellular distribution of Cd in T. caerulescens

We examined the importance of different leaf parts in Cd storage through a protoplasts extraction procedure. Cadmium distribution between apoplasm/cell walls, lower epidermis and mesophyll protoplasts was similar for the different concentrations tested (5, 10 and 50 µM Cd) and for both ecotypes (Figure 4.7), although the Cd content of the different subcellular fractions increased with increasing Cd treatments. The ranking with respect to Cd concentration was the following: apoplasm/cell walls> lower epidermis> mesophyll protoplasts. We measured in Ganges 5.4-fold more Cd in the apoplasm than in the lower epidermis and 4.0-fold respectively in Prayon. The concentration of Cd measured in the mesophyll protoplasts was negligible.

To further characterize the distribution of Cd between the apoplasm and the cell walls, the apoplast wash fluid was extracted by centrifugation and separated from the cell walls (Table 4.2). Since Lohaus *et al.* (2001) suggested that above 1000g serious cytoplasmic contamination would happen, three centrifugation speeds were tested: 200g to avoid cell damage, 500g to ensure a good extraction of the wash fluid and 4500g for comparison. The extent of contamination of the extract by cytoplasm products was assessed by measuring K concentration. In Ganges, K concentration was 3-fold higher at 500g and 6-fold higher at 4500g than at 200g. In Prayon, cytoplasmic contamination was larger with a 300-fold increase at 500g and 3000-fold at 4500g in K concentration compared to 200g. However even at 4500g the proportion of Cd found in these apoplastic solutes compared to Cd found in the whole leaf was negligible in both ecotypes. Concentration measured in the apoplasm fluid was 10^6-fold for Ganges and 10^8-fold for Prayon smaller than the Cd concentration in the whole leaf.

The cell wall extraction allowed the measurement of Cd concentration found in

cell walls and comparison to concentration of Cd found in the shoots or roots (Table 4.3). Concentrations in cell walls were similar to concentrations in whole shoots. However, concentrations found in whole roots were 4-fold higher than Cd concentrations found in cell walls. All together for shoots and roots of both ecotypes between 33 and 35% of the total Cd content was allocated to cell walls (Table 4.3).

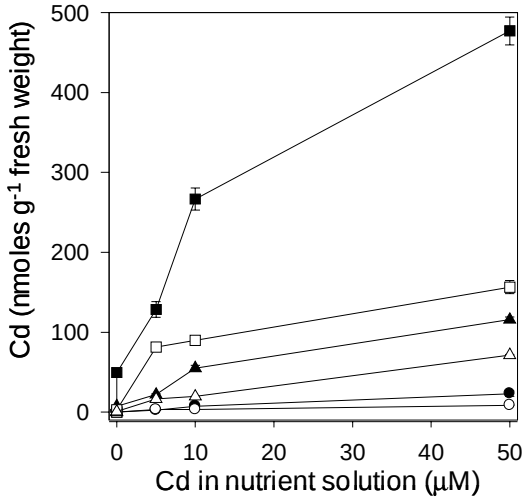

Figure 4.7: Cadmium amount measured in mesophyll protoplasts (circles), apoplasm and cell walls (squares) and lower epidermis (triangles) in Ganges (closed symbols) and Prayon (open symbols) leaves. Error bars do not extend outside some data symbols.

Table 4.2: Concentration of Cd and K measured in the apoplasm wash fluid and in the leaf extract in mg kg^{-1} per g of infiltrated leaf of *T. caerulescens* grown in hydroponics with 0.1 kBq mL^{-1} (= 2.2 10^{-3} µM) of ^{109}Cd. Samples were pooled before analysis.

	Apoplasm wash (mg kg^{-1})		Leaf extract (mg kg^{-1})	
Centrifugation (*g*)	Cd	K	Cd	K
Ganges				
200	0.3 10^{-6}	0.01	9.7	4.88
500	1 10^{-6}	0.03	4.0	5.46
4500	7.1 10^{-6}	0.06	3.5	4.51
Prayon				
200	0.3 10^{-8}	0.0002	1.0	5.33
500	9 10^{-8}	0.006	1.4	5.91
4500	51 10^{-8}	0.06	1.0	6.05

Table 4.3: Repartition of Cd in the cell wall isolates compared to the total Cd accumulation in the shoots and roots of *T. caerulescens* grown in hydroponics with 100 µM of Cd (n= 4, SD in parentheses). Samples were pooled before analysis.

	Cd (mg kg^{-1})		Biomass (%)	
	Ganges	Prayon	Ganges	Prayon
shoots				
Shoots	1122	920	100	100
Cell walls	1436	938	26	35
roots				
Roots	9947	2934	100	100
Cell walls	10030	2154	87	97

4.4.6 Screening of Cd-responsive genes

We tested on microarrays, the effect of 10 µM non labelled Cd on the genetic expression of both *T. caerulescens* and *A. halleri*, whereas 0.2 µM non labelled Cd was chosen for *A. thaliana*. At this concentration, Ganges and *A. halleri* presented similar concentration of Cd in shoots, and significantly higher concentration than Prayon. Cadmium concentration of 0.2 µM did not affect tremendously *A. thaliana* biomass, and concentration of Cd in leaves where below 100 mg kg^{-1}. The mRNAs were successfully extracted and purified from Ganges, Prayon, *A. halleri* and *A. thaliana* grown with or without Cd. In order to identify Cd-regulated mRNA populations, cDNAs were produced and hybridized on two different sets of microarray slides. When assessing the involvement of genes and proteins related to the ABC transporters in Cd transport (Figure 4.8), the highest alteration of expression pattern was observed in *A. thaliana*: several genes were up- and down- regulated, including genes known to be induced by oxidative stress. Nine ABC transporters were up-regulated and one was down-regulated in leaves. No or very few genes were induced in *T. caerulescens* and *A. halleri* compared to *A. thaliana*. Genes up-regulated in those plants nevertheless included oxidative stress genes.

A further approach was performed using commercially available high density cDNA microarray slides. The number of up-regulated genes obtained with the high density slides varied strongly with 1226 for Ganges, 194 for Prayon, 659 for *A. halleri* and 580 for *A. thaliana* (duplicate ESTs were not considered in this study). Results were slightly different between *T. caerulescens* and *Arabidopsis* leaves, whereas they were very similar in both genera (Table 4.4). The main difference was the number of miscellaneous (MISC; 28% and 32% for respectively Ganges and Prayon; 36% and 39% respectively for *A. thaliana* and *A. halleri*) and unknown (U; 51% and 48% for respectively Ganges and Prayon; 39% and 38% respectively for *A. thaliana* and *A. halleri*) genes. When comparing Ganges and Prayon, the former had a higher

alteration of transcription factors (TF) genes (7% compared to 6%), whereas the latter did not show induction of genes involved in sulfur metabolism (SM) and heat-shock proteins (HSP). Proteins involved in the cell wall organization (CW) were more up-regulated in *A. thaliana* (4%) than in *A. halleri* (2%), Ganges (1.8%) and Prayon (1.1%). In both *T. caerulescens* metal transporters (M-TR) were not induced whereas a slight induction was detected in both *Arabidopsis*.

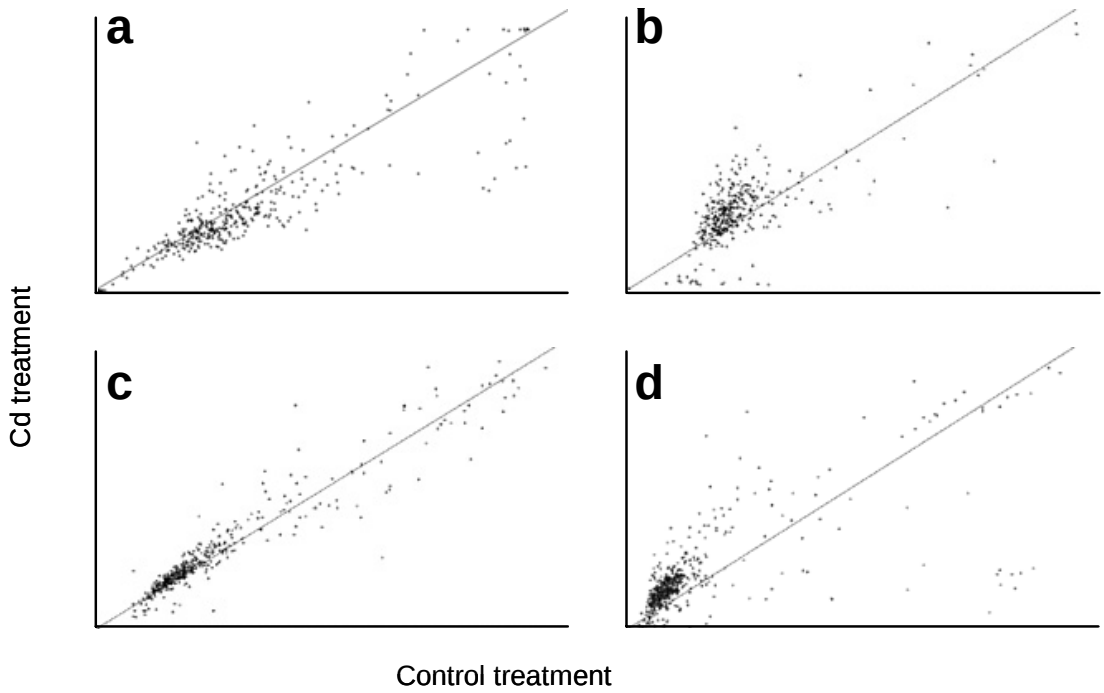

Figure 4.8: Effect of Cd on expression of genes related to ABC-transporters in leaves of a) Ganges, b) Prayon, c) *A. halleri* and d) *A. thaliana*. Dots represent the expression ratio of genes in control and Cd treated plants. The line (ratio= 1) represents unaltered gene expression. When the gene expression is altered, the dot is far from this line.

Table 4.4: Cd-responsive genes in Ganges, Prayon, *A. halleri*, and *A. thaliana*: leaf-induced genes were classified in 12 broad families of gene products and reported according to their percentage. (U: unknown genes; MISC, miscellaneous identified genes; TF: transcription factors; RED: genes involved in red-ox reactions; PK: protein kinases; CW: proteins involved in the cell wall organization; TR: general membrane transporters excluding ion and metal transporters; HSP, heat-shock proteins; I-TR: ion transporters; PP: protein phosphatases; SM, genes involved in the sulphur metabolism; M-TR: metal transporters).

Gene family	Ganges (%)	Prayon (%)	*A. halleri* (%)	*A. thaliana* (%)
U	51.3	48.3	38.1	39.4
MISC	28.1	31.7	39.1	36.1
TF	7.4	6.1	6.4	5.5
RED	4.0	5.5	4.3	5.9
PK	3.5	3.9	3.5	3.8
CW	1.8	1.1	2.3	3.5
TR	1.25	2.2	2.0	2.4
HSP	1.1	0.0	1.2	1.1
I-TR	0.8	0.6	0.8	1.35
PP	0.4	0.6	0.9	0.7
SM	0.2	0.0	0.9	0.3
M-TR	0.0	0.0	0.5	0.2

4.5 Discussion

4.5.1 Growth and metal uptake

Prayon was the most tolerant to Cd at low and medium Cd concentrations. However, Cd concentrations found in the leaves of Ganges at 10 µM Cd were already above 1000 mg kg^{-1} Cd without showing significant toxicity symptoms. This concentration was 10-fold larger than the threshold that defines Cd hyperaccumulation in natural environment (Brown *et al.*, 1994). At 50 µM Ganges and Prayon showed similar Cd concentrations and similar biomass reduction. Prayon does not usually hyperaccumulate Cd in the field (Lombi *et al.*, 2000), but it is known that plants in solution culture accumulate more Cd than the same ones in soil (Grant *et al.*, 1998). Similarly, *A. halleri* showed the highest Cd concentration at all concentrations tested and also the highest biomass loss. The relative higher tolerance of Prayon might then simply be related to its lower accumulation capacity resulting in lower Cd concentrations in the leaves. Nevertheless it can be concluded that at 50 µM Ganges performed better than Prayon since biomass losses were similar for similar concentration but only the latter showed necrosis.

Roosens *et al.* (2003) studied several populations of *T. caerulescens* in hydroponics and reported Cd concentrations in shoots of Ganges and Prayon in the

same order as the ones reported here. However, they reported that Ganges was more tolerant to Cd than Prayon. We have no explanation for this discrepancy, although it is possible that seeds from different populations may originate from the Ganges area.

The dramatic decrease in biomass production of both roots and shoots of *A. thaliana* observed in spite of a very low Cd concentration in the nutrient medium confirmed its high sensitivity to Cd (Murphy and Taiz, 1995). This sensitivity is probably related to a poor control of Cd uptake at the root level as Cd concentration in shoots at 0.2 µM Cd was indeed close to the treshold of 100 mg kg^{-1}.

4.5.2 Localization of heavy metals in leaves

To our knowledge, this is the first direct evidence of Cd distribution in leaves of *T. caerulescens*, although they are a few reports on the accumulation of Cd at specific sites in the plant body (Vázquez *et al.*, 1992a; Perronnet *et al.*, 2003). We observed that the duration of exposure to the metal had no influence on the Cd distribution pattern within the plant: after 3, 15, or 30 days the allocation of Cd remained similar in the leaves, as also reported by Weigel and Jäger (1980) for Cd in bean plants.

On the contrary, Cd localization in leaves of Ganges changed according to Cd concentration in the nutrient solution: at very low Cd concentration, the metal was preferentially located in the younger leaves, whereas at higher Cd concentration Cd was mainly located in the older leaves. Perronnet *et al.* (2003) similarly reported that in the Vivier population of *T. caerulescens* grown on contaminated soil the youngest leaves exhibited higher Cd concentrations than older leaves. The Cd concentrations found in shoots (600 mg Cd kg^{-1}) by Perronnet *et al.* (2003) are close to the Cd concentrations in shoots of Ganges (751±299 mg Cd kg^{-1}) grown at 5 µM Cd, that is low Cd for our experiment but already high for soil solution (Wagner, 1993; Knight *et al.*, 1997; Lombi *et al.*, 2001a). In Prayon no such difference was found between low and high Cd treatments and the highest Cd concentrations were always found in the bigger and mature leaves. Cadmium accumulation might thus be related to growth and thus more active metabolism.

The localization of Cd was highly uneven over *T. caerulescens* leaf surface in both ecotypes. Beside showing spots spread on the leaf surface and along the edge, the autoradiographs of adaxial and abaxial sides of the same leaf were not similar, indicating very localized Cd accumulation. In Prayon the spots of high Cd accumulation observed on the autoradiographs were directly correlated with the necrotic spots observed on the leaves. We believe that this indicates that the necrosis resulted directly from Cd accumulation. This view would be clearly distinct from the alternative idea that Cd results in unspecific necrosis, since in our experiment Cd accumulation obviously decreased cellular viability and finally resulted in cell death. Salt *et al.* (1995) also analyzed Cd localization in leaves of Indian mustard by autoradiography. They found that Cd toxicity induced chlorosis in young leaves and that Cd preferentially accumulated within these young leaves explaining the localization of the chlorosis. However contrary to our results with *T. caerulescens*, they found an even distribution over the leaf surface that they could correlate with trichomes localization. In a number of other annual hyperaccumulating and non-hyperaccumulating plants sequestration of metals in trichomes has been reported

(Küpper *et al.*, 1999; Küpper *et al.*, 2000; Zhao *et al.*, 2000; Choi *et al.*, 2001; Ager *et al.*, 2002). Trichomes are ideal organs for sequestration of heavy metals because of their isolation from plant bodies. However, *T. caerulescens* does not possess trichomes and mechanisms ought to be based on a different compartment or cell type.

4.5.3 Localization of Cd at the subcellular level

The SEM-EDXMA analysis confirmed that an important site of Cd allocation was at the edge of the leaves in both Ganges and Prayon. Additionally it showed that Cd was found mainly in the large epidermal cells and to a lesser extend in small epidermal cells, both inside the cells and in the cell walls. This was further confirmed by compartmental studies.

Mizuno *et al.* (2003) pointed out the importance of Ni allocation at the leaf edge of *T. japonicum*. They suggested that Ni was transported along with transpiration stream and that Ni in excess was excreted together with guttation. It was earlier reported that the guttation fluid commonly functions to exclude various substances including K, Mg, and Ca in sunflower, and recombinant protein in tobacco (Komarnytsky *et al.*, 2000; Tanner and Beevers, 2001). However, we obtained very low Cd concentration in the apoplastic wash fluid, it is thus questionable if this would be an acceptable hypothesis for Cd in *T. caerulescens*.

Cellular Cd is known to be preferentially accumulated within vacuoles in the leaves of a number of different plant species including tobacco, and *Silene vulgaris* (Vögeli-Lange and Wagner, 1990; Chardonnens *et al.*, 1998; Chardonnens *et al.*, 1999), and this has been often proposed as a possible mechanism of Cd tolerance in plants. In *A. halleri* and *B. napus* the mesophyll cells are thought to contain more Cd than the epidermal cells (Küpper *et al.*, 2000; Carrier *et al.*, 2003). Zinc accumulation in *T. caerulescens* leaves was also reported to be higher in vacuoles of larger epidermal cells than in mesophyll cells (Vázquez *et al.*, 1992a; Vázquez *et al.*, 1994; Küpper *et al.*, 1999; Frey *et al.*, 2000). Chardonnens *et al.* (1998; 1999) further showed in *S. vulgaris* that Cd accumulated mainly in the lower epidermis of leaves. There were no consistent differences either between the upper and lower epidermis, or between the upper and lower mesophyll cells in our study. The variation of the relative Cd concentration in the epidermal cells appeared to be mainly associated with the cell size similarly to Zn (Küpper *et al.*, 1999). This relationship suggests that vacuolation in the epidermal cells may be an important driving force for Cd sequestration in epidermal cells of *T. caerulescens* leaf. A significant role in Cd detoxification and tolerance might indeed be played by vacuolar compartmentation, which prevents the free circulation of Cd ions in the cytosol and forces them into a limited area. In addition, preferential distribution of Cd in the epidermis might help to protect mesophyll cells from build-up and toxicity of Cd, and maintain the functionality of mesophyll cells over a wide range of external Cd concentrations.

These results however differ from the ones obtained for Zn in *T. caerulescens* because Zn was never reported in the cell walls in *T. caerulescens*. The accumulation of Cd in the cell walls of epidermal and mesophyll cells seems then to be an important tolerance mechanism as it is the case for other organisms or plants. In mycorrhizal

fungi for example Cd was found to be bound to negatively charged sites associated with the cell walls such as cellulose, cellulose derivatives or to the outer pigmented layer of the cell walls (Galli *et al.*, 1994; Turnau *et al.*, 1994; Blaudez *et al.*, 2000). Nickel was also found to be associated with cell wall pectates in *Hybanthus floribundus* (Salt and Krämer, 2000), however the role of cell walls in Cd binding and storage in plants remains controversial. Vögeli-Lange and Wagner (1990) and Vázquez *et al.* (1992a) for example have failed to show substantial Cd binding in cell walls of tobacco and *T. caerulescens*. In contrast, in *Zea mays* cell walls were assumed to play the most important role in Cd accumulation (Khan *et al.*, 1984). The importance of Cd binding to cell walls and the limitation of its subsequent translocation into shoots has been demonstrated for root cells of non-hyperaccumulating plants (Wagner, 1993; Grant *et al.*, 1998) and has been recently described in *T. caerulescens* hairy roots (Boominathan and Doran, 2003). Cell walls can easily interact with metal ions because of the presence of many enzymes and negative charge sites at their surface (Wang and Evangelou, 1995), thereby lowering potentially toxic free Cd ions in the cytoplasm. The low Cd concentrations measured in the apoplasm fluid, could indeed be due to the efficient role of the cell walls in continuously reducing metal-ion activity in the intercellular spaces through tight metal binding (Bringezu *et al.*, 1999). At last, Carrier *et al.* (2003) reported that Cd was distributed both in vacuoles and in cell walls of *B. napus* leaves.

