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MARTA SOFIA
SOARES CRAVEIRO
ALVES MONTEIRO

FITOTOXICIDADE E TRANSFERÊNCIA TRÓFICA DE CÁDMIO PARA ISÓPODES

CADMIUM PHYTOTOXICITY AND TROPHIC TRANSFER TO ISOPODS

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CADMIUM PHYTOTOXICITY AND TROPHIC TRANSFER TO ISOPODS

dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Professora Doutora Maria da Conceição Lopes Vieira dos Santos, Professora Associada com Agregação da Universidade de Aveiro e do Doutor Reinier Mann, Investigador de Pós-Doutoramento do CESAM – Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro

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palavras-chave   Cádmio, citometria de fluxo, ecotoxicologia, fitotoxicidade, genotoxicidade, isópodes, *Lactuca sativa*, microsatélites, plantas, *Thlaspi arvense*, *Thlaspi caerulescens*, transferência trófica

resumo   O cádmio (Cd) é um metal não essencial e é considerado um poluente prioritário pela comunidade europeia. Este metal atinge o ambiente no decurso de várias actividades antropogénicas e tende a concentrar-se nos solos e sedimentos, onde está potencialmente disponível para as plantas, sendo posteriormente transferido através da cadeia trófica. Neste contexto, o principal objectivo da presente dissertação foi o estudo dos efeitos da assimilação e da acumulação de Cd em plantas e as suas consequências para animais consumidores. Numa primeira fase, foram estudados os principais efeitos fisiológicos e genotóxicos do Cd em plantas. As plantas de alface (*Lactuca sativa* L.) expostas a Cd apresentaram um decréscimo na eficiência fotossintética, aumento de peroxidação lipídica e alterações significativas na actividade de enzimas de stress oxidativo. Estas alterações culminaram num decréscimo do crescimento da parte aérea no final da exposição. As respostas obtidas pelos parâmetros bioquímicos sugerem que estes poderão ser utilizados como eventuais biomarcadores em testes ecotoxicológicos com Cd em abordagens integrantes em conjunto com parâmetros clássicos. Os efeitos mutagénicos de Cd foram avaliados através da determinação da instabilidade de microsatélites (IM). Não foi observada IM, nem nas folhas nem nas raízes de plantas de alface com 5 semanas de idade expostas a 100 μM Cd durante 14 dias, no entanto observou-se IM em raízes de alface exposta a 10 μM Cd durante 28 dias desde a germinação. A idade da planta e a maior acumulação de Cd nas raízes poderão explicar os resultados obtidos. A clastogenicidade de Cd foi analisada em três espécies vegetais com diferentes capacidades de destoxificação e acumulação de metais através de citometria de fluxo. Foram detectadas alterações significativas nos parâmetros analisados em raízes alface, mas não nas espécies *Thlaspi caerulescens* J & C Presl e *Thlaspi arvense* L. Estes resultados sugerem que o stress provocado pelo Cd originou clastogenicidade como consequência da perda de porções de cromossomas, uma vez que o conteúdo de ADN nuclear diminuiu. A transferência trófica através da cadeia alimentar permanece muito pouco estudada em termos ecotoxicológicos. A distribuição subcellular de metais num organismo pode ser utilizada para compreender a transferência trófica de um metal na cadeia alimentar. Como tal, numa última parte é estudado de que modo a distribuição subcellular do Cd em plantas com perfis de acumulação de Cd distintos afecta a biodisponibilidade e transferência trófica de Cd para isópodes. A distribuição de Cd entre as 4 fracções subcelulares obtidas através de centrifugação diferencial revelou a existência de diferenças significativas entre as espécies de plantas. Estes resultados em conjunto com a avaliação directa da eficiência de assimilação (EA) de Cd individual de cada uma das quatro fracções subcellulares das plantas em estudo, resultou em informação de grande relevância para a explicação das diferenças observadas na EA de Cd por parte de isópodes alimentados com folhas de diferentes espécies de plantas. Com base nos resultados obtidos, o Cd ligado a proteínas estáveis à temperatura (e.g. metaloteoninas e fitoquelatinas) é o menos biodisponível, sendo assim o que menos contribuiu para a transferência trófica, enquanto que o Cd ligado a proteínas desnaturadas pela temperatura foi a fracção mais disponível para transferência trófica de Cd ao isópode. Estes resultados realçam a relevância ecológica da distribuição subcellular de Cd em plantas que tem influência directa na tranferência trófica deste metal para os consumidores e ainda o facto de que alterações na distribuição subcellular de Cd em plantas devido a diferentes mecanismos de destoxificação poderá ter um impacto directo na transferência trófica de Cd para o animal consumidor.

abstract

Cadmium (Cd) is a non-essential metal and is considered a priority pollutant by the European Community. This metal is released to the environment as a consequence of several anthropogenic activities and tends to accumulate in soils and sediments where it is potentially available to rooted plants causing severe detrimental effects and then transferred to animals through the food chain. In this context, the main objective of the present dissertation is to study the effects of Cd uptake and accumulation in plants and its implications to animal consumers. First, the main physiologic and genotoxic effects of Cd to plants were examined. Cadmium-exposed lettuce (*Lactuca sativa* L.) plants displayed a significant decrease in photosynthetic efficiency, enhanced lipid peroxidation and alterations in the activities of antioxidant enzymes over the duration of exposure. These alterations culminated in reduced shoot growth at the end of the exposure. The aforementioned biochemical alterations are suggested to be used as plant biomarkers in integrative approaches with classical endpoints in future ecotoxicological tests with Cd. The mutagenic effects of Cd on plants were assessed examining microsatellite instability (MSI). No MSI was found neither in leaves nor roots of 5-week old lettuce plants exposed to 100 μM Cd, but MSI was found in roots of lettuce plants exposed to 10 μM for 28 days from seed germination. The age of the plant at the time and the higher accumulation of Cd in the roots might explain the results obtained. Clastogenic effects of Cd was examined in plants with different metal accumulation and detoxification capacities by flow cytometric (FCM). The endpoints analysed indicated significant alterations in lettuce roots but not in *Thlaspi caerulescens* J & C Presl and *Thlaspi arvense* L.. The results obtained suggested that Cd stress may have lead to clastogenic damage as a consequence of loss of chromosome portions because nDNA content was found to be diminished. Trophic transfer through the food chain remains a largely unexplored area of ecotoxicology. Subcellullar distribution of metal accumulated within an organism can be used to understand metal trophic transfer within a food chain. Thus, in a final stage we examined how Cd subcellular distribution in plants with different patterns of Cd accumulation can affect assimilation of Cd by the isopod. The distribution of Cd between the four different subcellular fractions obtained by differential centrifugation revealed significant differences between the plant species. This, together with the direct assessment of isopod Cd AE from individual subcellular fractions of the leaves of the three plant species, resulted in vital information to help explain the differences observed in Cd AE by isopods fed the different type of leaves. On the basis of our results, Cd bound to heat-stable proteins (e.g. phytochelatins and methallothionein) was the least bioavailable to isopods and contributed less to the trophic transfer of Cd, while Cd bound to heat-denatured proteins was the most trophically available to the isopod. These results point to the ecological relevance of the subcellular distribution of consumer and highlight that a shift in Cd subcellular distribution in plants due to different detoxifying mechanisms may have a direct impact in the trophic transfer of Cd to the animal consumer.