In the current literature however the emphasis is given to internal tolerance mechanisms, particularly vacuolar metal compartmentation and sequestration by phytochelatins. So far, less attention has been devoted to other tolerance mechanisms, although they could operate in combination (Baker, 1987). It is evolutionary conceivable that plants possess both external and internal mechanisms (e.g. extracellular cell wall compartmentation for external metal avoidance and intracellular vacuole compartmentation for internal metal tolerance) as it seems to be the case for Cd in *T. caerulescens*. It would seem to be an unwise evolutionary selection for higher plants to possess only internal tolerance mechanisms.

Roosens *et al.* (2003) measured Cd concentration in xylem sap of different *T. caerulescens* and showed that the population St-Félix (Cd hyperaccumulator) had 4-fold higher Cd concentration than Prayon, indicating that Cd reaches leaf cells mainly by the xylem. The fact that we could not detect Cd in the lateral veins by EDXMA can be a consequence of the elevated detection limit of the device or of the efflux of Cd in the apoplasm that could explain the light coloration of veins observed in some cases on the autoradiographs. Additionally the unevenly spot-like distribution of Cd renders its detection by SEM difficult.

Despite decades of research, little is known about the relative contribution of the symplastic and apoplastic pathways to the delivery of a particular cation, although this has important implication for identifying the targets for improving phytoextraction potential (White *et al.*, 2002). On a functional basis, the symplastic pathways involve specific transporters in the plasma membrane of cells and are therefore selective (Ernst *et al.*, 2002). On the opposite, the apoplastic cation flux is largely determined by the cation exchange properties of the cell walls and by water flows (White *et al.*, 2002). From our results, it appears that Cd is probably translocated both apoplastically with the transpiration stream towards the epidermal cells in leaves, and symplastically for short distance in the cells adjacent to the main vessels.

4.5.4 Screening of Cd-responsive genes

Our results obtained with the high density slides confirmed that ESTs from *A. thaliana* could hybridize genes from a close relative genus. Nevertheless, the differences in the number of genes up-regulated and miscellaneous and unknown genes between *Thlaspi* and *Arabidopsis* could be a direct consequence of cross-hybridization and be linked to the phylogenetic differences between the four plants.

The ABC-transporter is one of the largest protein families known (Martinoia *et al.*, 2002). In plants, it is strongly speculated that ABC transporters are able to transport phytochelatin (PC)-Cd and/or GS_2-Cd complexes into the vacuole by analogy with fission yeast and yeast (Ortiz *et al.*, 1995; Cobbett, 2000). However in plants the corresponding ABC transporter proteins have yet to be identified. Based on our results it is well possible than ABC transporters might have a role in Cd accumulation in *A. thaliana*. Recently, Bovet *et al.* (2003) similarly suggested that in *A. thaliana* ABC transporters were involved in Cd fluxes. However, the role of ABC-transporters is not obvious concerning *T. caerulescens* and *A. halleri*. Indeed very few or no ABC-transporters showed a change in regulation after Cd treatment, as shown on ABC sub-array slides and high density slides (data not shown). Besides, few or no genes related to sulfur metabolism were activated in *Thlaspi*, compared to *Arabidopsis*. Since plants synthesize PCs from glutathione (GSH) a sulfur-containing compound (Cobbett, 2000) an increase of expression of these genes would indicate the need for sulfur assimilated compounds to produce more GSH. Nevertheless, Ebbs *et al.* (2002) and Schat *et al.* (2002) have also shown that PCs do not seem to play a predominant role in Cd tolerance in *T. caerulescens*.

Interestingly when assessing the proteins involved in the cell wall organization, another site for Cd storage (see above), *A. thaliana* showed the highest gene expression alteration followed by *A. halleri* and at last *T. caerulescens*. This may indicate that there might have constitutive cell wall binding capacity in plants. There are indeed large variations in the binding capacity of the cell wall between plant species (Guo *et al.*, 1995) or genotypes of a given species (Florjin and Van Beusichem, 1993; Cakmak *et al.*, 2000), that might be an explanation for the differences in Cd uptake and distribution. Cadmium on the other hand induced transcription factors and protein kinase suggesting that plants respond to Cd by activating signal transduction pathways, which may include a protein phosphorylation cascade. Interestingly in both *Thlaspi* metal transporters (M-TR) were not induced, maybe indicating the existence of specific metal transporters in this species or alternatively that Cd concentration applied was not high enough to induce them. Some heat shock proteins (HSP), which may be a protection against Cd stress, were up-regulated in *Arabidopsis* but not in Prayon, thereby confirming the data published by Suzuki *et al.* (2001). All these differences between *Arabidopsis* and *Thlaspi* likely suggest the establishment of different detoxification and stress pathways. However, only functional analyses of the identified genes will reveal the molecular mechanisms of Cd response in hyperaccumulating plants. In addition, these results suggest also that oxidative stress is an important component of Cd toxicity in plant cells (Schützendübel and Polle, 2002).

4.6 Conclusion

Several different approaches were tested to examined the importance of different leaf parts in Cd storage. Due to methodological difficulties different Cd treatments were also performed on plants. However, the results of the different experiements were consistent. Furthermore, we demonstrated with the protoplast extraction procedure that although the Cd content of the different subcellular fractions increased with increasing Cd treatments, Cd distribution remained similar for the different concentrations tested.

This work has demonstrated that Cd is similarly allocated in leaves of Ganges and Prayon, although the latter does not hyperaccumulate Cd in the field. Metal allocation indicates that the principle of metal storage in metabolically inactive plant parts governs metal accumulation. However, the limited storage capacity of vacuoles and cell walls demands the involvement of the whole plant if survival on metal-enriched soils is to be realized. Metals are first bound to the cell wall, an ion exchanger of comparatively low affinity and low selectivity. Transport systems and intracellular high affinity binding sites then mediate and drive uptake across the plasma membrane. The complex mechanisms resulting in an apparently undeterminable pattern underlying the Cd accumulation in limbs remain nevertheless unclear. Based on our results, we suggest that PCs might have a secondary role in Cd tolerance in *T. caerulescens*, possibly related to sequestration of Cd in leaf cell vacuoles.

The mechanisms of metal uptake by hyperaccumulating plants and the basis of their metal specificity are poorly understood. To date two mechanisms have been proposed to partly explain the high uptake of metals by hyperaccumulators compared to non-hyperaccumulating plants: 1) enhanced absorption of metal into the roots (Lasat *et al.*, 1996) coupled with high rates of translocation of metal from roots to shoots (Shen *et al.*, 1997; Lasat *et al.*, 1998), and 2) foraging for metal by the roots, involving preferential allocation of root biomass into regions of metal enrichment (Schwartz *et al.*, 1999; Whiting *et al.*, 2000) and a large root system compared to shoot dry matter that might further favour soil prospecting as well as heavy metals uptake (Keller *et al.*, 2003). Kochian *et al.* (2002) hypothesized that this increased transport could include a stimulated metal influx across the leaf cell plasma membrane and an enhanced storage in the leaf vacuole. Based on our results it is well possible, since the only differences found between both ecotypes in this study were indeed concentrations of Cd found in the storage sinks.

We know still frustratingly little about the mechanisms of accumulation in plants. Real progress is likely to follow the cloning and molecular characterisation of a tolerance locus. Unfortunately, searching for mutants in *A. thaliana* is unlikely to be helpful in this case, since it appeared to have clearly different mechanisms than hyperaccumulating plants. However, none of the wild plants showing real tolerance have been sufficiently well studied molecularly that any strategy for the cloning of a tolerance gene suggests itself.

5

Hyperaccumulation of Cd and Zn in *Thlaspi caerulescens* and *Arabidopsis halleri* at the leaf cellular level

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5.1 Abstract

Vacuolar compartmentalization or cell wall binding in leaves could play a major role in hyperaccumulation of heavy metals. However, little is known about the physiology of intracellular Cd sequestration in plants. We investigated the role of the leaf cells in allocating metal in hyperaccumulating plants by measuring short-term ^{109}Cd and ^{65}Zn uptake in mesophyll protoplasts of *Thlaspi caerulescens* Ganges, *Arabidopsis halleri* both hyperaccumulators of Zn and Cd, and *T. caerulescens* Prayon accumulating at lower degree Cd. The effects of low temperature, several divalent cations, and pre-exposure of the plants to metals were investigated.

There was no significant difference between the Michaelis-Menten kinetic constants of the three plants. It indicates that differences in metal uptake can not be explained by different constitutive transport capacities at the leaf protoplast level and that plasma and vacuole membranes of mesophyll cells are not responsible for the differences observed in heavy metal allocation. This suggests the existence of regulation mechanisms before the plasma membrane of leaf mesophyll protoplasts. However, pre-exposure of the plants to Cd induced an increase in Cd accumulation in protoplasts of Ganges, whereas it decreased Cd accumulation in *A. halleri* protoplasts, indicating that Cd permeable transport proteins are differentially regulated. The experiment with competitors has shown that probably more than one single transport system are carrying Cd in parallel into the cell, and that in *T. caerulescens* Prayon Cd could be transported by a Zn and Ca pathway, whereas Cd in Ganges could mainly be transported by other pathways.

5.2 Introduction

Cadmium and zinc are two widespread harmful heavy metals, but there is no cost-effective mean to remove them from the soil. Although phytoextraction using

hyperaccumulator plants is seen as a promising technique, a lack of understanding of the basic physiological, biochemical, and molecular mechanisms involved in heavy metal hyperaccumulation prevents the optimization of the phytoextraction technique and its further commercial application. A research priority is therefore to gain basic information on the dynamics of metal movement into the cells, on their final allocation and their sink capacities in hyperaccumulating species.

Thlaspi caerulescens and *Arabidopsis halleri* are both plants able to hyperaccumulate Zn and Cd (Robinson *et al.*, 1998; Bert *et al.*, 2000). In *T. caerulescens* Zn seems to be preferentially sequestrated in vacuoles of epidermal cells in a soluble form (Küpper *et al.*, 1999; Frey *et al.*, 2000). In *A. halleri* leaves, Zn was found to be predominantly coordinated to malate (Sarret *et al.*, 2002) and accumulated in the mesophyll cells (Küpper *et al.*, 2000; Zhao *et al.*, 2000). An important trait of hyperaccumulating species might be enhanced translocation of the absorbed metal to the shoot. Time course studies of Zn accumulation revealed that *T. caerulescens* exhibited a 10-fold greater Zn translocation to the shoot as compared with *T. arvense* (Lasat *et al.*, 1996), which was correlated with a 5-fold increase of Zn in xylem sap (Lasat *et al.*, 1998). These authors have performed compartmentation studies and have found that ^{65}Zn uptake by leaf protoplasts is also stimulated in *T. caerulescens* as compared to *T. arvense*. These physiological evidences indicate that Zn hyperaccumulation in *T. caerulescens* is caused, in part, by an increased transport at multiple sites along the metal absorption and translocation pathway. Kochian *et al.* (2002) hypothesized that this increased transport could include a stimulated metal influx across the leaf cell plasma membrane and an enhanced storage in the leaf vacuole. Although vacuole and/or protoplast transport and storage of major elements have been studied for crop plants (Dietz *et al.*, 1992), Lasat's study is to our knowledge the only one trying to define the role of the leaf cells in uptake and storage of heavy metals in hyperaccumulator plants. Tolerance and accumulation in *T. caerulescens* have also been studied at the molecular level. Three ZIP-like (Grotz *et al.*, 1998) zinc transporters mainly overexpressed in the roots, and one ZAT-like (Van der Zaal *et al.*, 1999) zinc transporter mainly expressed in the leaves have been described (Pence *et al.*, 2000; Assunção *et al.*, 2001). Comparison with *T. arvense* (non accumulator) indicated that metal regulation of gene expression was altered in the hyperaccumulator, but not functionally different from the non-accumulator (Lasat and Kochian, 2000).

Contrary to Zn little information is available on Cd hyperaccumulation, and no putative high affinity transporter gene has been identified in plants yet. In *T. caerulescens*, Cd has been found in the apoplast as well as in the vacuole (Vázquez *et al.*, 1992a). It has been demonstrated that the physiological mechanism of Cd tolerance is not based on an enhanced synthesis of phytochelatins (Ebbs *et al.*, 2002; Schat *et al.*, 2002) but on a preferential compartmentation of the metal in the plant. Boominathan and Doran (2003) reported for example the ability of *T. caerulescens* hairy roots to hold most of the Cd in the cell walls. It is generally believed that Cd uptake by plants represents opportunistic transport via cation channels for Ca and Mg or via a carrier for other divalent cations such as Zn, Cu or Fe (Welch and Norvell, 1999). It has also been suggested that there exist common mechanisms of absorption and transport of Zn and Cd in *T. caerulescens* since Cd and Zn have a similar electronic structure (Baker *et al.*, 1994b). Bert *et al.* (2003) showed that in *A. halleri* Cd and Zn accumulation were positively correlated suggesting that the metals are

taken up, at least to a certain degree, by the same transporter(s) or are controlled by common regulators. There is evidence that the Zn transporter, ZNT1, recently cloned from the Prayon ecotype of *T. caerulescens* can also mediate transport of Cd, albeit with low affinity (Pence *et al.*, 2000). Zinc was found in some cases to depress Cd uptake indicating some kind of interaction between these metals (Lombi *et al.*, 2000; Lombi *et al.*, 2001b). However, because of differences in Cd uptake found between populations, it seems now that there might be differences between Cd and Zn for uptake and accumulation by *T. caerulescens* (Lombi *et al.*, 2000).

From these early results, it seems that removal of heavy metals from metabolically active cellular sites and subsequent storage in inactive compartments is the key to heavy metal tolerance, therefore vacuolar compartmentalization or cell-wall binding could play a major role in tolerance and hyperaccumulation.

The goal of this work was to understand some of the mechanisms involved in Cd and Zn uptake at the mesophyll protoplast level. Special attention was directed to the differences between *T. caerulescens* population Ganges, *A. halleri*, both hyperaccumulators of Zn and Cd, and *T. caerulescens* population Prayon accumulating at lower degree Cd. More in detail, we assessed 1) if differences in uptake at the plant level could be explained by an enhanced transport at the cell level, 2) if Cd and Zn compartmentation in *T. caerulescens* leaves was a passive or carrier-mediated mechanism and how it differed between *T. caerulescens* ecotypes and *A. halleri*, and 3) the rate of metal uptake in protoplasts and their storage kinetics for the three plants tested. Metal uptake experiments on mesophyll protoplasts were thus performed over time, and at various concentrations of Cd and the effect of plant pre-treatment with metals, the effect of low temperature, of several divalent cations, and of a competitor on protoplast uptake were assessed. Emphasis was put on Cd although Zn was also studied for comparison.

5.3 Materials and Methods

5.3.1 Plant material and culture

The plants studied were *T. caerulescens* ecotype Ganges (southern France) and *A. halleri* (northern France) both known for Zn and Cd hyperaccumulation (Robinson *et al.*, 1998; Bert *et al.*, 2000; Dahmani-Muller *et al.*, 2001), and *T. caerulescens* population Prayon accumulating at lower degree Cd (Lombi *et al.*, 2000).

Seeds were germinated in the dark on filters moistened with deionized water. Three-week-old seedlings were transferred to a 1-L pot (four plants per pot) filled with modified quarter-strength Hoagland's nutrient solution (Sigma, St Louis, USA) supplemented with 20 µM Fe-HBED (Strem chemical, Newburyport, USA). Fe(III)-HBED was prepared as described by Chaney *et al.* (1998) in such a way that all HBED was saturated with Fe. Plants were allowed to grow 2 weeks in hydroponics before treatment with metals was started. Five different treatments were then performed: control, 5 µM Cd, 10 µM Cd, 50 µM Zn and 500 µM Zn. Three pots per treatment were set up.

Germination and plant culture were performed in a climate chamber (day/night period 16/8 h, day/night temperatures 20°C/16°C, and a light intensity of 500 lux). The nutrient solution was renewed every week and aerated continuously.

5.3.2 Concentration of Cd and Zn in plants

The total concentration of Cd and Zn in leaves of *A. halleri,* Ganges and Prayon pre-treated with Cd (5 and 10 µM) and Ganges pre-treated with Zn (50 and 500 µM) and in control plants was measured. Four different 12-week-old plants grown in hydroponics (1 pot) were harvested and dried at 80°C for one week. Plants were individually weighted and hot-digested in HNO_3 65% sp. (Fluka, Buchs, Switzerland) and $HClO_4$ 70% p.a. (Fluka, Buchs, Switzerland). The concentrations in plants were then measured by ICP-AES (Perkin Elmer Plasma 2000, Wellesley, USA).

5.3.3 Preparation and purification of mesophyll protoplasts

Mesophyll protoplasts were prepared from leaves of 8 different 12-week-old plants (2 pots). Abaxial sides of leaves were pealed and placed in a cell wall digesting medium composed of sorbitol medium (500 mM sorbitol, 10 mM Mes, 10 µM $CaCl_2$, pH 5.3), 0.75% (w/v) Cellulase Y-C (Kikkoman, Tokyo, Japan), and 0.075% (w/v) Pectolyase Y-23 (Kikkoman, Tokyo, Japan). The leaves were incubated during 2 to 4.5 hours at 30°C until digestion was judged satisfactory but had not reached the epidermal cell layer yet. The resulting suspension was centrifuged at 400g for 7 minutes on top of a 100 % Percoll medium cushion (500 mM Sorbitol, 10 µM $CaCl_2$, 20 mM MES, pH 6 solubilized in Percoll (Sigma, St Louis, USA)). The supernatant was discarded and the layer of mesophyll protoplasts was resuspended in the residual liquid. Percoll medium 100% was added to the protoplasts mix to obtain a final Percoll medium 50% (1:1 (v/v) sorbitol medium with 100% Percoll medium), which was overlayed with a Percoll medium 40% (3:2 (v/v) sorbitol medium with 100% Percoll medium) and further with a layer of sorbitol medium. The gradient was centrifuged at 400g for 5 minutes. The protoplasts were collected from the upper interface. All centrifugation steps were performed at 4°C. Protoplasts were shortly kept in test tubes on ice until the uptake experiments were performed. A typical preparation of protoplasts is shown in Figure 5.1.

5.3.4 Determination of protoplast viability

The percentage of viable protoplasts in the stock was determined by staining with fluorescein diacetate (Fluka, Buchs, Switzerland) as described by Lasat *et al.* (1998). A stock solution of 7.2 mM FDA dissolved in acetone was prepared. Protoplasts were incubated 10 minutes in 36 µM FDA (final concentration), and inspected using a fluorescence microscope. Protoplasts showing bright fluorescence were counted as viable.

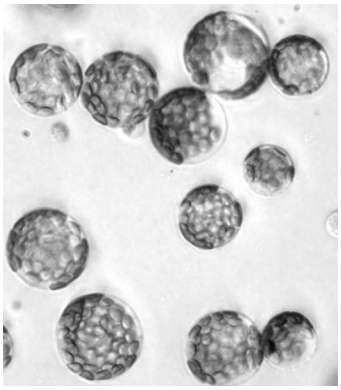

Figure 5.1: Purified mesophyll protoplasts from leaves of 12-week-old Ganges.

5.3.5 Uptake experiment with protoplasts: concentration and time-dependence

As a first step we studied the concentration-dependent kinetics of Zn and Cd. The protoplasts were diluted 1:4 (v/v) with betaine medium (500 mM, 10 µM $CaCl_2$, 20 mM MES, pH 5.5) prior to the uptake experiment. In standard conditions, the pH of the incubation medium had a strong effect on the uptake. Several pHs (4.5, 5.0, 5.5, 5.7, 6.2, 6.6 and 7.6) were tested for the betaine medium since it determines the final pH during incubation. Both Cd and Zn uptake in protoplasts was maximal at pH 5.5 (data not shown). In a further experiment pH was maintained at 5.5 with MES buffer, which is known to have minimal metal-complexing ability.