Aos meus pais e ao Edgar

"There is no simple answer to the basic questions - why and how?"

Z Krupa et al. (2002) Heavy metal interactions with plant nutrients. In: MNV Prasad and K Strzałka (Eds). Physiology and biochemistry of metal toxicity and tolerance in plants. Kluwer Academic Publishers, London, pp. 287-301.

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General Introduction

Chapter 1

Preamble

Cadmium (Cd) is a naturally occurring element, and its presence has been detected in more than 1,000 species of aquatic and terrestrial flora and fauna (Eisler, 1985). With one known exception, there is no evidence that Cd is biologically essential or beneficial; on the contrary, it has been implicated in several human health diseases and various deleterious effects in wildlife (Eisler, 1985). The exception is a Cd-dependent carbonic anhydrase found in the marine diatom *Thalassiosira weissflogii* (Lane and Morel, 2000; Lane et al., 2005); a similar role has been postulated for the metal hyperaccumulating plant, *Thlaspi caerulescens* (Liu et al., 2008). In all life-forms, including microorganisms, higher plants and animals (in particular humans), Cd is toxic when present in sufficient concentrations (Eisler, 1985).

The identification of Cd as a distinct element is relatively recent. The German scientist Friedrich Stromeyer was at the origin of the discovery of Cd in 1817 (Robards and Worsfold, 1991). Its toxicity was soon recognized and early recorded cases of Cd poisoning were generally as a consequence of industrial exposure involving inhalation of Cd dusts (Robards and Worsfold, 1991).

Pollution of the biosphere with this toxic metal has accelerated dramatically since the beginning of the industrial revolution (Nriagu, 1996) and Cd accumulation in soil and water now poses a major environmental and human health problem. In 1955 in Japan, Cd toxicity was found to be the cause of Itai–itai disease. For the first time, Cd pollution was shown to have severe consequences on human health. Cadmium contaminations were attributed to the effluents from a zinc mine located in the upper reaches of Jinzu river and profoundly affected the health of the human population living in that area (Inaba et al., 2005).

Historically, the study of metal uptake by plants has focused on micronutrient metals important in agricultural production, whereas non-essential metals, such as Cd, Hg and Pb, have generally received less attention. However, over the last three decades Cd has been the subject of several investigations in plant research mainly because of its potential for bioaccumulation through soil-plant-animal food-chain. For example, the consequences of soil contamination by Cd, through application of treated sewage sludge (biosolids) (McLaughlin et al., 2006) and Cd-enriched phosphate fertilizers (e.g. He and Singh, 1994b, a) to soils have been extensively studied. However, the driving force of this research area has been the concern for the risk to human health, not for the state of the plant itself.

Most of the research on Cd pollution focused on the processes involved in Cd accumulation in crop plants and on the consequences of this accumulation on human health (Wagner, 1993). Cadmium phytotoxicity is, however, a relevant problem, especially in some highly metal polluted regions, where a decrease in agricultural crop productivity has been observed (Vassilev and Yordanov, 1997). More recently, metal hyperaccumulator plant species have been used to re-examine mechanisms of metal uptake by plants in light of the potential for phytoremediation of metal-contaminated soils (Chaney et al., 1997; Pilon-Smits, 2005; Padmavathiamma and Li, 2007). On the other hand, Cd toxicity to plants has great impact and relevance not only for plants but also to the ecosystem, in which the plants form an integral component. Therefore, understanding Cd uptake and physiological responses of plants is critical to the long-term safety and conservation of agricultural resources and ecosystems. In addition, plants as sedentary organisms offer unique advantages for *in situ* monitoring of soil contamination (Grant, 1999) and can potentially be used as biomonitors of environmental quality through the use of biomarkers. In plants it is well known that Cd interferes with photosynthesis, respiration and nitrogen metabolism, induces oxidative stress and genotoxicity, all of which can culminate in poor growth and low biomass production (Sanitá di Toppi and Gabbrielli, 1999; Fodor, 2002). The biochemical pathways involved in these processes offer a battery of biochemical biomarkers that not only provide mechanistic endpoints of toxicity, but also improve our understanding on the toxic modes of action and exposure assessment. Hence, the purpose of the first part of the present dissertation is to contribute to a better understanding of the overall process of Cd-induced senescence, describing the cascade of events and the enzymatic protection strategies that plants can adopt against Cd-induced oxidative stress (Chapter 2) in order to shed light on a selection of relevant plant biomarkers for further use as biomonitoring tools in the assessment of environmental Cd pollution. Special emphasis is given to genotoxicity of Cd; in Chapter 3 the clastogenic and mutagenic effects of Cd in exposed plants are examined.

Another issue concerning Cd accumulation in plants centres on the fact that Cd could pose a risk to animal health if they consume plants contaminated with Cd, even if plant tissue concentrations are not generally phytotoxic (McLaughlin, 2002). Indeed, the ability of some plant species to uptake and hyperaccumulate Cd in edible parts increases the risk of Cd assimilation by animal consumers through trophic transfer. Because there is very limited knowledge regarding the trophic transfer of metallic contaminants between plants and consumers of plants, the second part of this dissertation presents an examination of the subcellular distribution of Cd within plant leaves, and subsequent

significance of that distribution on the bioavailability of Cd to a consumer - a detritivore isopod (Crustacea). A subcellular fractionation procedure developed by Wallace and co-workers (Wallace et al., 2003; Wallace and Luoma, 2003) that has been largely applied in the dietary accumulation of metals, particularly in marine food chains, was adopted to try to explain the variability observed in metal assimilation by isopods fed plants with different patterns of Cd accumulation. Indeed, this method is considered a simple and pragmatic approach in the prediction of trophic transfer of metals and a first step towards a practical tool that could explain most of the variability observed in metals accumulation and toxicity in organisms (Vijver et al., 2004).

Cadmium – a priority pollutant

What is Cd

Cadmium is an element that occurs naturally in the earth's crust as a result, for instance, of volcanic emissions. Pure Cd is a soft, silver-white metal, and it is not usually present in the environment as a pure metal, but as a mineral compound combined with other elements. Cadmium is most often present in nature as complex oxides, sulphides, and carbonates in zinc, lead and copper ores (ATSDR, 1999).

Cadmium is generally considered to be a so called "heavy metal" due to its high density (8.6 $g.cm^{-3}$), high atomic weight (112.4 $g.mol^{-1}$) or even for its toxic properties. However, many different definitions have been proposed for this commonly used term; some based on density, on atomic number or weight, and others on chemical properties or toxicity (Nieboer and Richardson, 1980). Despite early suggestions for the use of other nomenclature (Nieboer and Richardson, 1980), the term "heavy metal" has been widely used by scientific community over the past three decades. More recently the term "heavy metal" has been considered meaningless and misleading in an IUPAC technical report due to the contradictory definitions and its lack of a coherent scientific basis (Duffus, 2002). Therefore, in the present dissertation the term "heavy metal" is used restrictively, specifically when citing other works.

Sources of Cd pollution

Cadmium is included in the list of 33 priority pollutants established by European Community (2455/2001/EC, 2001) and is one of 129 priority pollutants listed by EPA

(Environmental Protection Agency, USA). The release of Cd into the environment constitutes a significant pollution problem. Cadmium occurs naturally in the environment. It is estimated that about 25,000 to 30,000 tons of Cd are released to the environment each year; about half from the weathering of rocks into river water; and a further proportion from forest fires and volcanoes (ATSDR, 1999). Release of Cd from human activities is estimated to be about 4,000 to 13,000 tons per year, with major contributions from mining activities, and burning of fossil fuels (ATSDR, 1999). The Cd-yellow oil colours used by landscape painters, including Claude Monet (Figure 1.1) is just one of the many valuable uses of Cd. Other important applications of Cd are in metallurgical industry and in the manufacture of nickel–cadmium batteries, pigments, plastic stabilizers and anti-corrosive products, phosphors for television sets, scintillation counters and X-ray screens, semiconductors and ceramic glazes (Robards and Worsfold, 1991). As a consequence of this widespread and diverse usage, large quantities of Cd end up in sewage.

Treated sewage sludge ("biosolids") and phosphate fertilizers (He and Singh, 1994b, a; Speir et al., 2003; McLaughlin et al., 2006; Singh and Agrawal, 2007) are important sources of Cd contamination in agricultural soils. The usage of Cd in developed countries has, however, begun to decline because of its toxicity. For instance, Cd is one of six substances banned by the European Union's Restriction on Hazardous Substances (RoHS) directive, which bans carcinogens in computers (2002/95/EC, 2002).
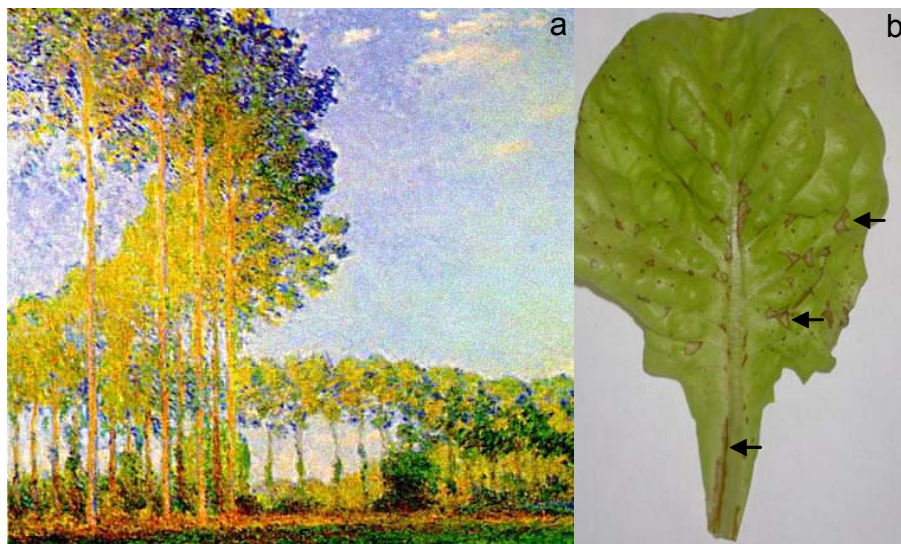
Figure 1.1 – Cadmium, a beautiful toxic colour. a) *Poplars in the sun* by Claude Monet, 1891 (www.monet-on-canvas.com/prod197.htm), showing the powerful use of Cd yellow pigments and b) a lettuce leaf reflecting the toxic effects of Cd exposure (arrow: Cd-induced necrosis).

Cadmium characteristics

The most important feature which distinguishes metals from other toxic pollutants is that they are not biodegradable and, once they become resident in the biosphere, they remain as a persistent pollutant. For instance, in estuarine coastal systems the residence time of Cd has been estimated as a relatively low 1-2 years (Robards and Worsfold, 1991). On the other hand, estimates of the residence time in ocean water range from 7,000 to 250,000 years (Robards and Worsfold, 1991). The bioavailability of metals and their subsequent toxicity to organisms within the biosphere is controlled largely by their physico-chemical form. Cadmium, among the metals, is likely to have high mobility in soils because it does not bind as strongly to organic matter as do metals such as Hg and Pb (Nelson and Campbell, 1991).