The time course of ^{65}Zn and ^{109}Cd uptake was initiated by the addition of $ZnCl_2$ spiked with 18.5 kBq mL^{-1} of $^{65}ZnCl_2$ (NEN Life Science Products, Boston, USA) for a final total Zn concentration of 3.4, 6.6, 9.6, 19.2, 28.2, 37.1, and 45.8 µM, or $CdCl_2$ spiked with 18.5 kBq mL^{-1} of $^{109}CdCl_2$ (NEN Life Science Products, Boston, USA) for a final total Cd concentration of 1.6, 3.2, 6.3, 9.6, 14.3, 19.3, 28.9, 38.3, 47.9, 96.1 and 186.8 µM. Uptake was measured until t =120 minutes for Ganges (Figure 5.2). Because the accumulation rate reached a plateau after 30 minutes (Figure 5.2), uptake was further measured only at t =1, 2, 5, 10 and 30 minutes for all the samples. At various time intervals, a 100 µL aliquot of the spiked protoplast suspension was sampled and placed on top of a discontinuous gradient consisting of 200 µL of silicon oil (AR 200, Fluka, Buchs, Switzerland) on top of 30 µL of 40% Percoll medium added in a 400 µL microcentrifuge tube. The microcentrifuge tubes were immediately centrifuged using a bench-top microcentrifuge (model 5417R, Eppendorf, Hamburg, Germany) at high speed for 20 secondes to pellet the protoplasts through the silicon oil layer. After centrifugation, tubes were frozen at -20°C. Tips containing the frozen protoplast pellets were cut off and placed in counting vials, 4 mL of scintillation solution (Ultima Gold LSC-cocktail, Packard Bioscience, Meriden, USA) was added. The uptake of ^{65}Zn and ^{109}Cd was quantified via scintillation counting (Packard, Tri-Carb, liquid scintillation analyser, Meriden, USA). The values obtained for t= 1 minute incubation were considered as a background coming from the thin film of spiked

solution sticking to the protoplasts surface. These values were systematically substracted from the following measures. All the fittings were calculated using SigmaPlot (Chicago, USA).

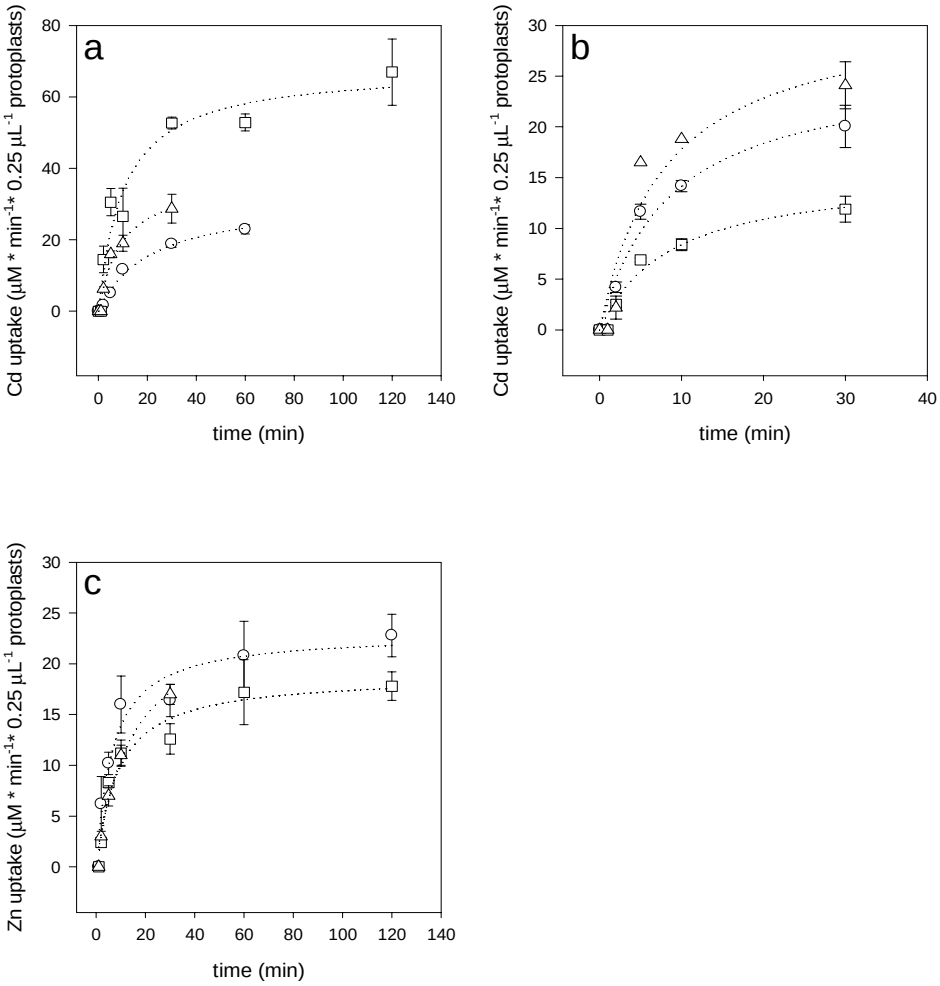

Figure 5.2: Time-dependent uptake of 10 µM Cd in a) Ganges mesophyll protoplasts or b) *A. halleri* mesophyll protoplasts. Plants were pre-exposed seven weeks to 0 (triangles), 5 (circles) or 10 (squares) µM Cd during growth in hydroponics. c) Time-dependent uptake of 10 µM Zn in Ganges mesophyll protoplasts. Plants were pre-exposed seven weeks to 0 (triangles), 50 (circles) or 500 (squares) µM Zn during growth in hydroponics. Error bars do not extend outside some data symbols. Error bars represent means (n=4) ± SD.

The protoplast volume collected after silicon oil centrifugation was quantified with ^{3}H-water (Hartmann analytic, Braunschweig, Germany). Hundred µL of the concentrated protoplast solution were incubated 10 minutes with 400 µL betaine medium and 18.5 kBq ^{3}H-water. The protoplast were pelleted and quantified via scintillation counting as described before. The method allowed a quick estimation of number of protoplasts in the preparation. The results obtained by this method and the total number of protoplast per mL in the concentrated preparation counted with a hemocytometer were compared and are presented in Figure 5.3. All the calculations were reported for 0.25 µL of protoplasts, which corresponds to $4.8 \cdot 10^{6}$ protoplasts per

mL. Additionally, since with this method the rate of uptake into cells is proportional to the surface area of the protoplasts, we measured with the software MetaView™ Imaging Sytem (Universal Imaging Corporation, WestChester, USA) the diameter of the protoplasts in order to estimate their surface (4 π radius^2).

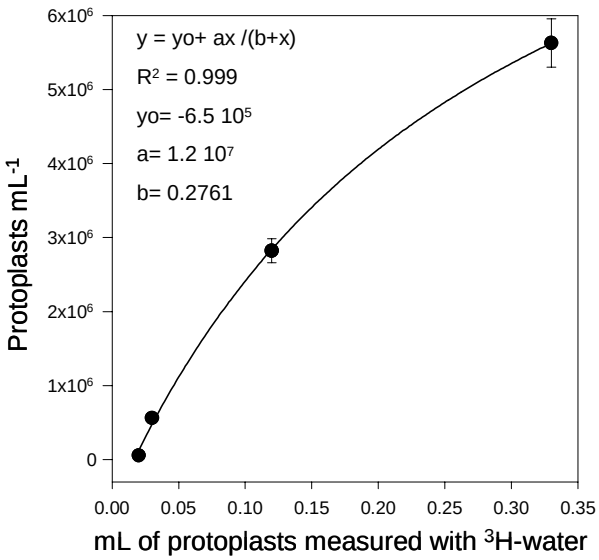

Figure 5.3: Relation between the µL of protoplasts measured with the ^{3}H-water method and the total number of protoplasts per mL counted with a hemocytometer. Error bars do not extend outside some data symbols. Error bars represent means (n= 4) ± SD.

5.3.6 Effect of plant Cd and Zn pre-exposure on protoplast uptake

The effect of Cd^{2+} (5 and 10 µM) or Zn^{2+} (50 and 500 µM) during growth of plants on heavy metal uptake in protoplasts was investigated in Ganges. The effect of plant pre-exposure to Cd^{2+} (5 and 10 µM) on Cd uptake in protoplasts was also studied in *A. halleri* to assess if Cd had a similar effect on the other Cd hyperaccumulating plant. Experiments were carried out as described above for time-dependent uptake in mesophyll protoplasts. This experiment was not carried out with the Prayon ecotype.

5.3.7 Effect of different protoplast treatments on Cd and Zn uptake

Several inhibitors or competitors (verapamil 100 µM; Ca^{2+} 50 and 200 µM; Cd^{2+} 50 and 200 µM; Mg^{2+} 50 and 200 µM; Zn^{2+} 50 and 200 µM; no addition and on ice) were added in the standard assay medium. Cadmium or Zn uptake in mesophyll protoplasts extracted from non-preexposed plants was measured as explained above. Verapamil was only tested on Ganges. Calcium and Mg^{2+} competition were not tested on *A. halleri*.

5.4 Results

5.4.1 Viability and homogeneity of protoplasts samples

The percentage of viable protoplasts was determined by staining with fluorescein diacetate (Fluka, Buchs, Switzerland). Protoplast viability ranged between 80% and 95%. Additionally, sizes of protoplasts were measured to assess homogeneity between the different samples. Protoplasts were similarly distributed for all the plants tested and the different treatments. Most of the protoplasts were found in the surface classes between 1000 and 2000 μm^2 (39±6% of protoplast number), and between 2000 and 4000 μm^2 (41±4%). Only 7±4% were smaller than 1000 μm^2 and 13±8% bigger than 4000 μm^2.

5.4.2 Effects of plant pre-exposure to Cd and Zn

To determine whether Zn and Cd accumulation in protoplasts was modified by exposure of the plant to heavy metals, we investigated the effect of plant pre-treatment with Cd or Zn on the time-dependent kinetics of heavy metal apparent uptake in protoplasts. The concentration of Cd and Zn measured in leaves and the aerial biomass of Ganges and *A. halleri* are presented in Table 5.1. No phytotoxic effect was visible on leaves of both pre-treated plants. No effect of Zn pre-exposure of the plants was observed on the Zn uptake capacities of protoplasts from Ganges, but protoplasts extracted from plants grown in 10 µM Cd solution accumulated 2.8-times more Cd than protoplasts extracted from plants grown in 5 µM or no Cd (Figure 5.2). On the contrary, protoplasts extracted from *A. halleri* grown in 10 µM Cd solution accumulated 2.1-times less Cd than protoplasts extracted from plants grown in 5 µM or no Cd (Figure 5.2). The differences were statistically significant for both plants (Student t-test $P<0.001$).

Table 5.1: Average biomass per plant and concentrations of Cd and Zn in leaves of 12-week-old Ganges, *A. halleri* and Prayon grown in hydroponics with or without heavy metals (n= 4, SD in parentheses).

	Ganges		*A. halleri*		Prayon	
Cd in nutrient solution (µM)	Cd (mg kg^{-1})	dry matter (mg)	Cd (mg kg^{-1})	dry matter (mg)	Cd (mg kg^{-1})	dry matter (mg)
0	3 (2)	205 (37)	3 (2)	334 (24)	4 (4)	235 (73)
5	795 (300)	138 (61)	1927 (623)	124 (36)	170 (11)	242 (5)
10	1274 (643)	131 (39)	1167 (913)	119 (24)	264 (44)	287 (52)

	Ganges	
Zn in nutrient solution (µM)	Zn (mg kg^{-1})	dry matter (mg)
0	78 (53)	205 (37)
50	7201 (1006)	268 (68)
500	12773 (2454)	284 (59)

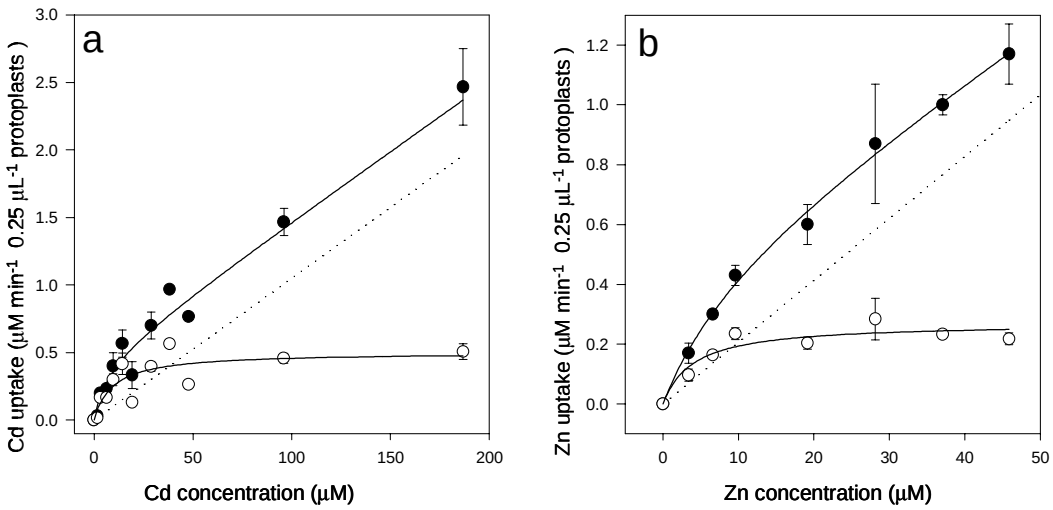

Figure 5.4: Concentration dependent kinetic at 30 minutes for a) Cd and b) Zn accumulation in 0.25 µL mesophyll protoplasts extracted from Ganges leaves. The linear (dashed line) and saturable (open symbols) component were derived from the experimental data (closed symbols) by computing the linear component from the regression line plotted through high-concentration points and substracting this contribution from a curve fit to the experimental data. Error bars do not extend outside some data symbols.

5.4.3 Cadmium and Zn accumulation in protoplasts of the two ecotypes of T. caerulescens *and* A. halleri

Plants were grown in absence of metals in the nutrient medium to avoid pre-exposure effects. When starting the study, we calculated kinetic constants for different times of exposure to find the most representative time of incubation for the calculations. Based on the time-dependent experiment (Figure 5.2), calculations of the Michaelis-Menten parameters v_{max} and K_m were done at t= 5 minutes (linear accumulation rate phase) and at t= 30 minutes (beginning of the plateau). After 5 minutes incubation, Ganges exhibited for Cd a v_{max} of 0.83±0.07 µM minute^{-1} 0.25 µL^{-1} protoplasts and a K_m of 2.58±1.03 µM, for Zn the v_{max} was 1.915±0.3 µM minute^{-1} 0.25 µL^{-1} protoplasts and K_m was 5.053±3.14 µM. Results at 30 minutes were a v_{max} of 0.50±0.08 µM Cd minute^{-1} 0.25 µL^{-1} protoplasts and a K_m of 9.68±5.78 µM Cd, v_{max} of 0.27±0.03 µM Zn minute^{-1} 0.25 µL^{-1} protoplasts and a K_m of 4.01±1.90 µM Zn. Because of high SD there were no significant differences in the calculated K_m between the two times of incubation and v_{max} values were in the same order of magnitude. Additionally, at 30 minutes reproductibility was better. We decided then to perform all the measurements and calculations at 30 minutes although the accumulation rate was not linear. The accumulation in protoplasts was shown to be concentration dependent for the 3 plants tested. Because ^{109}Cd and ^{65}Zn uptake was measured during a short

period only (30 minutes), the results mainly represent unidirectional influxes. The concentration-dependent accumulation kinetics for ^{109}Cd and ^{65}Zn was characterized by smooth non-saturating curves. The curves could be mathematically resolved into linear and saturable components (Figure 5.4) by applying a modified hyperbolic function defined as:

$$y = [(a \cdot x)/(b+x)]+(c \cdot x)$$

where x= Cd or Zn concentration in the incubation medium in µM, and y= Cd or Zn accumulation rate in protoplasts in µM minute^{-1} 0.25 µL^{-1} protoplasts, and a, b, and c were parameters determined by the curve fitting algorithm to best fit the data points. In all cases, the model fitted closely the experimental data as demonstrated by the R^2 values for the fitted curves ranging from 0.961 to 0.996 (Table 5.2). As calculated by Lasat *et al.* (1996) and Lombi *et al.* (2001b, 2002) substraction of the regression line plotted through high-concentration points leaves out the saturating Michaelis-Menten curve which allows determination of the kinetic constants K_m and v_{max} (Table 5.2). The remaining saturable component is the result of carrier-mediated transport across the plasma membrane. There were no significant differences in the calculated kinetic constants between the plants studied: mesophyll protoplasts of the three plants showed the same affinity and, when not preincubated, the same capacity to transport Cd or Zn into the cell.

Table 5.2: Parameters of the Michaelis-Menten model for Cd and Zn absorption by mesophyll protoplasts (n=4, SD in parentheses)

	Cd			Zn		
Plant used for protoplasts extraction	v_{max} (#)	K_m (µM)	R^2	v_{max} (#)	K_m (µM)	R^2
Ganges	0.50 (0.08)	9.68 (5.78)	0.976[a]	0.27 (0.03)	4.01 (1.90)	0.996[a]
Prayon	1.15 (0.14)	4.83 (2.11)	0.977[a]	0.53 (0.08)	12.30 (5.80)	0.961[a]
A. halleri	0.78 (0.10)	4.81 (2.13)	0.983[a]	0.05 (0.01)	3.35 (4.42)	0.983[a]

(#= µM minute^{-1} 0.25 µL^{-1} protoplasts; sign. a: $2\alpha<0.001$)

5.4.4 Effect of low temperature

We investigated the effect of low temperature on heavy metal uptake in protoplasts to further determine whether Cd and Zn accumulation in protoplasts was

caused by movement across the plasma membrane into the cytosol or by binding of heavy metal to negatively charged sites associated with the external face of the plasma membrane. The uptake was strongly inhibited when performed at ice temperature (2 °C) for both Cd and Zn for the three plants: after 30 minutes incubation with 10 µM Cd or Zn the cold treatment decreased total accumulation of Cd or Zn by at least 50 % compared with the controls (Figures 5.5 and 5.6).

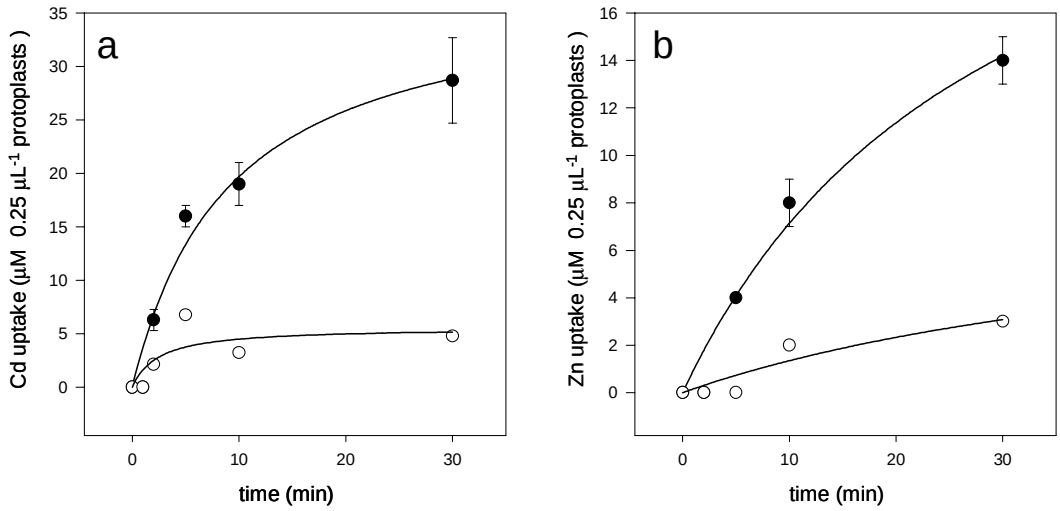

Figure 5.5: Time-dependent uptake of a) 10 µM Cd and b) 10 µM Zn in Ganges mesophyll protoplasts at room temperature (closed symbols) and at ice-cold temperature (open symbols). Error bars do not extend outside some data symbols. Error bars represent means (n=4) ± SD.

5.4.5 Effect of competitors

To determine whether Cd accumulation in protoplasts was due to opportunistic transport via channels or carriers for other cations, we investigated the effects of several cations and of a specific inhibitor of Ca channel, verapamil, on the kinetics of Cd and Zn apparent uptake in protoplasts. Figure 5.6 summarizes the results for the 3 plants. The addition of 100 µM verapamil had no effect on the apparent uptake of Cd or Zn in the Ganges ecotype (data not shown). In this ecotype addition of 50 µM Cd^{2+} or 50 µM Ca^{2+} decreased Zn transport by respectively 40% (Student t-test $P<0.0001$) and 15% ($P<0.001$), whereas only addition of 50 µM Zn^{2+} decreased Cd transport by 20% ($P<0.01$). However, 200 µM Ca^{2+} had also a 15 % ($P<0.001$) inhibitory effect on Cd^{2+} uptake. Fifty µM Cd^{2+} inhibited Zn uptake by 15% ($P<0.05$) in Prayon, but 50 µM Zn^{2+}, 50 µM Ca^{2+} and 50 µM Mg^{2+} inhibited Cd uptake by respectively 30% ($P<0.0001$), 15% ($P<0.0001$), and 25% ($P<0.001$). A competition between Cd and Zn

was also observed for *A. halleri*: 50 µM Cd^{2+} reduced Zn accumulation by 15% (P<0.001) and 50 µM Zn^{2+} reduced Cd accumulation by 10% (P<0.001).

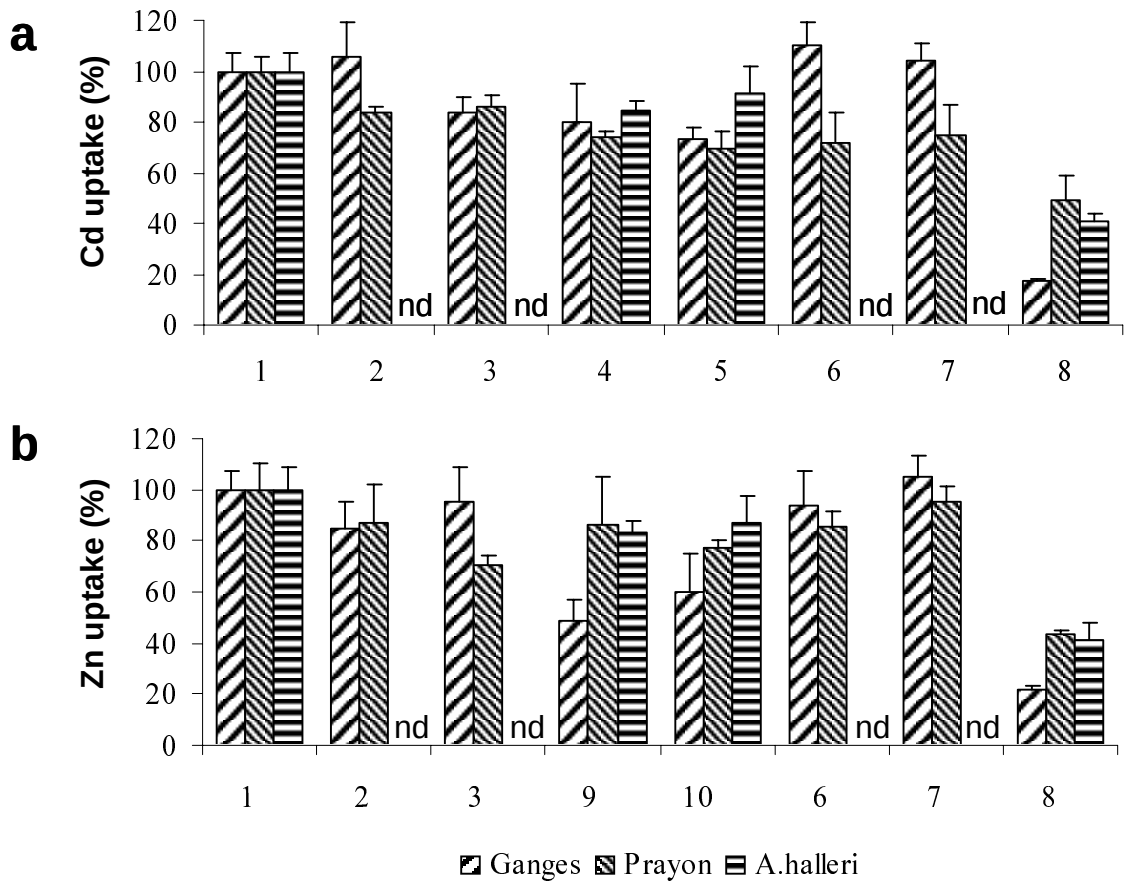

Figure 5.6: Mesophyll protoplasts apparent uptake (30 minutes) of 10 µM a) Cd, or b) Zn. Plants were grown without Cd. Effect of 1: control, addition of 2: Ca^{2+} 50 µM, 3: Ca^{2+} 200 µM, 4: Zn^{2+} 50 µM, 5: Zn^{2+} 200 µM, 6: Mg^{2+} 50 µM, 7: Mg^{2+} 200 µM, 8: ice-cold temperature, 9: Cd^{2+} 50 µM, 10: Cd^{2+} 200 µM, nd: not determined.

5.5 Discussion

5.5.1 Effects of plant pre-treatment

Laboratory studies using nutrient solutions are the only possible experiments allowing determination of the precise metal concentration in the growth medium: in soil it is necessary to base the discussion on total metal concentration or one of a variety of measures of the available metal concentration. Because of numerous parameters controlling soil metal bioavailability, the link between metal available to plant in soil and plant uptake is not obvious. On the other hand, in soils the metals are present from the beginning and during the plant growth and may have a significant

effect on plant uptake ability. To date most studies in nutrient solution have examined Cd transport at unrealistically high concentrations of metal and very short-time exposure, creating experimental conditions far from the soil conditions and limiting the possible impact of pre-exposure (Sanità di Toppi and Gabbrielli, 1999). It is therefore difficult to compare hydroponics studies with soil studies. We chose long time exposure and Cd concentrations in the range of concentrations that can be found in soil solution of contaminated soils. Additionally, concentrations were chosen in order to avoid visible phytotoxic symptoms, like necrosis or chlorosis, and indeed, no phytotoxic symptoms were observed on any of the treated plants compared to the controls except a loss in biomass (Table 5.1).

Ganges showed a higher uptake capacity in mesophyll protoplasts when exposed to 10 µM Cd during growth. It seems that a mechanism was switched-on in the treated plant above a determined threshold, since 5 µM Cd did not trigger an increased uptake. This mechanism may allow a faster removal of heavy metals from metabolically active cellular sites and subsequent storage in inactive compartments. No such effect was observed with Zn, which could indicate that either the mechanism for Zn uptake is regulated differently or the threshold has not been reached at 500 µM Zn. Further experiments with Zn would be needed to clarify this point, however this was not the purpose of the present work. *Arabidopsis halleri* on the contrary showed a decreased transport into protoplasts in Cd treated plants, indicating the establishment of a mechanism of Cd avoidance. Moreover, *A. halleri* seemed to be more affected than Ganges by Cd pre-treatments as shown by the larger loss in biomass (Table 5.1). An explanation to the establishment of a mechanism of Cd avoidance in *A. halleri* could be that a plant accumulating at high concentration Cd in the mesophyll cells would risk damaging its photosynthesis apparatus (Krupa and Baszynski, 1995; Horváth *et al.*, 1996; Geiken *et al.*, 1998). These results suggest that Ganges and *A. halleri* have a different mechanism regulating adaptation to Cd, despite the fact that mesophyll cells of the leaves have a similar constitutive Cd transport capacity as discussed below.

5.5.2 Cadmium and Zn uptake by the two ecotypes of T. caerulescens *and* A. halleri

The saturable nature of Cd and Zn accumulation in this study suggests that they are both taken up in protoplasts via a carrier-mediated system. There were no significant differences in the calculated kinetic constants between the 3 plants. In the three cases mesophyll cells were equally capable of transporting and accumulating the two metals. Nevertheless, *A. halleri* is known to hyperaccumulate Cd and Zn in the mesophyll cells (Küpper *et al.*, 2000; Zhao *et al.*, 2000), whereas *T. caerulescens* preferentially hyperaccumulates Zn in the epidermal cells (Frey *et al.*, 2000). Moreover, Prayon does not hyperaccumulate Cd in the field (Lombi *et al.*, 2000). Calculated kinetic constants were also similar for Cd and Zn in the three plants, again not reflecting the differences of accumulation known in Prayon ecotype for Zn and Cd. Because experimental procedures and conditions have a considerable influence on the kinetics of ion uptake, v_{max} and K_m reported by different authors are not strictly comparable. However, the K_ms estimated here for Zn in mesophyll protoplasts were in the same order of magnitude as those reported by several authors, ranging from 0.3 to 8 µM, for whole plants or roots of *T. caerulescens* (Lasat and Kochian, 1997; Pence

et al., 2000; Lombi *et al.*, 2001b; Kochian *et al.*, 2002; Lombi *et al.*, 2002). V_{max} values are even more difficult to compare because of the different units of measure used. However, we found a higher v_{max} value for Prayon than for Ganges for Zn uptake, whereas Lombi *et al.* (2001b) reported the opposite for roots of the same plants. This difference may indicate that different mechanisms underlie in the different steps along the way of the metal from the soil to the leaf cells, indicating that the mechanism responsible for metal uptake in hyperaccumulator plants is probably very complex. Lombi *et al.* (2001b; 2002) are the only authors who calculated Michaelis-Menten parameters for Cd in *T. caerulescens*: they reported values for apparent K_ms between 0.18 and 1.21 µM for Cd in roots of Ganges and Prayon. However, the studies were performed on 40-day-old seedlings of *T. caerulescens* thereby limiting the comparison with our work that was performed on leaf cells of 12-week-old plants. Besides it is questionable whether 40-day-old plants had reached their plain maturity, since they have a very slow growth: flowering usually takes place after at least 14 weeks in controlled conditions. Indeed, Bovet *et al.* (2003) showed that results obtained on Cd transport in *Arabidopsis thaliana* could not be extrapolated from seedlings to mature plants.

The physiological evidence obtained from this study (similar behavior at the protoplast level) suggests the existence of regulation mechanisms before the protoplast plasma membrane that direct the metals to their final location in plants. The cell walls could act as a selective barrier, considering their capacity to interact with metal ions and the presence of many enzymes at its surface (Wang and Evangelou, 1995). In mycorrhizal fungi, Cd was found to be bound to negatively charged sites associated with the cell walls such as cellulose, cellulose derivatives or to the outer pigmented layer of the cell wall (Galli *et al.*, 1994; Turnau *et al.*, 1994; Blaudez *et al.*, 2000). Nickel was also found to be associated with cell wall pectates in *Hybanthus floribundus* (Salt and Krämer, 2000). Metal binding to cell walls as a possible heavy metal tolerance mechanism has been proposed for various plants and metals (for a review see Wang and Evangelou, 1995). The importance of Cd binding to cell walls and the limitation of its subsequent translocation into shoots is well known for root cells of non-hyperaccumulating plants (Wagner, 1993; Grant *et al.*, 1998) and was recently described in *T. caerulescens* hairy roots (Boominathan and Doran, 2003). There are large variations in the retention of Cd in cells between plant species (Guo *et al.*, 1995) or genotypes of a given species (Florjin and Van Beusichem, 1993; Cakmak *et al.*, 2000): different binding capacity to the cell wall has been proposed by these authors as an explanation for the differences in Cd uptake and distribution. Polyvalent cations are more likely to interact with cell walls than monovalent cations, due to their stronger electrostatic attraction to cell wall negative charge sites. Most heavy metals are divalent or trivalent cations and therefore are expected to undergo adsorption/ exchange reactions with wall surfaces before they move to their final location (Wang and Evangelou, 1995). It is thus likely that variation in binding to leaf cell walls could explain the differences of heavy metal allocation described for the three plants studied here. Nevertheless since this study is based on short-term experiments because of the short life of extracted protoplasts, we can not totally exclude that in the long-term differences might appear, for example originating from the required time for synthesis of heavy metals complexing molecules or to reach the equilibrium between free and bound heavy metals.

5.5.3 Effects of cold and competitors on Cd and Zn uptake

Zhao *et al.* (2002) assumed that metabolically dependent uptake would be negligible at low temperatures. Thus the difference between results obtained at room temperature and on ice would represent the metabolically dependent uptake of Cd and Zn. In our experiment Cd and Zn accumulation in protoplasts was strongly decreased at low-temperature for the three plants, suggesting that it was a metabolically mediated process. This result together with the concentration-dependent results confirms a carrier-mediated pathway for both metals.

It has been shown that verapamil at µM concentrations inhibited voltage-dependent Ca and Cd influx in animal cells (Hinkle *et al.*, 1987), but the effects reported in plant studies seem inconsistent (Pineros and Tester, 1997): Ca influx into protoplasts from *Physcomitrella patens* (Schumaker and Gizinski, 1993), carrot (Graziana *et al.*, 1988), *Amaranthus tricolor* (Rengel and Elliot, 1992), tobacco (Volotovski *et al.*, 1998) and into plasma membrane vesicles isolated from oat roots (Gonzalez *et al.*, 1999) was reduced upon exposure to verapamil, although up to 100 µM verapamil did not block the calcium influx into plasma membrane vesicles isolated from wheat roots or oat seedlings (Huang *et al.*, 1994; Babourina *et al.*, 2000). Furthermore in aquatic plants it was found to inhibit Ca uptake, but to increase Cd uptake (Karez *et al.*, 1990; Tripathi *et al.*, 1995). In epidermal peels of *A. thaliana* 250 µM verapamil was found to inhibit the stomatal closure induced by Cd (Perfus-Barbeoch *et al.*, 2002). Interpretation of these pharmacological results is difficult as the effect of verapamil on other experimental variables (e.g. membrane potential) is unknown (Pineros and Tester, 1997; Babourina *et al.*, 2000). In this study, verapamil at 100 µM was found to have no effect on the accumulation of Cd or Zn by Ganges.

Cadmium could be taken up in plants by carriers or cation channels for other cations such as Zn^{2+}, Fe^{2+}, Ca^{2+} or Mg^{2+} (Welch and Norvell, 1999). There are numerous studies showing inhibitory effects of those divalent cations on Cd uptake by higher plants or algae (Smeyer-Verbeke *et al.*, 1978; Cataldo *et al.*, 1983; Karez *et al.*, 1990; Costa and Morel, 1993; Tripathi *et al.*, 1995; Hart *et al.*, 2002). In non accumulating plants, Clemens *et al.* (1998) showed that a Ca transport pathway could be involved in the uptake of Cd, albeit with low affinity. Members of the ZIP gene family were shown to be capable of transporting transition metals including Fe, Zn, Mn, and Cd (Guerinot, 2000). The Fe transporters such as IRT1 (ZIP) and Nramp have been shown to be able to transport several metals including Cd in *A. thaliana* (Korshunova *et al.*, 1999; Thomine *et al.*, 2002) and in *T. caerulescens* (Zhao *et al.*, 2002). All these data are consistent with the reciprocal uptake inhibition between Cd and Zn in the two ecotypes of *T. caerulescens* and *A. halleri* that we observed in our work: we also observed an inhibition by Ca^{2+} and Mg^{2+} for Prayon, whereas Ganges was less sensitive to Ca^{2+} and not at all to Mg^{2+}. Although a definite type of transporter could not be discriminated from our experiment, it seems that we are in presence of different Cd transporters for the two ecotypes of *T. caerulescens*. In Prayon Cd could be transported by a Zn and Ca pathway, whereas in Ganges Cd could mainly be transported by a different pathway than Zn and Ca. Considering the multiple effects observed with the different cations tested here, it is however likely that more than one single transport system are carrying Cd into the cell.

5.6 Conclusion

The main objectives of the study were to characterize the uptake at the leaf cell level of Cd and Zn in two contrasting ecotypes of *T. caerulescens* and in *A. halleri*. In particular the kinetics of Cd and Zn uptake, and the effects of Ca^{2+}, Ca^{2+} channel blocker, and several other divalent cations, as well as the impact of plant pre-exposure to metals on Cd and Zn uptake in mesophyll protoplasts were investigated.

Despite the similarity between Cd and Zn in their electronic structure, it seems that there are differences between these metals in terms of their accumulation by the plants. Nevertheless, we found that Zn depressed Cd uptake and reciprocally in the three plants, indicating some kind of interaction between the uptake mechanisms of these two metals. This study demonstrated that the cellular uptake of Cd and Zn in leaf cells is a carrier-mediated mechanism in hyperaccumulating plants and that differences in uptake between Ganges, Prayon and *A. halleri* can not be explained by different transport capacities at the protoplast level in leaves. There must therefore exist regulation mechanisms before the plasma membrane of the leaf cell that direct the metals to their final location in plants. The cell walls could play a role in this regulation. Protoplasts accumulation capacities were nevertheless modified after the plants pre-exposure to Cd, indicating that above a determined Cd concentration threshold different transport mechanisms were induced in leaf cells.

6

Localization and effects of Cd in leaves of tolerant willow (*Salix viminalis*)

Combined version of two papers in preparation for Plant, Cell and Environment[1]

Localization and effects of cadmium in leaves of tolerant willows (*Salix viminalis* L.)-

Part I. Macrolocalization and phytotoxic effects of cadmium
COSIO Claudia, VOLLENWEIDER Pierre and KELLER Catherine

Part II. Microlocalization and cellular effects of cadmium
VOLLENWEIDER Pierre, COSIO Claudia, GÜNTHARDT-GOERG Madeleine S., and KELLER Catherine

6.1 Abstract

Cadmium accumulation and its effects on morphological parameters was monitored in *Salix viminalis* (Clone n°78198) grown in nutrient medium containing varying concentrations of Cd (0, 3, 5, 10, 20, 50, 100, 200 µM). A reduction in roots and shoots biomass and growth was observed with increasing Cd concentrations. Cadmium accumulation and the intensity of the visible phytotoxic symptoms, such as chlorosis and necrosis, increased until 50 µM Cd followed by a decline at 100 and 200 µM Cd. Willow was surprisingly tolerant to Cd, with only a 18 % shoot biomass reduction at 20 µM Cd and no significant root biomass reduction. Although Cd accumulation was higher in roots, Cd found in the shoots exceeded 100 mg kg^{-1}.

Cadmium localized by autoradiography was found mainly in the tips, and the edge of the younger leaves. This localization coincided with some of the necrotic spots also observed at the margin of the leaves. In older leaves Cd was mainly located at the base of the leaves. The different Cd localization between younger and older leaves was further confirmed by microlocalization (physical development) of Cd and was attributed to a physiological difference between these leaves. In young leaves where vein storage is smaller, Cd was increasingly deposited as spots inside the leaf blade. In older leaves collenchym maturation seemed to play a central role in Cd storage around the veins. At the cell level Cd showed a preferential accumulation

1.For citation please use the papers and not the thesis reference «Microlocalization of Cd»: Part II; others paragraphs: Part I.

in cell walls, indicating that vacuolar sequestration was not the main mechanism of Cd accumulation in *S. viminalis* leaves.

6.2 Introduction

Metal concentrations found in contaminated soils can reach levels at which they threaten soil fertility. Cadmium is one of the most widespread and toxic heavy metals, but there is no cost-effective mean to remove it from the soil. Since Cd tends to adsorb on topsoil, phytoextraction has been proposed as a low cost technique. Nevertheless, the complex nature of plant response to heavy metal toxicity and more specifically the mechanisms inducing tolerance to Cd are still not clearly understood and may be one of the factor limiting phytoextraction efficiency.