Besides of a long environmental persistence, Cd has a long biological half-life, which accounts for its bioaccumulation in individuals (John and Leventhal, 1996). For example, in man Cd accumulates in the liver and kidneys and has a long biological half-life ranging from 17 to 30 years (Goyer, 1997). Existing data on Cd bioaccumulation in a range of animals and plants (Robards and Worsfold, 1991; Greger, 1999) verify the ability of these species to amplify the concentration of Cd relative to their environment. Bioaccumulation occurs within an organism and is the increase in concentration of a substance in an individual's tissue as a consequence of uptake from their environment, including diet (Connell et al., 1999). The extent of contaminant bioaccumulation, which is the net outcome of two competing processes, uptake and depuration, is related to the level of environmental contamination and depends upon a number of physico-chemical (e.g. chemical speciation, partitioning) and environmental factors (e.g. season, temperature) and biological variables (e.g. specie, feeding habitat, physiology) that may alter the distribution and bioavailability of individual contaminants (Robards and Worsfold, 1991; Connell et al., 1999). Among the factors that can affect Cd bioaccumulation are its physico-chemical form, the presence of other metals, pH, salinity, temperature, season, cation-exchange capacity of soils and the species taking up the Cd (Robards and Worsfold, 1991 and references therein).

Metal biomagnification is defined as the progressive accumulation of a metal with increasing trophic levels towards higher consumers (Connell et al., 1999). Among metals, this type of amplification at higher trophic levels was previously thought to occur only for mercury, but it has also been demonstrated in a few studies with Cd (Croteau et al., 2005). Croteau and co-workers (2005) have demonstrated that Cd was progressively enriched

among trophic levels in two discrete epiphyte-based food webs composed of macrophyte-dwelling invertebrates or fishes. Cadmium concentrations were biomagnified 15-fold within the span of two trophic links in both food webs.

Cadmium uptake: from soils to plants

Cadmium in soils

Among metals, Cd is of particular concern because of its mobility in the plant-soil system. Wagner (1993) estimated that non-polluted soil solutions contain Cd concentrations ranging from 0.04 to 0.32 µM. Cadmium concentrations in nonpolluted soils are however highly variable, depending on sources of minerals and organic material. For instance, Eisler (1985) reported Cd concentrations of 0.01-1.00 mg/kg in soils of nonvolcanic origin and up to 4.50 mg/kg in soils of volcanic origin. Soil solutions which have a Cd concentration varying from 0.32 to about 1 µM are considered as moderately polluted (Sanitá di Toppi and Gabbrielli, 1999). Topsoil concentrations are often more than twice as high as subsoil levels as the result of atmospheric fallout and contamination (Pierce et al., 1982). Cadmium levels up to 800 mg/kg have been reported for soils in polluted areas (IARC, 1993). Jung and Thornton (1996) have found Cd concentrations up to 40 mg/kg in surface soils taken from a mining area in Korea; and more recently, Cd contaminated river water (65-240 µg/l, 0.58-2.13 µM) downstream from a mining area in Bolivia has increased the soil concentration of Cd to 20 mg/kg and the concentration of Cd in soil solutions to 27 µg/l (0.24 µM) (Oporto et al., 2007).

Contamination of topsoil is likely the most important route for human exposure to Cd, mediated through uptake of soil Cd into edible plants (IARC, 1993). Cadmium concentrations of 0.5 mg/kg or more have been found in rice grown in Cd-polluted areas of Japan (Nogawa et al., 1989) and China (Cai et al., 1990). Furthermore, in a recent field study in Europe performed by Peris et al. (2007) the Cd content in edible parts of vegetables such as lettuce were found to be above the maximum levels established by the Commission Regulation no. 466/2001 for horticultural crops (466/2001/EC, 2001).

The Cd concentration of 100 µM to grow/contaminate plants hydroponically was chosen for use in all different approaches of the present dissertation. Previous studies have used similar approaches and similar concentrations (Azevedo et al., 2005a; Azevedo et al., 2005b, c). Also, this concentration is twice the maximum permitted concentration in irrigation water by Portuguese legislation (0.05 mg/l) (Decreto-Lei, n.º236/98), and therefore represents worst case scenario.

Uptake and transport of Cd by plants

Cadmium accumulation by higher plants can occur through foliar or root uptake. However, the primary point of entry for Cd into plants is through the roots. Cadmium uptake by plants grown in contaminated soils has been extensively studied, particularly in sludge-amended soils (e.g. Jackson and Alloway, 1991; Speir et al., 2003; McLaughlin et al., 2006; Singh and Agrawal, 2007) and in soils treated with Cd-enriched phosphate fertilizers (Crews and Davies, 1985; He and Singh, 1994b, a; Huang et al., 2003). In general, metals have to be in an available form to be taken up by plants. Alternatively plants must have mechanisms to make the metals available. The degree to which higher plants are able to take up Cd depend on its concentration in the soil and its bioavailability. Cadmium bioavailablity in soils is modulated by the presence of organic matter, pH, redox potential, temperature, light intensity, cation exchange capacity and concentrations of other elements (He and Singh, 1993; Greger, 1999; Sanitá di Toppi and Gabbrielli, 1999). In particular, Cd ions seem to compete with other micro and macro-nutrients such as calcium and zinc for the same transmembrane carriers (Sanitá di Toppi and Gabbrielli, 1999), which might lead to plant nutrient deficiencies (Krupa et al., 2002). As is the case for other metals, Cd uptake tends to be reduced at low pHs because of competition with H^+ ions at root uptake sites; however, Cd bioavailability increases with decreasing pH in soil (Greger, 1999). The presence of colloids from which there is a release of metals at low pH, increases the metal concentration in pore water and thus also in the roots (Greger, 1999). For instance, acid rain and the resulting acidification of soils and surface waters are known to increase the geochemical mobility of Cd (Campbell, 2006). Cadmium uptake also appears to be decreased in the presence of dissolved organic matter because ligands on the organic matter effectively bind Cd ions (He and Singh, 1993; Prasad, 1995). Chloride levels would also be expected to affect Cd availability as soil sodium chloride has an antagonistic effect on metal toxicity (Bhartia and Singh, 1994).

In the present dissertation hydroponic culture of plants was chosen as the most suitable culturing method because it avoids taking in account the above factors that can alter bioavailability of Cd in soils for plant uptake. Hence, hydroponics provides the most consistent and reproducible levels of contamination required for the present objectives. In addition, a previous study demonstrated that >90% of the Cd remained in solution in the Hoagland's nutrient solution used in almost all experiments in this work, and was therefore available for uptake (Mann et al., 2005).