Cadmium intoxication disturbs the leaf physiology and causes visible symptoms. It causes leaf roll and chlorosis, and reduces growth both in roots and stems (Sanità di Toppi and Gabbrielli, 1999; Kabata-Pendias and Pendias, 2001). It interacts with the water balance (Barcelo and Poschenrieder, 1990; Poschenrieder and Barcelo, 1999), damages the photosynthetic apparatus (Krupa and Baszynski, 1995; Horváth *et al.*, 1996; Geiken *et al.*, 1998), and inhibits the stomatal opening (Barcelo and Poschenrieder, 1990; Perfus-Barbeoch *et al.*, 2002). Cadmium produces oxidative stress (for a review see Schützendübel and Polle, 2002) and induces premature senescence in leaves (Vázquez *et al.*, 1989; Ouzounidou *et al.*, 1997; McCarthy *et al.*, 2001). On the other hand, Cd ions can either inhibit or stimulate the activity of several enzymes, including antioxidative enzymes (Ernst, 1980; Sanità di Toppi and Gabbrielli, 1999; Schlicker and Caspi, 1999; Schützendübel and Polle, 2002).

The distribution of metals within plant organs and tissues can indicate detoxification and tolerance mechanisms employed by plant species. Unfortunately due to low Cd concentrations found in plants, and methodological difficulties, the link between the toxicity symptoms and the presence of Cd in the organ has not been made, although this would enhance the knowledge on Cd tolerance and accumulation mechanisms. Physical methods such as atomic absorption spectroscopy (AAS) after tissue disruption (Brown *et al.*, 1995; Chardonnens *et al.*, 1998), secondary ion mass spectroscopy (SIMS) imaging (Lazof *et al.*, 1996), or short term desorption with radiotracer (Lasat *et al.*, 1996; Blaudez *et al.*, 2000) are appropriate to spot compartments, but not to localize metals precisely. EDXMA, ESI and EELS have been used with some success to plot metals subcellular distribution (Nassiri *et al.*, 1997; Bringezu *et al.*, 1999; Küpper *et al.*, 1999; Frey *et al.*, 2000; Zhao *et al.*, 2000), but lack of sensitivity and interference with other cations (e.g. K) limit Cd detection, and do not allow visualization of the Cd distribution over the whole leaf surface. Little information on the longitudinal accumulation of metals is indeed available, although uneven metal distribution at the leaf level might be expected (Küpper *et al.*, 1999; Frey *et al.*, 2000). Additionally the techniques described above, which offer insight into plant uptake and heavy metal accumulation have often been performed either on hyperaccumulating plants, or on crop species following short time exposure to abnormally elevated external levels of metals, not comparable to chronic ‘natural-field-condition’ stress (Sanità di Toppi and Gabbrielli, 1999). There is a need for a simple

low-cost method, which enables direct visualization of metals in leaves, cells and subcellular organelles *in situ*, without extensive sample preparation to elucidate relationships between metal localization and mechanisms of tolerance. Autoradiography that uses radioactive tracers has been used in the past to localize metal accumulation in vertebrate tissues (Takeda *et al.*, 2000) and in plant tissues (Crafts and Yamaguchi, 1964; Van Balen *et al.*, 1980; Salt *et al.*, 1995). The technique is very precise and the radioactive emission enables detection of extremely low metal concentrations (Gahan, 1972). Alternatively, histochemical detection by the silver sulphide method (physical development; Danscher 1981) is also highly sensitive with a theoretical detection limit of 10 atoms (Litwin, 1985) and thus allows detection of very small metal concentrations at the ultrastructural level (Lindsey and Lineberger, 1981; Vázquez *et al.,* 1992b; Heumann, 2002).

The success of phytoextraction of metal-polluted soils depends on the availability of high biomass plants able to concentrate Cd to high levels within their shoots. Willows have been shown to be promising for Cd phytoextraction (Granel *et al.*, 2002; Hammer *et al.*, 2003, Klang-Westin and Eriksson, 2003; Pulford and Watson, 2003). Punshon and Dickinson (1997) showed that acclimatizing willows to toxic metals could be achieved by increasing gradually concentration of heavy metals in the nutrient solution. Additionally the authors reported that leaf morphological alteration occurred within the lifetime of a clone following exposure to Cd, highlighting the capacity of willows to adapt to their environment. Preliminary laboratory experiment with different species of the genus *Salix* have single out *S. viminalis* (clone n°78198) that tolerates and accumulates large concentrations of Cd (Landberg and Greger, 1996). Field trials have shown that this clone has a high biomass production combined with large Cd and Zn accumulation (Kayser *et al.*, 2000; Hammer *et al.*, 2003). Several authors reported that Cd concentration was the highest in leaves of *S. viminalis* (Punshon *et al.*, 1996; Punshon and Dickinson, 1997; Klang-Westin and Perttu, 2002). Hammer *et al.* (2003) found in the field that leaves represented 15 to 19% of the total biomass produced in mature *S. viminalis* plants but yielded 34 to 37% of the total Cd output per year. However, knowledge about Cd storage location in tissues or cells of *S. viminalis* is necessary to evaluate the full potential of using *S. viminalis* for Cd phytoextraction.

To assess the tolerance and the allocation of Cd in *S. viminalis,* we performed experiments with *S. viminalis* grown in hydroponics and treated with increasing concentrations of Cd. The effect of Cd on the biomass, the leaf aspect, the leaf area, the root length and root branching of *S. viminalis* were recorded and Cd concentrations were measured in the whole plants as well as in individual leaves. Macro-autoradiographies of *S. viminalis* were performed to visualize the localization of Cd within the leaves. Autoradiographs were then confronted to visual symptoms and to the parameters describing tolerance and metal accumulation in the different plant compartments to assess the effect of Cd localization on plant performance. Distribution of Cd at the macroscopic level was completed by the microlocalization of Cd at the tissue level using physical development and light microscopy.

6.3 Materials and Methods

6.3.1 Plant material and culture

The plant tested was a Swedish clone of *S. viminalis* (Clone n°78198) found to accumulate large concentrations of Zn and Cd in its shoots (Landberg and Greger, 1996). Ten-cm long stem cuttings were rooted and grown in 100-mL pot (25 cm height, 3 cm diameter, one plant per pot) filled with modified quarter-strength Hoagland's nutrient solution (Sigma, St Louis, USA) supplemented with 20 µM Fe-HBED (Strem chemical, Newburyport, USA). Fe(III)-HBED was prepared as described by Chaney *et al.* (1998) in such a way that all HBED was saturated with Fe. Plants were allowed to grow 2 weeks in hydroponics before the Cd treatment was started.

To determine tolerance and accumulation in the plants a first trial with four different treatments (0, 3, 10, and 20 µM Cd) was performed during 45 days. After observing the surprisingly good tolerance of the selected clone to Cd, we tested in a second 45 days trial 50, 100 and 200 µM Cd. Finally a third trial with a longer growth period was performed during 3 months (0, 5, 10, 50 and 200 µM Cd). For visualization of Cd in plants by autoradiography five different treatments were performed (0, 5, 10, 50, and 200 µM Cd) after 3, 15, 30 and 45 days of exposure to the metal. All nutrient solutions were spiked with 0.1 kBq mL^{-1} (= 2.2 10^{-3} µM) of $^{109}CdCl_2$ (NEN Life Science Products, Boston, USA). Four pots per treatment were set up. Physical development was performed on plants from the third trial during the 12th week of Cd treatment.

Plant culture was performed in a climate chamber (day/night period 16/8 h, day/ night temperatures 20°C/16°C, and a light intensity of 500 lux). The nutrient solution was renewed from twice a week at the beginning to every two days at the end.

6.3.2 Assessment of Cd phytotoxic symptoms

To assess Cd toxicity and tolerance, different tests were performed on willows. The biomass of shoots and roots (dry weight) was measured in the 3 trials. The total root length, the distribution of root diameter (16 classes from 100 to 3000 µm) and the number of forks, as well as the surface area of the 10 first leaves from the top (counting the first clearly identifiable leaf from the top of the stem as n°1) were measured with the software winRHIZOPro 5.0a (Regent Intruments, Quebec, Canada) coupled with a STD1600 desktop scanner (Epson) provided by Regent Instruments. All measurements were carried out at a resolution of 400 dpi. If necessary, root samples were divided into sub-samples for more accurate measurement. Leaf surface areas of one single 45-day-old plant previously used for autoradiography and root parameters of only two 45-day-old plants previously used for autoradiography were analysed for each Cd concentration tested.

6.3.3 Concentration of Cd in plants

The total concentration of heavy metals in leaves and roots of *S. viminalis* was measured in the 3 trials. Shoots were washed thoroughly with deionized water before drying. To remove cations slightly absorbed on the root surface, the roots from the first

trial (3 to 20 µM Cd, 45 days) were washed 5 minutes with 20 mM Na ethylenediamine tetraacetic (EDTA), and next rinsed quickly with deionized water. Roots and shoots were then dried at 80°C for a week till constant weight was reached, and weighted. Shoots were divided into leaves and stems that were then individually ground in a tungsten Retsch mill (Haan, Germany) and hot-digested in HNO_3 65% sp. (Fluka, Buchs, Switzerland) and $HClO_4$ 70% p.a. (Fluka, Buchs, Switzerland). The root biomass of the plants grown with 200 µM Cd was too small and the 4 root samples were thus pooled before digestion. The EDTA solution from root washing was recovered, filtered and the concentration of Cd desorbed was measured. Cadmium concentrations in all extracts and digests were measured by inductively coupled plasma atomic emission spectrometry (ICP-AES; Plasma 2000; Perkin Elmer, Wellesley, USA) or by graphite furnace atomic absorption spectrometry (GF-AAS; 5100PC atomic absorption spectrometer equipped with a HGA 600 graphite furnace, a Zeeman-corrected furnace module, and a AS-60 Furnace autosampler; PerkinElmer, Wellesley, USA) when needed. Standard reference plant material was used to assess the accuracy of the measurements. To homogenize datas all root Cd concentrations presented in the tables of this paper are concentrations of roots not washed with EDTA. Data of roots washed with EDTA are presented in the text only.

6.3.4 Visualization of Cd in plants

6.3.4.1 Autoradiography

Shoots and roots were collected separately, quickly rinsed with deionized water and then carefully wiped up. The samples were arranged as flat as possible and wrapped in a single layer of thin PE film. To obtain autoradiographs of adaxial and abaxial sides, samples were disposed 48 hours between 2 X-OMAT AR-5 autoradiography films (Kodak, Rochester, USA) at room temperature. Films used for samples harvested after 3 days in Cd solution were exposed 6 weeks due to their very low Cd content. In all cases control plants grown without ^{109}Cd were processed in parallel with radioactive samples to detect possible artefacts on the autoradiographs. Autoradiographs were developed with an automatic film-processor SRX-101A (Konica, Tokyo, Japan) and subsequently numerised on a Tango digitalisator (Linotype-Hell Heidelberg, Kiel, Germany). Localization of Cd in leaf was determined by examining the exposed film. Subsequently, for each concentration tested, leaves of one single plant were individually dried at 80°C, weighted, digested in HNO_3 65% sp. (Fluka, Buchs, Switzerland) 1 hour at 90°C and Cd concentration in each leaf was measured as described above.

Leaves could be separated in 3 groups of approximately the same size according to their position on the stem. The first group corresponded to young and still-growing leaves with increasing dry weight. The second group comprised fully expended leaves of almost identical weight. And the third group corresponded to leaves of the lower shoot section showing decreasing weight with increasing leaf age. These groups are further referred to as young (Y), mature (M) and old leaves (O).

6.3.4.2 Physical development

Cadmium was cytochemically detected, adapting Danscher's (1981) physical development method to fresh sections of plant tissues. Shortly after hand-microtoming 60 µm thick sections were dipped 7 minutes in 0.1 % Na_2S in 0.067 M Soerensen buffer pH 7.0 prior to 30 minutes fixation in glutaraldehyde 2.5 % in the same buffer. After two hours rinsing in buffer, sections were mounted on gelatine coated slides in one drop of 0.5 % gelatine and let to dry. Two sections from the middle portion of one young (from leaf n°1 to n°4) and one mature leaf (from leaf n°11 to n°20) from one 3-month-old plant per treatment (0, 10 and 50 µM Cd) were developed either 15 minutes (lighter staining) or 30 minutes (stronger staining). Pretreatment and physical development were conducted in the dark. Gelatine was removed in a 40°C water bath prior to mounting in water. All sections were observed in an optic microscope Leitz DM/RB (Leica, Solms, Germany). Micrographs were taken with the micrograph system Wild MPS 48/52 using Ektachrome 400 Asa films (Kodak , Tokyo, Japan).

Table 6.1: Effect of Cd on biomass repartition and Cd concentration: a) first and second trials with 45-day-old, and b) third trial with 3-month-old *S. viminalis* grown in hydroponics with or without Cd. Similar letters mean no difference between the means and different letters mean significant differences between means with $P<0.05$ (n= 4, SD in parentheses).

a	Cd in nutrient solution (µM)	Shoot:root weight ratio	Cd (mg kg^{-1})		Leaf:root Cd ratio
			Leaves	Roots	
	0	5.01 (0.32)a	0.9 (0.5)a	0.4 (0.3)a	2.24 (1.25)a
	3	5.63 (0.86)a	39 (13)b	313 (96)b	0.13 (0.04)b
	10	4.95 (0.96)a	90 (30)c	706 (306)c	0.13 (0.05)b
	20	5.08 (0.85)a	201 (21)d	1197 (159)d	0.17 (0.02)b
	50	4.62 (1.46)a	519 (105)e	4048 (946)e	0.13 (0.03)b
	100	8.30 (1.53)b	260 (90)f	798 (289)f	0.33 (0.12)c
	200	12.91 (3.13)c	52 (21)bc	1310†	0.04 (0.00)d

†Roots were pooled prior to analysis.

b	Cd in nutrient solution (µM)	Shoot:root weight ratio	Cd (mg kg^{-1})		Leaf:root Cd ratio
			Leaves	Roots	
	0	2.25 (0.38)a	1 (0.5)a	6 (6)a	0.16 (0.16)a
	5†	2.52	100	495	0.20
	10	2.86 (0.56)ab	153 (32)b	520 (57)b	0.29 (0.04)a
	50	3.19 (0.35)b	584 (159)b	3554 (534)c	0.16 (0.03)a
	200	13.37 (3.01)c	181 (79)c	13114‡	0.01 (0.01)b

† one plant only

‡ Roots were pooled prior to analysis.

6.4 Results

6.4.1 Cadmium concentration in whole plants

Cadmium concentrations followed a similar trend in leaves and roots (Table 6.1). The concentration of Cd in 45-day-old willows increased from 0 to 50 μM Cd (leaves 519±105 mg kg^{-1} and roots 4048±946 mg kg^{-1}), then declined at 100 μM (leaves 260±90 mg kg^{-1} and roots 798±289 mg kg^{-1}), and 200 μM Cd (leaves 52±21 mg kg^{-1} and roots 1311 mg kg^{-1}). Cadmium concentrations in shoots were linearly related to Cd concentrations in roots (r= 0.906; sign. $2\alpha<0.001$). The analyses showed that Cd was preferentially accumulated in the roots in the three trials (Table 6.1). At 200 μM Cd the leaf:root Cd ratio (0.04±0.01) was nevertheless significantly smaller ($P<0.05$) than the ratios calculated for the other treatments (between 0.13 and 0.33). When Cd adsorbed onto the roots was removed by EDTA washing about half (46±2%) of Cd was removed from the roots of 45-day-old willows grown with 3, 10 or 20 μM Cd, but the same linear correlation between roots and shoots concentration was observed (r= 0.986; sign. $2\alpha<0.001$). However, the mean corrected leaf:root Cd ratio reached 0.54±0.11, indicating that Cd concentrations in roots were twice higher than in leaves.

Cadmium concentrations measured in the leaves and roots after 3-month exposure, with an exception for 200 μM Cd, were in the same order of magnitude as those found after 45-day exposure (Table 6.1) although the biomass had increased (Figure 6.1). The Cd total amount in the plant was consequently larger. On the contrary at 200 μM Cd, Cd concentrations were resp. 3.5-fold and 10 fold larger in leaves and roots after 3 months than after 45 days even though the total biomass and shoot:root biomass ratio remained similar.

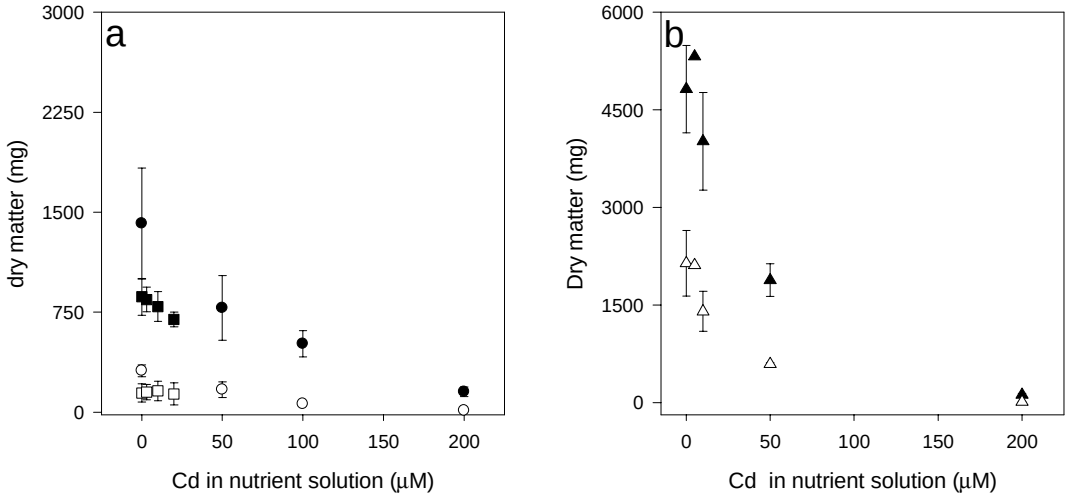

Figure 6.1: Effect of Cd on biomass production: average biomass of shoots (closed symbols) and roots (open symbols) of a) first trial (squares) and second trial (circles) of 45-day-old *S. viminalis* grown in hydroponics with or without Cd, and b) third trial (triangles) of 3-month-old *S. viminalis* grown in hydroponics with or without Cd. In the third trial one plant only was analysed for the 5 μM Cd treatment. Error bars do not extend outside some data symbols (n= 4± SD).

6.4.2 Assessment of Cd visible phytotoxic symptoms

Plants were observed to detect visible toxicity symptoms resulting from Cd exposure. The most obvious symptom of toxicity was the reduction of plants growth that will be detailed in the next section. Additionally, increasing chlorosis and necrosis were observed on leaves of the Cd-treated plants with increasing concentration until 50 µM Cd (Figure 6.2, Table 6.2). Symptoms were reduced at 200 µM Cd (Table 6.2): leaves were less chlorotic than leaves of plants grown at lower concentrations and no necrosis was detectable although plants were significantly smaller. Symptoms increased with time exposure. Another visible phytotoxic symptom was leaf rolling at 20 and 50 µM Cd. The symptom appeared specifically at those concentrations in 45-day-old as well as 3-month-old willows excluding a pathogenic origin. The plants that were grown at high Cd concentrations (100 and 200 µM Cd) did not exhibit this symptom. At the root level, roots turned brownish with increasing Cd concentration. Root hairs were not found in any treatment with or without Cd.

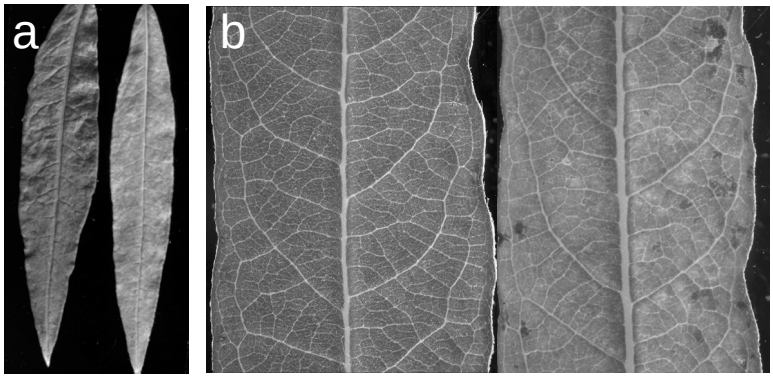

Figure 6.2: Symptoms of *S. viminalis* grown in hydroponics following exposure to 50 µM Cd (right) compared to the control (left) plants: a) chlorosis after 20-day exposure, and b) necrosis after 68-day exposure.

Table 6.2: Effect of Cd on leaves of 45-day-old *S. viminalis* grown in hydroponics with or without Cd. Symptoms intensity is given in brackets. The first clearly identifiable leaf from the top of the stem is counted as n°1. Leaves of one single plant were measured for each treatment.

Cd in nutrient solution (µM)	Morphological symptoms of leaves	Area (cm^2)		
		10 first leaves	1st leaf	10th leaf
0	Normal	18.4	0.29	3.30
5	Chlorosis (+)	14.4	0.27	3.27
10	Chlorosis (++), necrosis (+)	20.2	0.50	3.63
50	Chlorosis (+++), necrosis (++), leaf rolling	13.0	0.36	2.39
200	Chlorosis (+/-)	4.4	0.63	0.16

6.4.3 Biomass production

To assess *S. viminalis* (clone n°78198) tolerance to Cd, we first measured the biomass (Figure 6.1). *Salix viminalis* was found to be surprisingly tolerant to Cd with only an 18% shoot biomass reduction (Student t-test $P<0.05$) at 20 μM Cd and no significant root biomass reduction. It further showed a 45% shoot biomass reduction at 50 μM Cd ($P<0.01$), and a 90% reduction at 200 μM Cd ($P<0.001$) with a severe 96% root biomass reduction ($P<0.001$). At the highest concentrations an increase in shoot:root biomass ratio was also observed, reaching 8.3 for 100 μM Cd ($P<0.05$), and 12.9 for 200 μM Cd ($P<0.01$), whereas it was between 4.6 and 5.6 for all the other treatments (Table 6.1).

In general, losses of biomass as compared to the control plants were larger after 3 months than after 45 days (Figure 6.1). For example at 50 μM Cd the shoot biomass loss increased from 45±17% at 45 days to 61±5% at 3 months, and the root biomass loss from 46±19% to 72±1%. On the other hand the total biomass was larger at 3 months than after 45-day exposure (Figure 6.1). Additionally, if 200 μM Cd was excluded from the calculations, the average shoot:root biomass ratio decreased from 5.6 after 45 days to 2.7 after 3 months, indicating a proportionally greater development of the root system during the 2nd and 3rd months of growth (Table 6.1).

6.4.4 Leaf area

A reduction in leaf area was observed in 45-day-old plants treated with 50 and 200 μM Cd (Table 6.2). The sum of the area of the 10 first leaves on the stem gave a leaf surface reduction of 30% at 50 μM Cd and 76.5% at 200 μM Cd compared to the control. Nevertheless, when leaves of the treated and control plant were compared one by one according to their position on the stem, no clear pattern was observed. For all treatments the biggest 1st leaf was observed in the 200 μM Cd treatment, but the smallest 10th leaf was also observed in the 200 μM Cd treatment (Table 6.2), indicating that the reduction in leaf size has to be seen as a general pattern of toxicity.

6.4.5 Root length and diameter

Additionally to the biomass, roots showed a reduction of 65±6% of total root length at 50 μM Cd, and of 96±3% at 200 μM Cd (Table 6.3) after 45 days. Figure 6.3 shows the distribution of root diameter classes in percent of the total root length for the different Cd treatments. Most of the roots were found in the smallest diameter classes (0-100 and 100-200 μm) for all the treatments. For the control plants there was a sharp decrease of root length in the classes between 200 and 800 μm with almost negligible proportion of roots of bigger diameter. In the Cd treated plants there was also a sharp decrease of the proportion of roots in the classes between 200 and 500 μm, but followed by an increased proportion of the 500-900 μm classes. We summed the root length of the classes between 0-200 μm and compared it with the cumulative root length of the classes between 500-900 μm (Table 6.3). Compared to the control, at 5 μM Cd the length of finer roots diminished by 18±5%, whereas length of the 500-900 μm classes increased by 49±14%. At high Cd concentration (50 μM and 200 μM Cd) growth of both classes were inhibited compared to the control although finer roots

were more affected than the 500-900 μm classes. With increasing Cd concentrations laterals developed therefore less although their number per cm of roots remained between 3.8 and 4.9 forks per cm of roots (Table 6.3).

Table 6.3: Effect of Cd on root growth: total root length per plant (m), number of forks per cm of roots and root length per plant (m) of fine roots (diameter classes 0 to 200 μm) and coarse roots (diameter 500 to 900 μm) of 45-day-old *S. viminalis* grown in hydroponics with or without Cd. Values represent means of two plants.

Cd in nutrient solution (μM)	Total root length (m)	Forks (cm^{-1})	Root length (m)	
			Fine roots	Coarse roots
0	2.64	3.77	1.60	0.29
5	2.42	4.02	1.32	0.44
10	2.16	3.76	1.26	0.36
50	0.90	4.14	0.38	0.24
200	0.08	4.88	0.04	0.03

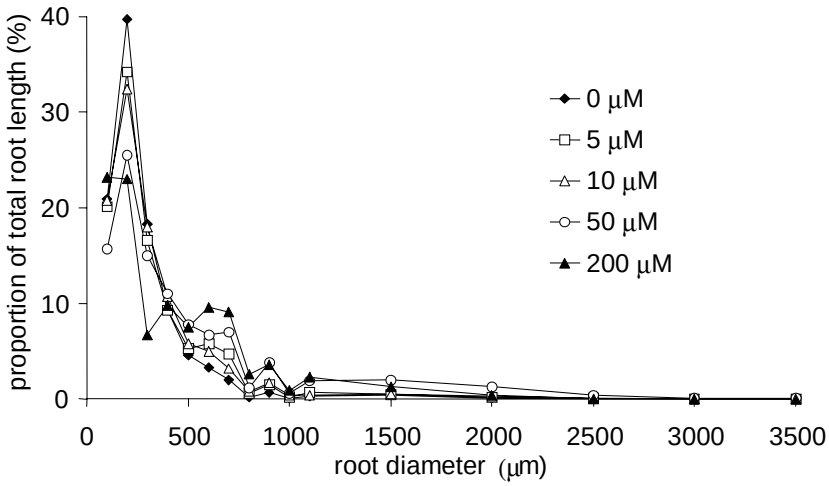

Figure 6.3: Effect of Cd on root diameter: proportion of the various root diameter classes accounting for the total root length in 45-day-old *S. viminalis* grown in hydroponics with or without Cd. Values represent means of two plants.

6.4.6 Nutrient solution consumption

Plants treated with 5 to 50 μM Cd had a higher requirement in nutrient solution than control plants. At the opposite, there was only a small reduction of the solution volume for pots containing plants grown in 100 and 200 μM Cd. Although no exact measurement was done, the difference was obvious when changing the nutrient solution. Additionally plants treated with 5 to 50 μM Cd developed water stress symptoms. At 5 μM Cd for example only one plant survived in the 3-month trial whereas the three other plants died of drought although they had nutrient solution.

6.4.7 Visualization of Cd in plants

The precise localization of Cd within the leaves was assessed by autoradiography using labelled Cd. Observations performed at various Cd concentrations and times of exposure gave information on the dynamic of Cd accumulation in the plant. The technique could not discriminate between metal adsorbed onto and located into the roots (data not shown), but it was very efficient at visualising Cd distribution in leaves.

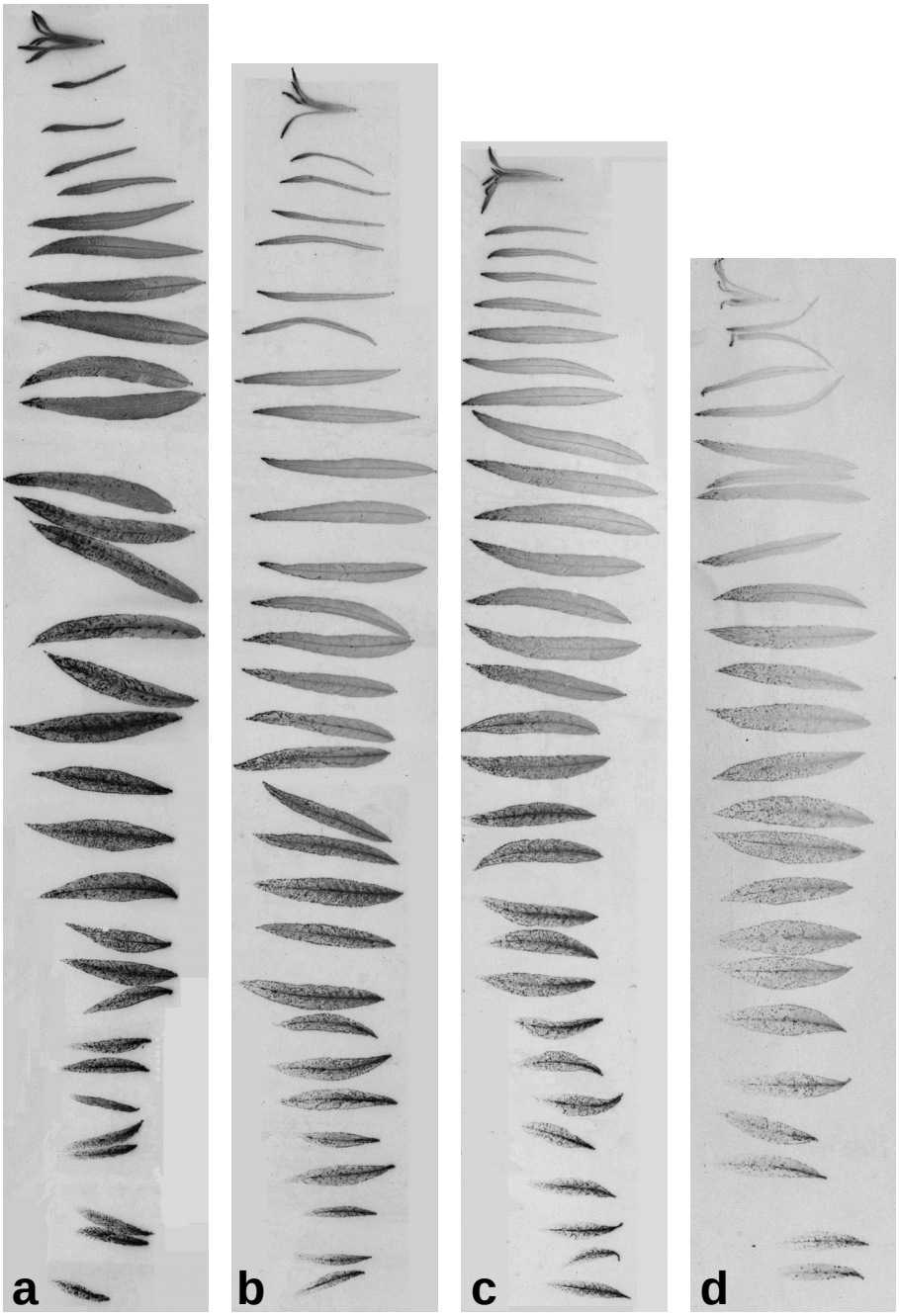

Figure 6.4: Cadmium visualization in leaves: autoradiographs of leaves of 45-day-old *S. viminalis* grown in hydroponics with 2.2 10^{-3} µM of ^{109}Cd and a) 0 µM, b) 5 µM, c) 10 µM, and d) 50 µM Cd. Leaves are ordered according to their position on the stem. The youngest leaf is at the top and the oldest at the bottom. Films were exposed 48 hours and are represented at the same scale.

The darkness of the autoradiographs depends on the ratio ^{109}Cd/total Cd. Concentration of ^{109}Cd remained constant (2.2 10^{-3} µM ^{109}Cd) in all solutions whereas total Cd concentration increased from 2.2 10^{-3} µM to 200 µM. As a consequence, the ^{109}Cd activity present in the samples proportionally decreased and autoradiographs became lighter with increasing total Cd concentrations (Figure 6.4). Ultimately the autoradiographs of plants grown with 200 µM Cd were so light that we could not analyse them (data not shown). Thus the intensity of the darkening does not reflect the total Cd concentrations in the leaves that are reported in the next section.

Fifteen, 30 or 45 days of exposure to Cd did not modify the Cd distribution patterns within the leaves, although Cd concentration increased as reflected by the increasing darkness of the autoradiographs (Figure 6.5). The same Cd distribution pattern was also observed at all concentrations tested (Figure 6.4): Cd was present in point-like accumulations in the tips, and along the edge of the younger leaves. In mature leaves the point-like accumulation spread to the whole limb of the leaves. In older leaves Cd was mainly located at the base of the leaves. Cadmium was also found in the central vein in young, mature and old leaves although more markedly in older leaves. After 3 days of exposure to Cd, concentration of ^{109}Cd was too low in plants to be clearly visible on the autoradiographs even after 10 days of exposure of the films, however the accumulation in the central vein of leaves and the differences between young and old leaves were already visible (autoradiographs not shown).

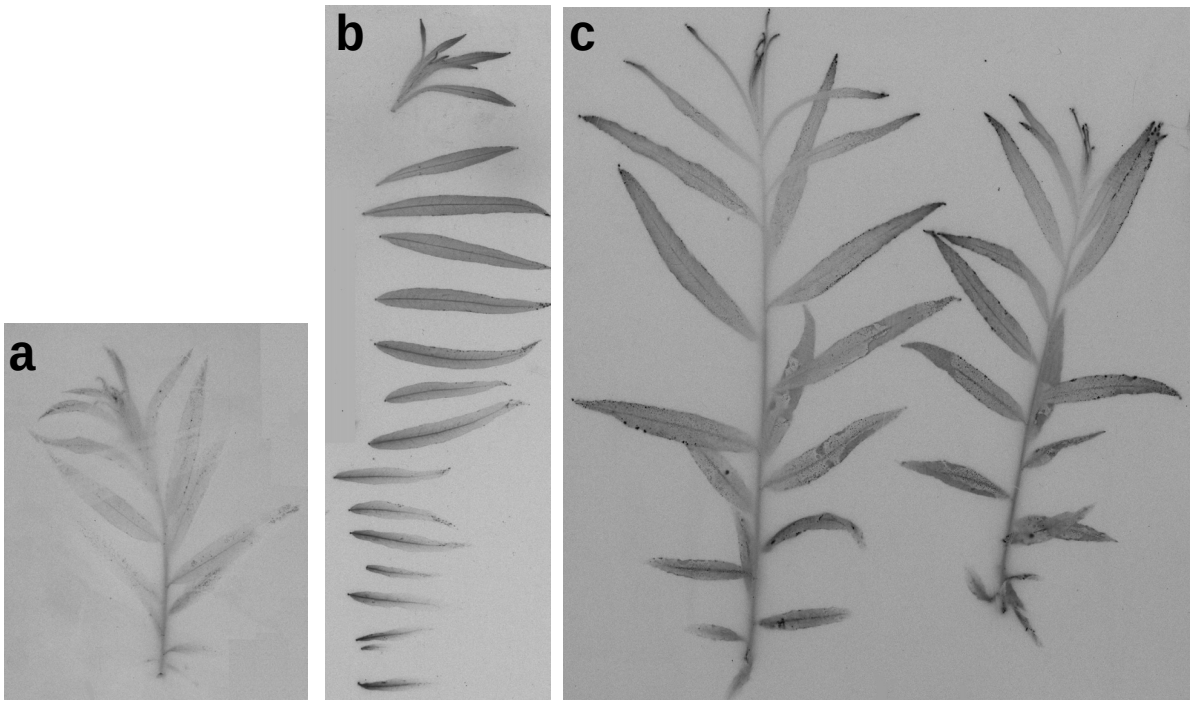

Figure 6.5: Cadmium visualization in leaves: autoradiographs of leaves of *S. viminalis* grown in hydroponics with 5 µM Cd spiked with 2.2 10^{-3} µM of ^{109}Cd at a) 15 days, b) 30 days and c) 45 days. Films were exposed 48 hours and are represented at the same scale.

6.4.8 Cadmium accumulation in individual leaves

To assess if differences between young and old leaves seen on the autoradiographs were reflected by different Cd concentrations in leaves, we measured the Cd concentration in individual leaves (Table 6.4). Data for leaves of willow grown with 5 µM Cd are detailed in Figure 6.6 and given according to their position on the stem. Although it seemed that Cd concentrations were higher in mature leaves in the 5 µM (P<0.0001) and the 50 µM Cd (P<0.0001) treatments, in the 10 µM Cd treatment on the contrary larger Cd concentration were measured in the oldest leaves (P<0.0001). Therefore globally no clear relation between the position of the leaves and their Cd concentration was found. Cadmium concentration seemed to be correlated mostly with leaf weight with r values ranging from 0.782 to 0.958 (2α<0.001) for the 5 µM, 10 µM and 50 µM Cd treatments, although the r value obtained for the 200 µM treatment was only 0.306 (not sign.).

Table 6.4: Cadmium effect on leaf biomass and Cd concentration of young (Y), mature (M) and old (O) leaves of *S. viminalis* grown 45 days in hydroponics with 0, 5, 10, 50 or 200 µM Cd spiked with 2.2 10^{-3} µM of ^{109}Cd. (SD in parentheses; <d.l.: below detection limit; n.d.: not determined; r= correlation factor between the dry weight and the Cd concentration of all leaves (n); similar letters mean no difference between the means and different letters mean significant differences between means with P<0.05 for one parameter). One single plant was analysed for each concentration tested.

Cd in nutrient solution (µM)†	n	Cd concentration (mg kg^{-1})			Dry weight (mg)			Cd amount (µg)			r
		Y	M	O	Y	M	O	Y	M	O	
0	27	<d.l.	<d.l.	<d.l.	8.0a (4.2)	11.6c (1.4)	3.4g (2.2)	<d.l.	<d.l.	<d.l.	n.d.
5	21	126a (9)	166b (21)	131c (15)	6.6a (3.1)	13.7d (2.4)	6.1a (3.0)	0.8a (0.4)	2.3b (0.5)	0.8a (0.4)	0.958‡
10	26	219d (24)	249e (41)	319f (47)	8.5a (3.6)	13.6e (1.6)	5.1h (1.8)	1.9b (0.8)	3.4c (0.6)	1.6b (0.6)	0.911‡
50	24	262g (31)	415h (88)	345i (136)	6.2a (1.8)	8.9f (1.2)	6.4a (1.8)	1.6b (0.5)	3.6c (0.7)	2.3b (1.1)	0.782‡
200	7	102j (9)	107j (12)	108j (35)	3.8b (0.6)	3.2b (0.1)	2.7b (0.3)	0.4a (0.0)	0.3a (0.0)	0.3a (0.1)	0.306

† only the concentration of ^{112}Cd is indicated. All nutrient solutions were spiked with 2.2 10^{-3} µM of ^{109}Cd.
‡ sign. 2α<0.001.
n= total number of leaves.

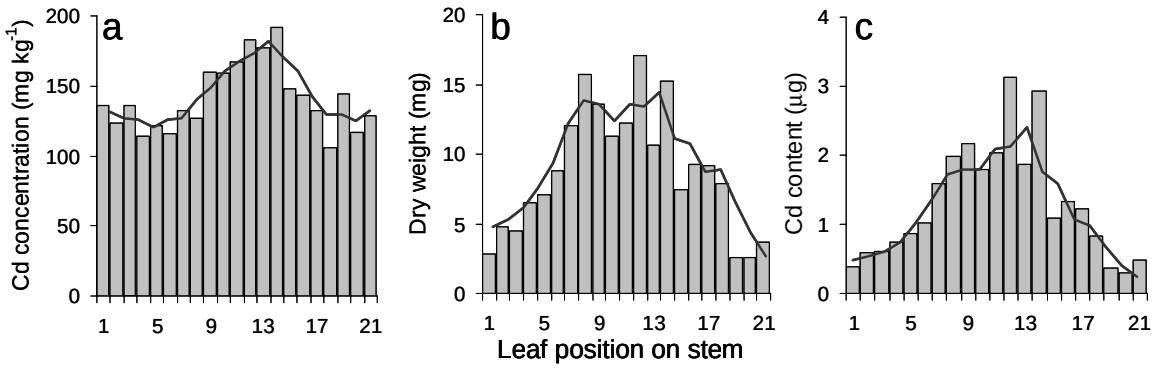

Figure 6.6: Cadmium effect on leaf biomass and Cd concentration: a) Cd concentration (mg kg^{-1}), b) weight (mg) and c) Cd content (µg) of leaves of *S. viminalis* grown 45 days in hydroponics with 5 µM Cd according to their position on the stem from the top (counting the first clearly identifiable leaf from the top of the stem as n°1). The line represents the mobile mean of 3 values. One single plant was analysed.