Cadmium is believed to enter the root through the cortical tissue till the stele either by apoplastic and/or a symplastic pathway (Sanitá di Toppi and Gabbrielli, 1999). The apoplast continuum of the root epidermis and cortex is readily permeable to solutes. The cell walls of the endodermal cell layer act as a barrier for apoplastic diffusion into the vascular system. In general, solutes have to be taken up into the root symplasm before they can enter the xylem (McLaughlin, 2002). The cell membrane plays a key role in metal homeostasis, preventing or reducing entry into the cell. However, examples of exclusion or reduced uptake mechanisms in higher plants are limited (Benavides et al., 2005). The mechanism for metal transport across the plasma membrane to the stele still not completely understood (McLaughlin, 2002). For all cationic metals, such as Cd, the main route for uptake across the plasma membrane is the large negative electrochemical potential produced as a result of the membrane H^+ translocating adenosine triphospatase (ATPases) (McLaughlin, 2002). Costa and Morel (1994) reported that in lettuce grown in hydroponic solution with Cd concentrations from 0.05 µM to 5 µM, high amounts of Cd in roots were correlated with high contributions from H^+-ATPase in the active process of Cd uptake. Other authors contend however, that the main route for uptake of divalent metals is via ion channels, such as Cd^{2+} and Mg^{2+} channels (McLaughlin, 2002 and references therein). Subsequent to metal uptake into the root symplasm, three processes govern the movement of metals from the root into the xylem: sequestration of metals inside root cells, symplastic transport into the stele and release into the xylem (Clemens et al., 2002).

During their transport through the plant, metals become bound to cell walls, which can explain why normally Cd^{2+} ions are mainly retained in the roots, and only small amounts are translocated to the shoots (Cataldo et al., 1983; Greger, 1999). But once loaded in the xylem sap, Cd is translocated to the aerial parts of plants through the transpiration stream, where they might be present as a divalent ion (Greger, 1999) or complexed by several ligands, such as amino acids, organic acids and/or, perhaps, phytochelatins (Salt et al., 1995; Briat and Lebrun, 1999; Sanitá di Toppi and Gabbrielli, 1999; Gong et al., 2003).

Phytotoxicity effects of Cd as environmental markers of Cd stress

General effects

Plants can play a crucial role in the monitoring and assessment of environmental metal pollution. Plants respond to metal accumulation by expressing various

manifestations of toxicity that can be detected and analyzed at various levels of organization ranging from gross morphology to cellular, biochemical or molecular levels, and thus can be useful to monitor as well as assess environmental metal pollution. Moreover, the sedentary nature of plants is a major advantage of plant-based assays for monitoring toxic chemicals in the environment.

Cadmium is a toxic element without any known physiological function in plants that can affect plants on various organizational and functional levels. Several symptoms of Cd stress have been described in plants and they include chlorosis, necrotic lesions, wilting, reddish coloration and growth reduction (Prasad, 1995; Hagemeyer, 1999; Sanitá di Toppi and Gabbrielli, 1999). Disturbances in plant water relations are widely known as one of the first effects of Cd toxicity. Indeed, some authors have proposed that water stress caused by Cd is the beginning of the cascade of physiological and metabolic processes, including photosynthesis impairment (Barceló and Poschenrieder, 1990).

The photosynthetic apparatus is particularly susceptible to Cd toxicity. Photosynthesis can be inhibited at several levels: CO_2-fixation, stomatal conductance, chlorophyll synthesis, electron transport and enzymes of the Calvin cycle (Mysliwa-Kurdziel and Strzalka, 2002). One of the most usual symptoms of Cd stress is chlorosis of the leaves due to an impairment of photosynthetic pigment biosynthetic pathways (Mysliwa-Kurdziel and Strzalka, 2002), but also to a strong interaction between Cd and Fe that reduces uptake of Fe and causes Fe deficiency in leaves (Krupa et al., 2002). Cadmium can alter both chlorophyll biosynthesis by inhibiting protochlorophyllide reductase and the photosynthetic electron transport by inhibiting the water-splitting enzyme located at the oxidising site of photosystem II (Mysliwa-Kurdziel et al., 2002; Mysliwa-Kurdziel and Strzalka, 2002). Moreover, Cd^{2+}, like other metals, can interfere with photosynthetic pigments through the substitution of the Mg^{2+} ion in the chlorophyll molecules by Cd^{2+} (Mysliwa-Kurdziel and Strzalka, 2002). These substituted chlorophylls have much lower fluorescence quantum yields when compared to Mg-chlorophylls (Krupa et al., 2002). Several authors have reported decreased levels of chlorophyll pigments in different plant species due to Cd stress (e.g. Krupa and Moniak, 1998; Lagriffoul et al., 1998; Láng et al., 1998; Chugh and Sawhney, 1999). Since the chlorophyll concentration may directly influence the functioning of the photosynthetic apparatus and thus affect overall plant metabolism, it is considered a key factor when assessing the impact of Cd stress (Fodor, 2002). The ratio Chl *a*/Chl *b* is another related endpoint relevant for Cd toxicity assessment. Although, there is no known direct influence of metal ions on the process of transformation of Chl *a* to Chl *b*, changes on Chl *a*/Chl *b* ratio are commonly

reported on metal stressed plants (Mysliwa-Kurdziel and Strzalka, 2002). Both increases and decreases in this ratio have been found in plants treated with Cd^{2+} (Mysliwa-Kurdziel and Strzalka, 2002 and references therein).

The reduction in photosynthetic rate is a common response in plants exposed to several metals (Mysliwa-Kurdziel and Strzalka, 2002). Profound anatomical changes in leaves and structural disorganization of chloroplasts are the basis of the inhibition of photosynthesis (Mysliwa-Kurdziel et al., 2002). The maximum photochemical efficiency of photosystem II (PSII) was found to be reduced in different plant species exposed to Cd (Chugh and Sawhney, 1999; Linger et al., 2005; He et al., 2008). Dark-adapted values of F_v/F_m (F_v, variable fluorescence; F_m, maximal fluorescence induction) reflect the potential quantitative efficiency of PSII and are used as a sensitive indicator of plant photosynthetic performance (Maxwell and Johnson, 2000).

Another unfavourable effects of toxic metals on plants are the inhibition of the normal uptake and utilization of mineral nutrients (Fodor, 2002). One of the crucial factors of Cd^{2+} influence on plant metabolism and physiological processes is its relationship with other mineral nutrients. As mentioned above, Cd^{2+} transport across cell membranes is most likely facilitated by metal transporters that normally act to mobilize essential metals. Thus, by substituting for essential divalent cations, Cd^{2+} limits their uptake. Alternatively, Cd^{2+} may bind to specific groups of proteins and lipids or channel proteins of membranes, thereby inhibiting transport and disturbing the uptake of many macro and micronutrients. Furthermore, destruction of the cell membranes can also alter the ratio of essential elements and cause the decrease in their content, thereby inducing nutrient deficiencies (Cseh, 2002).