6.4.9 Microlocalization of Cd

Cadmium localization at the subcellular level was further assessed. The method was most suitable to compare relative cell and tissue Cd level in the whole leaf, but tended to slightly shrink tissues without thickened cell walls. As in Danscher (1981), physical development revealed that Cd was in the cell structures in the form of a homogenous and rather brownish staining (Figures 6.7 and 6.8). At higher magnifications brown granules of silver were visible after 30 minutes of development in places with a high metal content. However besides Cd (in 0 to 50 µM: 0.9±0.5 to 519±105 mg Cd kg^{-1}), the physical development could also reveal the leaf Zn (in 0 to 50 µM: 34 to 77 mg Zn kg^{-1}) and Fe (in 0 to 50 µM: 87 to 39 mg Fe kg^{-1}) absorbed from the nutrient solution. Careful controls were therefore performed to verify that the deposits really contained Cd. The physical development is however a qualitative method that visualizes metal sinks rather than allows comparison of metal abundance between treatments. The best comparative indication was given by the shade of the stain at a given development time (Figure 6.7). According to development time and apparent Cd concentration yellow shades developed to a brown and finally to a black staining. A metal signal was observed in leaves of all Cd treatments after 30-minute-revelation time and only for the 10 µM and 50 µM after 15-minute-revelation time. Tissue-specific apoplastic or symplastic microlocalizations of Cd were observed throughout the leaves (Figure 6.7 and 6.8). Apparent metal concentration progressively increased from the 0 µM to the 50 µM treatment for both revelation times and with minor variation between plant replicates inside each treatment. With darker-brown stained cell walls, vein collenchym looked like the main Cd sink in the leaves (Figure 6.7). Cell wall-bound Cd was observed in decreasing amounts in collenchym >pith >cortical parenchyma >xylem (Figure 6.7). Middle lamella presented more Cd than other cell wall layers. Symplasmic Cd was observed in the phloem. The relative stain intensity of this tissue scaled up between those observed in the pith and cortical parenchyma. Black stacks of Cd were only occasionally observed in the veins of older leaves of the 50 µM treatment after 30 minutes revelation time (Figure 6.8).

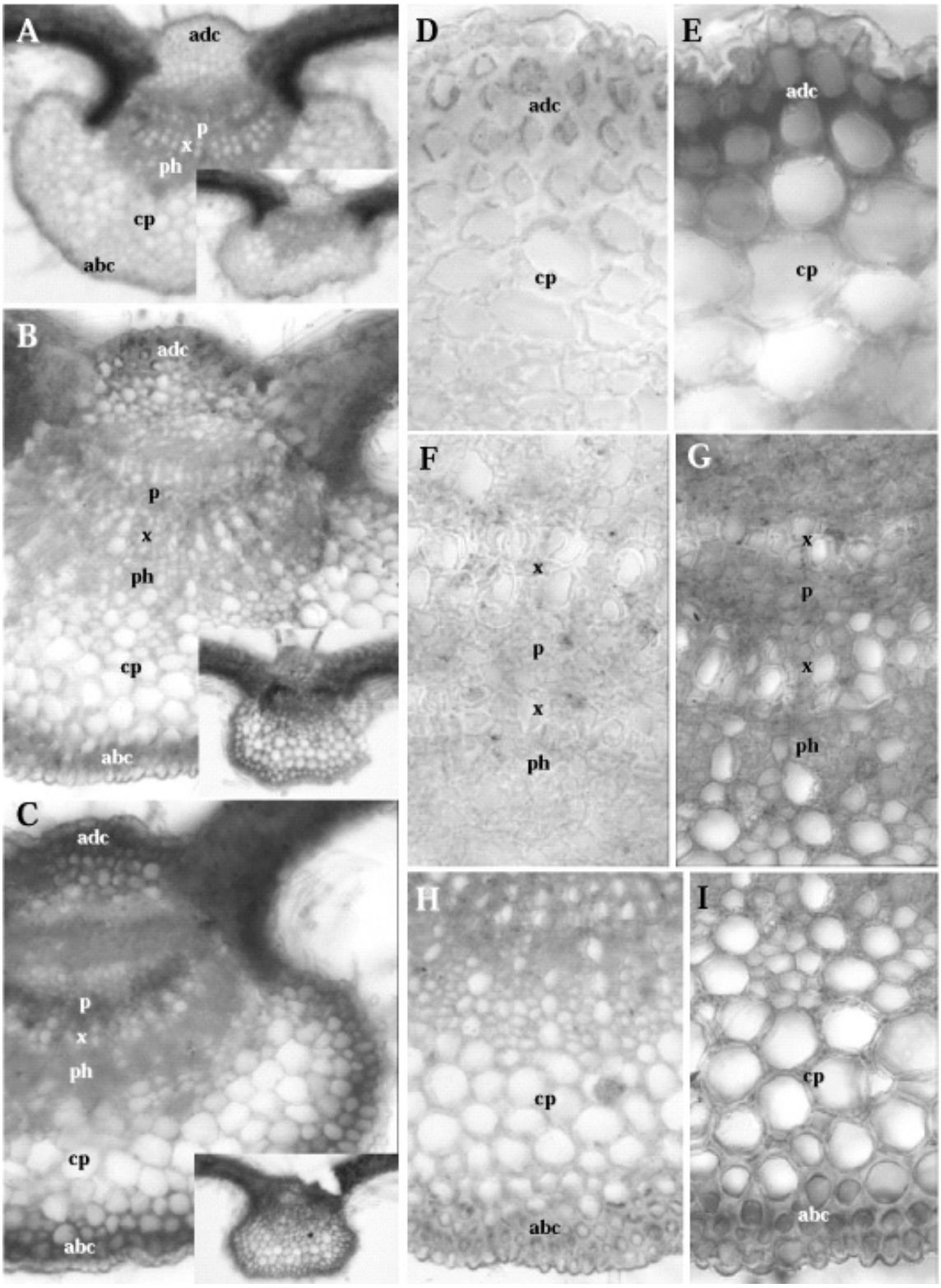

Figure 6.7: Microlocalization of Cd in the leaf veins with the physical development method. After 15 minutes development, Cd signal increased from 0 µM (A, no signal), to 10 µM (B) and 50 µM (C). Allocation trends were the same in older and younger leaves (smaller inserted pictures). Tissue allocation of Cd after 30 minutes of development in controls (D,F,H), 10 µM (G, I) and 50 (E) treatments. Metal signal was visible in adaxial (adc) and abaxial (abc) collenchym > pith (p) > phloem (ph) > cortical parenchyma (cp) > xylem (x). A weak signal was observed in the controls.

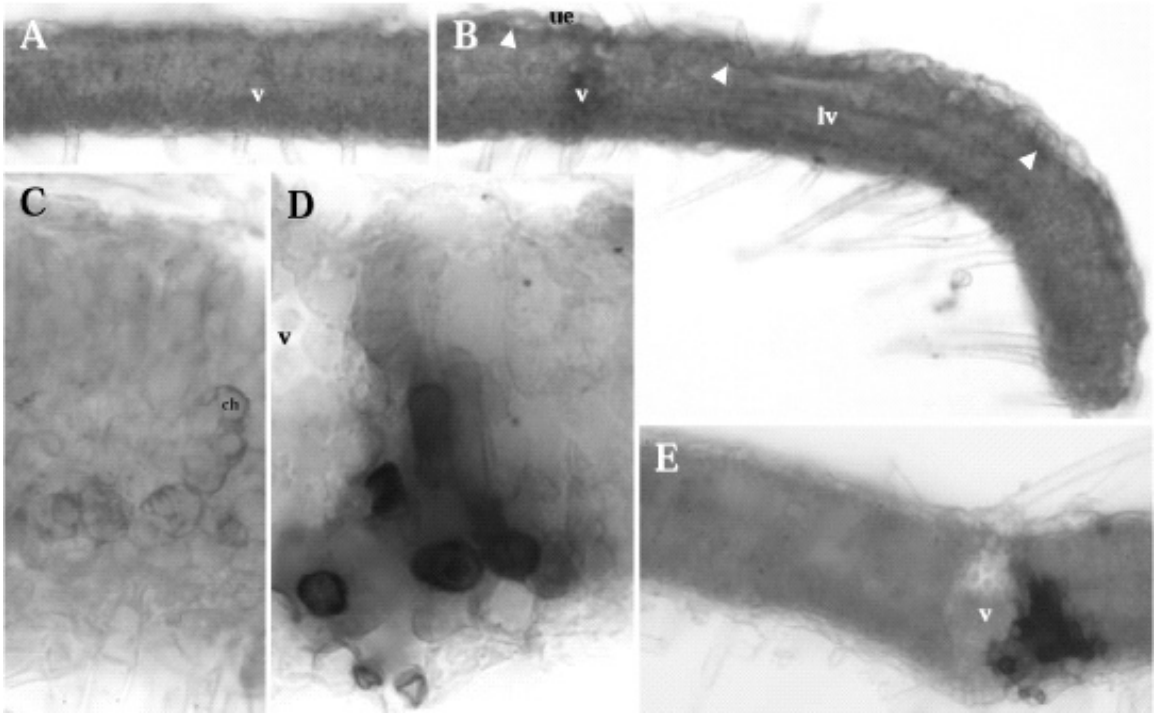

Figure 6.8: Microlocalization of Cd in the leaf limb with the physical development method. In comparison to the control (A) a brown metal signal was visible in the veinlets (v and lv: longitudinally cut veinlet) and the periclinal cell wall of the upper epidermis (ue; arrowheads) in leaves treated with 10 µM Cd (B). C) in the mesophyll cells with symplasmic Cd (50 µM Cd), less metal was visible in the chloroplasts than in the surrounding cytoplasm. D-E) Cd overflow in the 50 µM treatment. Stacks with high Cd concentrations were randomly formed in the mesophyll often next to a vein and without any apparent allocation order. Development times: 15 minutes (D, E) and 30 minutes (A-C).

Cadmium storage was more heterogeneous in leaf blade than in vein. Black Cd stacks appeared already after 15 minutes development in both younger and mature leaves treated with 50 µM Cd (data not shown). They were scattered throughout the limb, generally in connection with a secondary vein both in the upper or lower mesophyll symplasm and apoplasm. No stacks were observed in the 10 µM treatment. Cadmium also tended to accumulate more towards leaf rims where gradients were observed in the upper epidermis. In this tissue, periclinal and lower anticlinal cell walls facing the mesophyll displayed more intense red-brown shades starting from the stained distal veins (Figure 6.8). Leaf hairs covering the lower leaf side did not play a role in metal storage. Cytoplasmic Cd was not found inside the choroplasts (Figure 6.8).

6.5 Discussion

6.5.1 Cadmium concentration in whole plants

Cadmium concentration after 45 days of exposure followed a similar trend in

shoots and roots: it increased in tissues with increasing Cd concentration in the nutrient solution until 50 μM Cd and then decreased at 100 and 200 μM Cd. The incorporation of Cd into willow plants is then governed by processes that are independent of biomass production, since biomass decreased with increasing Cd concentration in the nutrient medium.

Cadmium concentration found in *S. viminalis* was higher in roots than in leaves as reflected by the leaf:root concentration ratio. In soils however, Cd concentration was shown to be higher in leaves (Punshon *et al.*, 1996; Hammer and Keller, 2002; Rosselli *et al.*, 2003). Therefore the higher concentrations in roots observed here were probably an artefact due to hydroponics and might not be limiting for the use of willows in phytoextraction. Nevertheless it indicates that pulling out roots at the end of the remediation process might allow to extract an additional amount of Cd adsorbed (Dickinson, 200.).

Cadmium concentration in leaves of *S. viminalis* already reached 100 mg Cd kg^{-1} with the 10 μM Cd treatment without the plant showing any significant toxicity symptoms. This concentration is the threshold that defines Cd hyperaccumulation in natural environment (Brown *et al.*, 1994). Punshon and Dickinson (1997) reported Cd concentration in the same range in leaves of willows grown in hydroponics with Cd concentrations up to 13 μM Cd. However 10 μM Cd (1.12 mg Cd L^{-1}) is already an elevated concentration for a soil solution (Wagner, 1993; Knight *et al.*, 1997; Lombi *et al.*, 2001a) and it is known that plants in solution culture accumulate more Cd than those in soil (Grant *et al.*, 1998; Dickinson, 200.). It is thus more likely that Cd concentrations into *S. viminalis* grown in the field may be above 10 mg Cd kg^{-1} rather than above 100 mg Cd kg^{-1} (Dickinson, 200.). Thus results obtained at 10 μM Cd are probably more relevant to real situation. Higher concentrations may allow prospection on the extent of *S. viminalis* tolerance to Cd.

The pattern of uptake can also be interpreted as an indication that the toxicity threshold has not been exceeded after 45 days of exposure even at high Cd supply, since the metabolic control of Cd uptake is not lost (Dan *et al.*, 2002). Control of Cd movement from roots to shoots in tobacco plants and inscented geranium was found to be dependent on the Cd concentration released by the root (Grant *et al.*, 1998; Dan *et al.*, 2002). We suggest that in the case of willow a similar scenario exists, wherein metal accumulation in the leaves is correlated ($2\alpha<0.001$) to the one transported to the roots. On the opposite, after 3 months at 200 μM Cd the dramatic increase in Cd concentration in roots might reflect the loss of metabolic control, happening when the threshold for acute Cd stress has been overpassed. It is well possible that as a result of Cd toxicity, roots lost not only their capacity for nutrient uptake, leading to growth retardation at the whole-plant level, but also their capacity to restrain accumulation of the metal (Schützendübel and Polle, 2002).

The results also highlight that the time of exposure to the metal is an important criteria for metal extraction: after 3 months of growth Cd concentrations in leaves were in the same order of magnitude than after 45 days whereas the biomass was larger. On the other hand, the biomass loss at 3 months significantly increased compared to 45-day-exposure indicating that tolerance in the long-term might be smaller than on short-term exposure. It is thus necessary to test Cd tolerance of *S. viminalis* during at

least one growing season.

6.5.2 Toxic symptoms on leaves

Visible Cd toxic symptoms on leaves followed a similar trend as Cd accumulation. The symptoms of chlorosis and necrosis were most extensively observed at the same concentration (50 μM Cd) as the highest accumulation of Cd in shoots, whereas at 200 μM Cd a decrease in visible leaf symptoms was observed together with a decrease in Cd concentration in leaves. This indicates that necrosis and chlorosis observed in our work resulted from Cd accumulation. Salt *et al.* (1995) similarly found that Cd toxicity produced chlorosis in young leaves of *Brassica juncea*. They observed that Cd preferentially accumulated within these young leaves explaining the localization of the chlorosis. Chlorosis reflects chlorophyll loss (McCarthy *et al.*, 2001; Yoshida, 2003) which could be a consequence of a premature senescence induced by Cd (Vázquez *et al.*, 1989; Ouzounidou *et al.*, 1997; McCarthy *et al.*, 2001).

6.5.3 Tolerance of *S. viminalis* clone to Cd: effect of Cd on biomass

A reduction in biomass and size was observed with increasing Cd concentrations. Willow was nevertheless surprisingly tolerant to Cd with biomass values not significantly different from the control until 20 μM for shoots and 50 μM for roots. For comparison Landberg and Greger (2002) observed that the most resistant clone of *S. viminalis* tested in their assay had a 19% decrease in biomass shoots after a 7 μM Cd treatment. We found for our clone a similar value at 20 μM Cd. Punshon and Dickinson (1999) tested a clone of *S. viminalis* with 10 μM Cd and obtained a 44% reduction in biomass. In wheat seedlings biomass reduction of shoots and roots at 45 μM Cd were respectively 74% and 77% (Shukla *et al.*, 2003). All these values are a lot larger than the values reported here for *S. viminalis* clone n°78198.

At 20 μM Cd the decrease in biomass was more pronounced for shoots than for roots. Cielinski *et al.* (1996) similarly found that Cd affected more leaf weight than root weight of strawberry plants, although Cd was mainly accumulated in roots. The authors concluded that leaf dry weight was the best indicator of Cd toxicity (Cieslinski *et al.*, 1996). But at high Cd concentrations (100 μM Cd and 200 μM Cd) roots clearly suffered more than shoots from Cd toxicity as reflected by a larger decrease in root biomass in agreement with other authors (Shukla *et al.*, 2003; Sottnikova *et al.*, 2003). Additionally the shoot:root biomass ratio significantly increased, indicating that Cd altered patterns of biomass allocation. It highlights the fact that Cd enters first the roots, which are thus likely to experience first severe damages at toxic Cd concentration. However total root length started decreasing at lower Cd concentration than root biomass, indicating that root growth inhibition caused by Cd stress may have been compensated by production of bigger roots. Indeed we observed an increased proportion of roots of larger diameter (500-900 μm) with increasing Cd concentrations. Box and Ramseur (1993) also found that root length showed more statistical differences between experimental treatments than total root weight in the case of wheat and Nye and Tinker (1977) that it was more important for solute uptake than root weight.

The absence of effect of Cd on root branching was unexpected, although the same phenomenon had already been reported in maize by Seregin and Ivanov (1997): Cd failed to stop root branching, although the main root growth was strongly inhibited. Another root toxicity symptom observed here was root browning. It has been reported that root browning was due to an enhanced suberization or lignification of roots tips that consequently lost their capacity for nutrient uptake (Kahle, 1993; Hagemeyer and Breckle, 1996; Schützendübel and Polle, 2002). Although Cd probably has significant effect on root hairs (Gussarson, 1994), this was not observed here as we did not find root hairs in any of the treatment with or without Cd, most probably as a consequence of hydroponics (Hagemeyer and Breckle, 1996).

6.5.4 Effect of Cd on plant water balance

Several authors reported that plants exposed to concentrations clearly above the critical toxicity level exhibited increased stomatal resistance beside low transpiration rates (Barcelo and Poschenrieder, 1990; Perfus-Barbeoch *et al.*, 2002). The reduced uptake of nutrient solution that we observed in plants grown at high Cd concentrations (100 µM Cd and 200 µM Cd) may be due to a diminished water flow into and within the roots because of both an alteration of roots anatomy (see above) and a reduced transpiration rate at the plant level.

Cadmium has been additionally found to diminish water stress tolerance in bean plant: turgor loss occurred at higher relative water concentration in Cd-treated plants than in control plants (Barcelo and Poschenrieder, 1990; Poschenrieder and Barcelo, 1999). This last fact was similarly observed in our 3-month-old plants grown with 5, 10 and 50 µM Cd. For example in the 3-month trial only one plant survived at 5 µM Cd whereas the three other plants died of drought although they still had nutrient solution. It is known that Cd interferes with water balance even in hydroponics where water availability is not expected to be limiting (Poschenrieder and Barcelo, 1999; Perfus-Barbeoch *et al.*, 2002) because of both a decrease in root length and a root tips alteration (Barcelo and Poschenrieder, 1990). Several authors explained also the leaf roll symptom as a consequence of a decreased water transport and the suppression of cell elongation and/or division because of an irreversible inhibition exerted by Cd on the proton pump responsible for the process (Hagemeyer and Breckle, 1996; Poschenrieder and Barcelo, 1999; Sanità di Toppi and Gabbrielli, 1999). However, since we only observed the leaf roll symptom at 20 and 50 µM Cd while willows were showing a higher need in nutrient solution, we suggest that it might rather be a symptom of a diminished tolerance to water stress, because Cd concentrations in leaves were similar for 20 µM and 100 µM Cd treatments.

6.5.5 Toxicity levels

Acute Cd stress seemed to appear above 50 µM Cd (100 and 200 µM) as shown simultaneously by an increase in shoot:root biomass ratio, a significant decrease in shoot biomass, in leaf area, in root biomass, in total root length and a reduction of water uptake. These symptoms have been reported for plants exposed to Cd concentration above the critical toxicity level (Barcelo and Poschenrieder, 1990, Poschenrieder and Barcelo, 1999; Sanità di Toppi and Gabbrielli, 1999). Below 50 µM we observed a small loss in root biomass and length compensated by the production

of bigger diameter roots, a small loss in leaves biomass and area, and a decrease in water stress tolerance. In conclusion these symptoms are likely to be those observed in moderately contaminated soils if Cd is the only contaminant.

6.5.6 Cadmium localization in leaves

The accumulation of Cd was uneven at the leaf surface. The spots of high Cd accumulation observed on the autoradiographs were localized in similar areas as the necrotic spots observed on the margin of the leaves. To our knowledge, this is the first direct evidence of Cd distribution in leaves of willow. Salt *et al.* (1995) analysed Cd localization in leaves of Indian mustard by autoradiography. They found an even distribution at the leaf surface and clearly showed that Cd was accumulated preferentially in trichomes. In a number of other annual plants sequestration of metals in trichomes has been reported (Küpper *et al.*, 1999; Choi *et al.*, 2001; Ager *et al.*, 2002). Trichomes are ideal organs for sequestration of heavy metals because of their isolation from plant bodies. Although *S. viminalis* possesses trichomes they did not accumulate Cd. Indeed accumulation in trichomes would result in an even distribution at the leaf surface (Salt *et al.*, 1995). This observation was further confirmed by Cd microlocalization.

As a perennial plant, *Salix* may have developed different mechanisms of detoxification. Additionally willow is a peculiar woody species with the rather uncommon habit of having no-predefined growth program when regularly coppiced (observation from authors). Willow might then react differently to cumulative stress than other species. For example, differences in Cd concentrations between young and old leaves were reported in several species. Cadmium accumulated preferentially in the youngest leaves of both *Brassica juncea* and *Thlaspi caerulescens* (Salt *et al.*, 1995), but on the contrary Cd was found at higher concentration in the older leaves in *Silene vulgaris* (Chardonnens *et al.*, 1999), *Empetrum nigrum* (Uhlig *et al.*, 2001) and *Armeria maritima* ssp. *halleri* (Dahmani-Müller *et al.*, 2000). As a consequence Dahmani-Müller *et al.* (2000) proposed leaf-fall as a metal detoxification mechanism for *Armeria maritima* ssp. *halleri*. Our results at the leaf level (no clear correlation between leaf position and Cd concentration) can be explained as a consequence of the indefinite growth. Based on our results we can unfortunately not determine if leaf fall is a detoxification mechanism in willow. Nevertheless, since large concentrations of Cd occurs in leaves, the harvest of leaves would be valuable when *Salix* is grown on metal-contaminated substrate.

In contrast with the visible toxicity symptoms reported before, which intensity varied according to Cd concentration in the leaves, the localization pattern of Cd in leaves was the same for the different Cd concentrations and times of exposure tested. A differential Cd localization between young and older leaves was observed. We excluded that this was a consequence of differences between leaves formed before Cd treatment and during Cd treatment because differences were already visible after 3 days of exposure to Cd in plants of which all leaves had been formed before Cd treatment. We believe that these differences are related to a physiological difference between young and old leaves. Young leaves develop their own photosynthetic machinery and thus have a higher demand for nutrients than older leaves (Yoshida, 2003). The localization in the tips and at the edge of the young leaves can thus be

explained by a greater transpiration stream driving Cd at the terminal sites of the transpiration stream (Marschner, 1995). Furthermore, the photosynthetic apparatus is mainly located in the limb and it is known to be particularly susceptible to Cd toxicity (Krupa and Baszynski, 1995; Horváth *et al.*, 1996; Geiken *et al.*, 1998). The accumulation of Cd mainly at the edge of the young leaves could thus help to protect the photosynthesis apparatus. On the contrary in older leaves the transpiration stream into leaves is reduced (Patrick, 1988) and Cd does not reach the edges anymore. Such a difference in flow has been demonstrated in poplar by transpiration measurement between young and old leaves (Siebrecht *et al.*, 2003). On the other hand, a reallocation of Cd with leaf aging can not be ruled out because although the plants develop, the same pattern of Cd distribution along the main stem is observed meaning that previous young leaves with accumulation at the tips has become mature leaves with less Cd at the tip. Furthermore Cd was found by physical development in the phloem. Patrick (1988) has indeed shown that reallocation of several cations (e.g. Ca) occurs through the phloem. It has also been hypothesized that Cd accumulation in developing fruits occur via phloem-mediated transport (Hart *et al*., 1998). In our case, the phloem and especially its conducting cells were damaged in the samples showing Cd accumulation. Its transport capacities might therefore be reduced.

Apparent Cd abundance depended on ontological cell and tissue development. Cadmium storage in leaf veins increased where larger and thicker veins provided an increased storage capacity. In mature leaves vein bases had thus more histochemically-detected and radioactively-labelled Cd than vein tips. Maturation of collenchym seemed to play a central role in the vein storage capacity of Cd. Apparent Cd abundance particularly increased in vein collenchym with thicker cell walls in mature leaves. In places where vein storage diminished, Cd was increasingly deposited as spots inside the leaf blade. In apexes such spots were sometimes grouped along arched lines probably indicating locations next to secondary veins.

The results obtained here indicated that Cd reached leaf cells mainly by the veins and was rapidly removed from metabolically active cellular sites for subsequent storage in less sensitive compartments. The accumulation of Cd in the cell wall of veins and cells is indeed an important tolerance mechanism. Cell walls can easily interact with metal ions (Wang and Evangelou, 1995), thereby lowering potentially toxic-free Cd ions in the cytoplasm. In mycorrhizal fungi for example Cd was found to be bound to cell wall negatively charged sites such as cellulose or cellulose derivatives (Ernst, 1980; Galli *et al.*, 1994; Turnau *et al.*, 1994; Blaudez *et al.*, 2000). The importance of Cd binding to cell walls has been demonstrated for root cells of several plants by limiting its subsequent translocation into shoots (Wagner, 1993; Grant *et al.*, 1998). Khan *et al.* (1984) also assumed that cell walls played the most important role in Cd accumulation in *Zea mays*. However, the role of cell walls in Cd storage in plants remains controversial. Vögeli-Lange and Wagner (1990) for example failed to show substantial Cd binding in cell walls of tobacco. To conclude, the results obtained here indicated that vacuolar sequestration of Cd was probably not the main mechanism of Cd accumulation in *S. viminalis* leaves.

6.6 Conclusion

Salix viminalis was surprisingly tolerant to Cd. Although Cd concentrations were higher in roots than in leaves, Cd found in leaves of *S. viminalis* exceeded 100 mg kg^{-1}. The results presented here showed that a longer time of exposure to the metal would allow increasing total Cd uptake as long as toxicity threshold is not overpassed. Fifty μM Cd was the threshold for acute toxicity, whereas 200 μM Cd really impaired survival. Observation of the establishment of toxicity symptoms showed that it was not simultaneous but progressive with increasing Cd concentration. A striking correspondance was established between the spots of high Cd accumulation observed on the autoradiographs and the necrotic spots observed on the margin of the leaves. Cadmium was localized mainly at the tips and around the veins. The localization was modulated according to the age of the leaf. Distribution of Cd at the cell level in *S. viminalis* showed a preferential accumulation in cell walls.

7

General conclusion

A major aim of this study was a better understanding of the mechanisms involved in Cd storage at different levels, from the whole plant physiology to single cell analysis and gene expression. In particular we aimed to assess the differences between *Thlaspi caerulescens* Ganges (Cd hyperaccumulating ecotype) and plants accumulating at a lower degree, especially the high biomass crop *Salix viminalis*. Although both plants have often been studied because of their high potential to extract heavy metals from soil, the aspects related to accumulation have been seldom studied comparatively on the same scientific basis (apart from total uptake comparison): whereas mechanisms have been emphasized for hyperaccumulating plants, high biomass crops have always been used on a more applied way, assuming a knowledge based on agronomic practices. To be able to find out the extent of a possible improvement of phytoremediation efficiency, it is necessary to understand the degree and type of differences between these two groups of plants.

Salix viminalis was surprisingly tolerant to Cd and to high Cd concentration in shoots. In hydroponics, willow performed better than Ganges. At 10 μM Cd and 3-month growth for example, willow showed a 17% loss in biomass and 153±32 mg Cd kg^{-1} in shoots. Ganges in the same growth conditions showed a 36% biomass decrease and accumulated 1091±160 mg kg^{-1} in shoots. When the yield was calculated for these specific conditions, Ganges extracted 143 μg Cd whereas *S. viminalis* extracted 4-fold more Cd (615 μg). This highlights the high potential of *Salix* for Cd phytoextraction, as 10 μM Cd (1.12 mg Cd L^{-1}) is already an elevated concentration for a soil solution (Wagner, 1993; Knight *et al.*, 1997; Lombi *et al.*, 2001a). However it is known that plants accumulate more Cd when cultivated in solution culture than in soil (Grant *et al.*, 1998; Dickinson, 200.). Consequently results in hydroponics might not strictly reflect the accumulation capacities of the plants in the field. Additionally soil contamination is rarely restricted to a single metal (McGrath, 1998), thus it would be necessary to test tolerance of the plants to the other contaminants found in the site to be remediated before choosing a plant species.

Generally speaking, accumulation of a given metal is a function of uptake capacity and intracellular binding sites (Clemens *et al.*, 2002). In a multicellular organism, the situation is complicated by tissue- and cell-specific differences and also by intracellular transport, mobilization and uptake from the soil, compartmentation and sequestration within the root, efficiency of xylem loading and transport, distribution between metal sinks in the aerial parts, sequestration and storage in leaf cells. At every level, concentrations and affinities of chelating molecules, as well as the presence and selectivity of transport activities, affect metal accumulation rates

(Clemens *et al.*, 2002). So far, the mechanisms of metal uptake by plants and the basis of their metal specificity have been poorly understood. Nevertheless, two main compartmentation mechanisms have been often proposed to explain the accumulation of metals by plants in roots and shoots: 1) binding of metals to cell walls, and 2) compartmentation of metals in the vacuoles (Wang and Evangelou, 1995).

The same range of Cd concentrations (0-100 µM) was tested on both *T. caerulescens* and *S. viminalis*. Autoradiographs showed similarities between both plants in Cd storage, although the Cd concentration found in leaves differed greatly and the former is a biannual plant and the latter a perennial plant with no-predefined growth (P. Vollenweider, personal communication). We observed both point-like accumulation and accumulation at the edges of the leaves. In both species a link could be established with visible symptoms, that were also similar (necrosis) for both plants. At last differences in Cd localization between young and mature leaves were observed. In both species, these differences were attributed to physiological differences between leaves. *Salix viminalis* and *T. caerulescens* were both able to reduce Cd toxicity by allocating Cd in less sensitive tissues. We demonstrated that Cd was found inside the cells and in the cell walls (ca. 35% of total Cd) of the leaves of *T. caerulescens*. On the other hand in *S. viminalis*, Cd was found mainly in cell walls and to a lesser extent in the symplasm. Unfortunately, we did not quantify the percentage of Cd stored in cell walls of *S. viminalis* as was done for *T. caerulescens*. Metal allocation in both plants indicated that the general plant growth strategy and the metal storage in metabolically inactive plant parts governed metal accumulation. It also seemed that movement of Cd from roots to shoots occurred via the xylem and was driven by transpiration stream.

This study confirmed that despite the similarity between Cd and Zn in their electronic structure, there are differences between these metals in terms of their mechanisms of accumulation by *T. caerulescens.* Cadmium was found for example in the cell walls and also around the vein. Cadmium and Zn nevertheless are both similarly accumulated in the epidermis probably protecting the photosynthesis apparatus. Competition experiments also suggested that the metals are, at least to a certain degree, taken up by the same transporter(s) or are controlled by common regulators.

The involvement of several sinks in our study was in accordance with the suggestion of Baker (1987) that proposed a “syndrome of tolerance” including several mechanisms operating to different extents in different species. Indeed, the limited storage capacity of each compartment probably demanded the involvement of other compartments or even of the whole plant. Only part of the Cd taken up by a plant was deposited in each compartment, but when added these percentages probably decreased significantly perceived internal concentration of the metal and is most certainly necessary for plant survival. It is more likely that a series of events rather than a single limiting event control Cd tolerance and accumulation (Baker, 1987; Wang and Evangelou, 1995; MacNair, 1997). It would indeed seem to be a risky evolutionary selection for higher plants to possess only one single tolerance mechanism. However, it does not explain why hyperaccumulating plants are able to accumulate specific metals to high levels in leaves even when growing in environments with low metal status. Besides, little attention has been devoted to other tolerance mechanisms, like the guttation excretion (Mizuno *et al.*, 2003) or reallocation

through the phloem (Hart *et al.*, 1998) that are also likely to play a role in metal tolerance and/or accumulation. Further work is then needed to assess many enigmatic steps of the trafficking of metals. In our plants the similarities observed may have led to the establishment of tolerance in all the treatments performed. The localization of Cd in leaves performed with non hyperaccumulating and less Cd-tolerant plants like *T. perfoliatum* or *Arabidopsis thaliana* would probably help to point out the specificities of the highly tolerant plants mainly tested in this study.

To date, two mechanisms have been proposed to explain the high uptake of metals by hyperaccumulators: 1) an enhanced absorption of metal into the roots (Lasat et al., 1996) coupled with high rates of translocation of metal from roots to shoots (Shen et al., 1997), 2) a preferential allocation of root biomass into regions of metal enrichment (Schwartz *et al.*, 1999; Whiting *et al.*, 2000), and a large root system compared to shoot dry matter that further favour soil prospecting as well as heavy metals uptake (Keller *et al.*, 2003). This nevertheless does not explain the metal specificity of hyperaccumulation. Besides uptake has mainly been studied at the root level, and few data are available on Cd uptake in leaves. Kochian *et al.* (2002) hypothesized that the increased transport observed at the root level could also concern leaf cell through a stimulated metal influx across the leaf cell plasma membrane and an enhanced storage in the leaf vacuole. This is supported by the fact that Prayon stored Cd in the same pattern as Ganges when grown in hydroponics. Indeed the only differences found between both populations at the leaf level in this study was concentration of Cd in the storage sinks. However, when investigating Cd uptake in mesophyll protoplasts of Ganges, Prayon and *A. halleri* we did not find significant differences between the Michaelis-Menten kinetic constants of the three plants. This indicated that differences in metal uptake could not be explained by different constitutive transport capacities at the leaf protoplast level and that plasma and vacuole membranes of mesophyll cells were not responsible for the differences observed in heavy metal allocation. However, pre-exposure of the plants to Cd induced an increase in Cd accumulation in protoplasts of Ganges, whereas it decreased Cd accumulation in *A. halleri* protoplasts, indicating that Cd permeable transport proteins were differentially regulated. Additionnally, the Cd amount accumulated in the different *T. caerulescens* populations studied seemed to be linked to the Cd exposure of the mother plant. It would therefore be interesting to assess precisely the genetic basis (constitutive or inducible) of Cd uptake and accumulation in *T. caerulescens*. The study performed here confirmed that Zn hyperaccumulation is constitutive in this species, but the results with Cd seemed more complex. Although it has been shown that tolerance and hyperaccumulation traits were genetically independent in plants (Baker *et al.*, 1994b; Meerts and Isacker Van, 1997; MacNair *et al.*, 1999), it is difficult to discriminate between both when looking at the mechanisms. The understanding, characterization and differentiation of metal tolerance and hyperaccumulation mechanisms are of great practical importance for phytoextraction.

Several of the methods performed in this study were derived from methods developed for other plants species and were unfortunately seldom adaptable to *S. viminalis*. The genetic expression study for example was unfeasible with *S. viminalis* because the identity to *A. thaliana* genes was too low. We were also unable to isolate protoplasts from our *S. viminalis* clone. Other authors similarly reported that they failed to extract protoplast of several different *S. viminalis* clones, but succeeded with other clones (Vahala and Eriksson, 1991). However, the origin of this phenomenon

was not identified. In addition it was not possible to extract the required quantity of epidermal cells from *T. caerulescens*, therefore the experiment was conducted on protoplasts isolated from mesophyll cells of leaves only. Although it has been reported as the main site of accumulation in *A. halleri* (Küpper *et al.*, 2000), it was shown in our study not to be a main site of accumulation in *T. caerulescens*. It would therefore be interesting to assess Cd localization in *A. halleri* in order to confirm the Cd accumulation in mesophyll cells and to assess the storage at the subcellular level. SEM-EDXMA was eventually not performed on *S. viminalis* due to the high detection limit of the device that did not allow detection of Cd concentration in the range of those found in willow leaves. On the other hand, willow leaves allowed the use of other methods less suitable to Brassicaceae leaves. For example, we are currently working in *S. viminalis* on the assessment of cellular toxicity symptoms (Vollenweider *et al.*, 200.). Analysis at the cell level with a microscopical approach should help to distinguish between direct and indirect effect of Cd (Vollenweider *et al.*, 2003). The link between these toxicity symptoms and the presence of Cd in the organ need to be made to enhance the knowledge on Cd tolerance and accumulation mechanisms. Indeed the distribution of metals within plant organs and tissues is an indicator of detoxification and tolerance mechanisms employed by plants.

Based on our results and the methods developed in this work, several steps could be further studied to address key questions on the mechanisms of heavy metal uptake in plants. The objectives could include the following:

- assessment of Cd movement and translocation in leaves by doing only a single short exposure to ^{109}Cd and subsequently following Cd movements in leaves throughout time,
- assessment of the cell wall components responsible of Cd binding by digesting sequentially the cell walls with different enzymes,
- assessment of Cd distribution in leaves of plants grown in pots,
- assessment of Zn distribution in *T. caerulescens* and *S. viminalis*.

Heavy metal transport is a very exciting and developing field in plant biology. We now have some understanding of the key processes in metal homeostasis in general, and we are beginning to understand the trafficking of metals at the cellular level and the roles of chelators and increased uptake rates in hyperaccumulation. We are probably close to the discovery of a range of new ion transporters that will undoubtedly change our concepts of metal nutrient acquisition in higher plants. However, there are numerous steps along the way of a transition metal from the soil to the storage sites in the leaf that remain to be fully characterized in order to explain the unusual behaviour of hyperaccumulating plants.

We have to admit that a complex picture has emerged from our study. Real progress in understanding hyperaccumulation mechanisms is likely to follow the cloning and molecular characterisation of a tolerance locus. Unfortunately, searching for mutants in *A. thaliana* is unlikely to be helpful in this case, since it is not known to evolve true tolerance, though small differences in susceptibility can be found (Murphy and Taiz, 1995), and it appeared to use different mechanisms than those used by hyperaccumulating plants. On the other hand, none of the wild plants showing real tolerance have been sufficiently well studied molecularly that any strategy for the

cloning of a Cd tolerance or accumulation gene suggests itself yet. Besides it seems unlikely that regulation of single genes will be sufficient to convert non-accumulators into metal hyperaccumulators. If whole suites of genes must be transferred, then somatic hybridisation between *T. caerulescens* and the high biomass crop *Brassica napus* offers another route to understand which genes are involved in hyperaccumulation (Brewer *et al.*, 1999). There is no doubt that in the coming years the outcomes of multi-approaches (physiological, biochemical and molecular) research will provide a sound basis for the future implementation of phytoextraction of Cd-contaminated soils. Understanding plant metal accumulation determinants will have numerous additional implications. For instance health-threatening human deficiencies in trace metals (e.g. Zn) appear to be widespread in developing countries and possibly worldwide (Hambidge, 2000). Conversely, most of the toxic non-essential elements such as Cd enter the human body via plant-derived material (Wagner, 1993). Identification of mechanisms governing these accumulation processes could result in the development of crops either enriched or with reduced metal content depending on the nutrition needs (Guerinot and Salt, 2001; Hacisalihoglu *et al.*, 2001).

8

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