One of the most important mechanisms for impairment of the uptake of nutrients by Cd is via the inhibition of Fe transport into the shoot, which has a pronounced effect on many aspects of the structure and function of the photosynthetic apparatus (Krupa et al., 2002). The induced iron shoot deficiency reduces the pool of Fe-containing electron carriers in the photosynthetic electron transport chain, causes disorganization of the chloroplast structure and even reduces RuBisCO (ribulose 1,5-bisphosphate carboxylase/oxygenase) content (Siedlecka and Krupa, 1996). Cadmium is also known to cause other important disturbances in nutrient levels that can severely affect normal plant metabolism. Specifically it can decrease the levels of Mg, K, P, Ca and Zn, and increase Mn content (Krupa et al., 2002).

Oxidative stress

A common consequence of most abiotic and biotic stresses is that they result, at some stage of exposure, in an increase in reactive oxygen species (ROS) (Mittler, 2002). The ROS intermediates are partially reduced forms of atmospheric oxygen (O_2); they typically result from the excitation of O_2 to form a singlet ($^1O_2$), or from the transfer of one, two or three electrons to O_2 to form, respectively, a superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) or a hydroxyl radical (HO^-) (Mittler, 2002). Metals have been demonstrated to stimulate the formation of ROS, either by direct electron transfer involving metal cations, or as a consequence of metal-mediated inhibition of metabolic reactions (Dietz et al., 1999).

Although Cd is known to produce oxidative stress, in contrast with other metals, it does not seem to act directly on the production of ROS (via Fenton and/or Haber Weiss reactions) (Dietz et al., 1999). Metals without redox capacity, such as Cd can enhance the pro-oxidant status of a plant by reducing the antioxidant glutathione (GSH) pool, activating calcium-dependent systems and affecting Fe-mediated processes (Dietz et al., 1999). These metals can also disrupt the photosynthetic electron chain, leading to the production of ROS (O_2^- and $^1O_2$) (Dietz et al., 1999).

The ROS are generated in plant cells during normal metabolic processes, such as respiration and photosynthesis (Mittler, 2002). Although some of them function as important signalling molecules that alter gene expression and modulate the activity of specific defence proteins, all ROS can be extremely harmful to organisms at high concentrations (Apel and Hirt, 2004). Reactive oxygen species may lead to the oxidation of proteins, lipids and nucleic acids (this particular aspect is discussed further in section 3.3), often leading to lipid peroxidation, membrane damage, mutagenesis and inactivation of enzymes, thus affecting cell viability (Apel and Hirt, 2004). As a consequence, tissues injured by oxidative stress generally contain increased concentrations of carbonylated proteins and malondialdehyde (MDA) (Apel and Hirt, 2004).

The balance between the steady-state levels of different ROS are determined by the interplay between different ROS-producing and ROS-scavenging mechanisms (Mittler, 2002; Apel and Hirt, 2004). One of the plant responses to ROS production is the increase in anti-oxidant enzyme activities providing protection from oxidative damage induced by several environmental stresses (Apel and Hirt, 2004). A variety of proteins function as scavengers of superoxide and hydrogen peroxide. Among the major ROS-scavenging enzymes in plants are catalase (CAT), peroxidase (POX) and superoxide dismutase

(SOD) (Mittler, 2002). The superoxide released by processes such as oxidative phosphorylation is first converted to hydrogen peroxide and then further reduced to give water. This detoxification pathway is the result of multiple enzymes, with superoxide dismutases catalysing the first step and then catalases and various peroxidases removing hydrogen peroxide (see 1.2). In addition, the anti-oxidative enzymes are supplemented with non-protein scavengers, including ascorbate and glutathione (Mittler, 2002).

$$O_2 \longrightarrow \cdot O_2^- \xrightarrow{\textbf{Superoxide dismutase}} H_2O_2 \xrightarrow{\textbf{Peroxidases Catalase}} H_2O$$

Oxygen   Superoxide   Hydrogen peroxide   Water
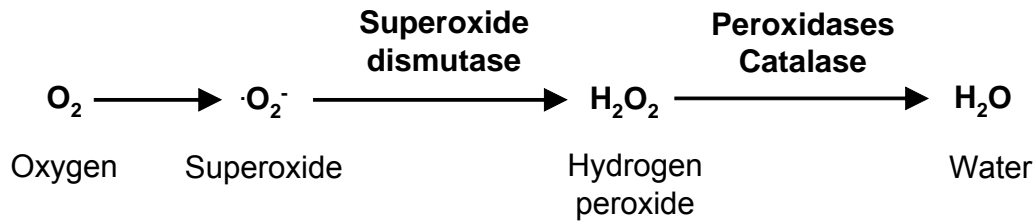
Figure 1.2 - Enzymatic pathway involving anti-oxidant enzymes for detoxification of ROS (adapted from Apel and Hirt (2004)).

As for other stresses, activation or inhibition of anti-oxidative enzymes due to metal stress depends not only on stress intensity and duration but also on the tissue type and the age of the plant (Dietz et al., 1999). Cadmium can inhibit and/or stimulate the activity of several anti-oxidative enzymes (Dietz et al., 1999). Several studies demonstrated that Cd stress induced antioxidant enzymes, whereas some others showed that exposure to high concentrations of Cd resulted in a decrease in antioxidant capacities (Schützendübel and Polle, 2002 and references therein). For instance, catalase activity has been shown to be suppressed in diverse plant species exposed to Cd (Chaoui et al., 1997; Chaoui and El Ferjani, 2005).

Finally, available data suggest that Cd, when not detoxified rapidly enough, may trigger, via the disturbance of the redox control of the cell, a sequence of reactions leading to growth inhibition, stimulation of secondary metabolism, lignification, and subsequent cell death (Schützendübel and Polle, 2002). Thus, in the present dissertation the antioxidant capacities of the plant were chosen as an appropriate endpoint for the assessment of Cd stress in plants.

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Genotoxicity effects of Cd in plants

In addition to the various biological effects referred above, Cd exposure may induce genotoxicity in plants; i.e. like other metals, Cd can damage the genome or DNA of plants (Panda and Panda, 2002). There are different types of genotoxic effects:

mutagenesis, which is a permanent change in DNA sequence within a gene; clastogenesis that refers to a damage in chromosome structure, usually resulting in a gain, loss or rearrangement of chromosome pieces within the genome; aneugenesis, which refers to the gain or loss of one or more chromosomes (aneuploidy) or to a complete haploid set of chromosomes (euploidy) (Panda and Panda, 2002).

The genotoxic effects of Cd have been extensively studied in mammals and particularly in humans. Cadmium and its compounds were classified as Category 1 human carcinogens by the International Agency for Research on Cancer (IARC, 1993); exposure to this metal has been linked to several types of cancer, such as lung, prostate and renal cancer, and has been shown to induce tumours in experimental animals and exposed human cell lines (Waalkes, 2003) and to induce large deletion mutations in mammalian cells (Filipic et al., 2006). However, the molecular mechanisms underlying the genotoxic and carcinogenic potential in organisms are still not well understood. Two models are currently favoured (Figure 1.3).
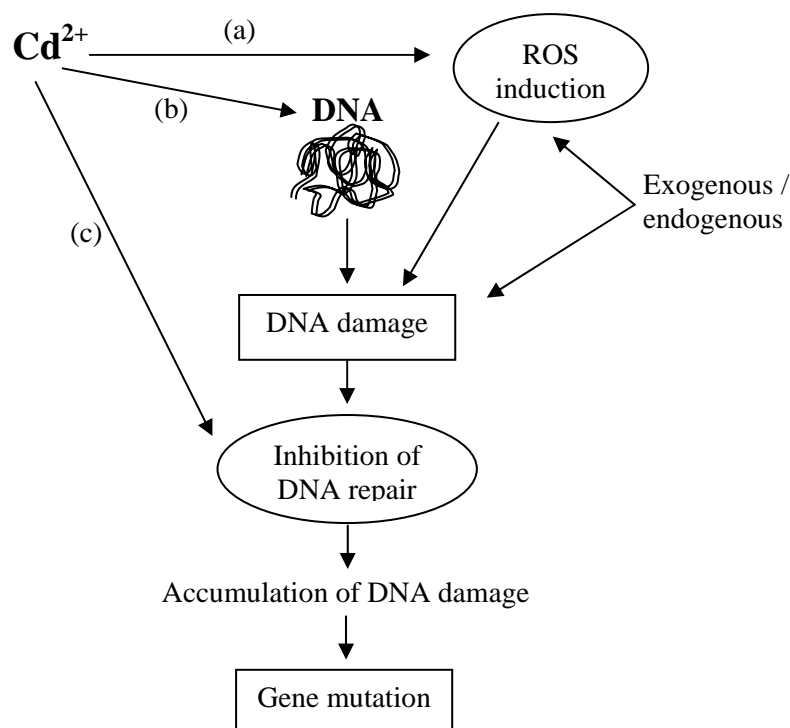
Figure 1.3 - Proposed mechanisms of Cd induced mutagenesis (adapted from Filipic et al., 2006). Cadmium can initiate genotoxicity: (a) by induction of intracellular ROS formation, which would directly produce critical mutations, (b) by direct binding of Cd^{2+} to DNA, possibly at guanine, adenine and thymine centres thereby damaging DNA (Hossain and Huq, 2002) or (c) by interference with DNA repair mechanisms, which would increase the level of spontaneous and/or exogenously produced mutations.

According to one, Cd may interfere with DNA repair (Hartwig, 1994) acting as a mutagen by direct inhibition of an essential DNA mismatch repair, resulting in a high level of genetic instability (Hartwig, 1994; Jin et al., 2003; Slebos et al., 2006). Alternatively, genotoxicity may be induced indirectly by promoting the production of reactive oxygen species (ROS e.g. O_2^{2-}, H_2O_2 and OH^-), which may then damage nucleic acids (Hartwig, 1994; Valverde et al., 2001; Apel and Hirt, 2004). Furthermore other authors have also shown that Cd^{2+} can directly damage DNA, binding to DNA, possibly at guanine, adenine and thymine centres (Hossain and Huq, 2002) (see Figure 1.3).

As in animals, continuous exposure to Cd might then significantly contribute to the inherited change of many phenotypic traits in the progeny of exposed plants. Thus, evaluation of the mutagenicity and/or induced genetic instability in plants by this metal is of the utmost importance in environmental studies. Furthermore, plants have been shown to provide ideal models for genotoxicity assays for screening as well as monitoring of environmental mutagens or genotoxins (Grant, 1994; Knasmuller et al., 1998; Grant, 1999). Several different techniques have been used in plant bioassays for the detection of environmental metal pollution, such as micronucleus (MCN) induction, chromosome aberration, comet assay, sister chromatid exchange (SCE), random-amplified polymorphic DNAs (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence repeats or microsatellite markers (SSRs). A brief compilation of relevant works and their main achievements in the study of Cd genotoxicity in plants is presented in Table 1.

Some of these plant assays, such as the *Allium cepa* chromosome aberration, micronucleus tests and the *Tradescantia* tests have relatively low sensitivity and they cannot provide information on the effects of toxicity at the DNA level (Panda and Panda, 2002).

Chapters 3.1 and 3.2 present an evaluation of Cd genotoxic effects on plants using simple sequence repeats or microsatellite markers (SSRs) to assess genetic instability. Microsatellite markers are tandem repeats of DNA sequences of 1-6 base pair (bp) long units spread throughout the genome. These markers have a high abundance, random occurrence and are highly polymorphic, and thus extremely useful for fine-scale genetic analysis (Gupta et al., 1996; Tóth et al., 2000); they can be used in the detection of genomic DNA damage and/or mutational events (e.g. deletions, insertions, point mutations) (Tóth et al., 2000). Microsatellite markers are likely to be one of the most reproducible techniques, especially when compared to RAPDs, which has the main disadvantage of low reproducibility with a consequent inconsistency of results (Powell et al., 1996; Jones et al., 1997).

Table 1 - A brief review of genotoxicity studies in Cd exposed plants.

Species studied	Method used	Genotoxicity effect (organ)	References
Allium cepa *Vicia faba* and *Tradescantia*	Micronucleus	Yes (root tips and pollen mother cells)	Steinkellner et al. (1998)
Bacopa monnieri	Comet assay	Yes (+ roots and - leaves)	Vaipayee et al. (2006)
Allium cepa	Chromosome aberrations	Yes (root)	Borboa and de La Torre (1996)
Helianthus annuus	SSRs[3]	No (root and leaves)	Gomes et al. (2005)
Helianthus annuus	Flow cytometry	No (roots and leaves)	Azevedo (pers. comm.)
Hordeum vulgare	RAPD[1]	Yes (root tips)	Liu et al. (2005)
Nicotiana tabacum	Comet assay	Yes (roots); No (leaves)	Gichner et al. (2004)
Oryza sativa	AFLP[2]	Yes (roots)	Aina et al. (2007)
Oryza sativa	RAPD[1]	Yes (root tips)	Liu et al. (2007)
Phaseolus vulgaris	RAPD[1]	Yes (seedlings)	Enan (2006)
Pisum sativum	Flow cytometry	Yes (roots)	Fusconi et al (2006)
Thlaspi caerulescens and *Thlaspi arvense*	SSRs[3]	No (roots)	Paiva (2008)
Vicia faba	Micronucleus	Yes (root tips)	Beraud et al. (2007)
Vicia faba	Comet assay	Yes (leaves)	Lin et al. (2007)

[1]RAPD - Random Amplified Polymorphic DNA.
[2]AFLP - Amplified Fragment Length Polymorphism.
[3]SSRs - Microsatellites or Simple Sequence Repeats.

Because of these advantages, SSRs have already been used to study genotoxic effects in several animal species (e.g. Zienolddiny et al., 2000; Jin et al., 2003; Ohshima, 2003; Slebos et al., 2006). For instance, some metals have been found to induce microsatellite instability (MSI). Nickel (Ni) has been reported to promote genetic instability in hamster (Ohshima, 2003) and human (Zienolddiny et al., 2000) cell lines, and exposure of human cell lines to environmentally relevant quantities of Cd led to statistically significant increases in MSI (Jin et al., 2003; Slebos et al., 2006). In plant research, SSRs are already a powerful tool in taxonomy (e.g. Prasad et al., 2000) genetic mapping (Gupta and Varshney, 2000; Ma et al., 2004) and environmental population genetics focusing on the relationships between environmental selective agents (stressors) and genotypic variability of plant natural populations (D'Surney et al., 2001; Mengoni et al., 2001; van Rossum et al., 2004; Berckmoes et al., 2005). Furthermore, SSRs have the potential to be used in the surveying of plant genomic DNA for evidence of genetic instability as in a

genotoxic bioassay for the detection of DNA damage induced by environmental contaminants. However, apart from a survey performed by Kovalchuck et al. (2000) using SSRs to monitor germline mutations in plants upon chronic exposure to ionizing radiation produced by the Chernobyl accident, the application of SSRs in higher plant bioassays remains unexplored. Therefore, in Chapters 3.1 and 3.2 these molecular markers were applied as a technique for the assessment of genetic instability at the level of DNA in plants exposed *in vivo* to Cd.

Conventional cytogenetic studies, such as chromosome aberration and micronucleus tests are very elaborate and time consuming. Flow cytometry (FCM) is largely used in health and biological research for many different purposes and appeared as a relatively rapid test applicable to any organism or tissue from which cellular or nuclear suspensions can be obtained. A FCM assay has been developed to detect the changes in nuclear DNA that result from the breakage of chromosomes providing a quantitative measurement of genetic damage at the cellular level (Otto and Oldiges, 1980). This technique has the potential to detect minute differences in nuclear DNA (nDNA) content and chromosomal damage produced by clastogenic agents through the quantification of the increase of the coefficient of variation (CV) of the G_0/G_1 peak (Otto and Oldiges, 1980). Flow cytometry measurement of the dispersion in the nDNA content as induced by the interactions of DNA with environmental agents, emerged then as a powerful tool in cytogenetic investigations and in genotoxicity testing (Otto et al., 1981). This technique has subsequently been successfully employed in both laboratory and field studies with several animal species (e.g. Otto et al., 1981; Bickham et al., 1998; Matson et al., 2005; Oliveira et al., 2006; Barbee et al., 2008). However, the use of FCM as an assay for the assessment of genotoxicity in plants remains much less common; it has been used to detect genotoxic effects in maize plants exposed to coal fly ash (McMurphy and Rayburn, 1993) and to the fungicides captan (Rayburn et al., 1993) and triticonazole (Biradar et al., 1994).

More recently, FCM has been used to assess metal genotoxicity in plants; Rayburn and Wetzel (2002) found an increase in the CV values of the G_0/G_1 peak in maize and wheat plants grown in soil with high levels of aluminium, and Citterio et al. (2002) reported that the exposure of *Trifolium repens* to Cd and Cr resulted in a decrease in the DNA index with increasing concentrations of Cr, and to an increase of debris background at the highest concentrations of Cd and Cr. Preliminary FCM assays performed in our laboratory revealed no genotoxic effects in lettuce plants exposed to Cd; no changes in nDNA content and in CV values were detected neither in five-week-old

lettuce plants exposed to 100 µM Cd for 14 days (Monteiro et al., 2004) nor in lettuce plants germinated and grown for 2 months in 10 µM Cd and analysed every 15 days (Monteiro et al., 2005). In Chapter 3.3 a FCM assay is used to detect putative genotoxic effects (e.g. clastogenic effects) in three plant species germinated and exposed to Cd for 28 days to increasing concentrations of Cd.

Mechanisms of tolerance

Metal-binding ligands

Plants, like all living organisms, have evolved a suite of mechanisms that control and respond to the uptake and accumulation of both essential and nonessential metals. These mechanisms include the chelation and sequestration of heavy metals by particular ligands and, in some cases, the subsequent compartmentalization of the ligand-metal complex in vacuoles.

The vacuole of plant cells plays an important role in the homeostasis of the cell (Barkla and Pantoja, 1996). In most plant cells the vacuole comprises more than 80-90% of the cell volume and acts as a central storage compartment for ions, amino acids, sugars and CO_2 in the form of malate and also play a key role in the sequestration of toxic ions and xenobiotics (Barkla and Pantoja, 1996; Briat and Lebrun, 1999). The vacuolar membrane, named tonoplast, functions as an effective and selective metal diffusion barrier (Briat and Lebrun, 1999). Vacuolar compartmentalization prevents the free circulation of Cd ions in the cytosol and forces them into a limited area (Sanitá di Toppi and Gabbrielli, 1999). Several studies have shown that the vacuole is the site of accumulation of a number of metals including Cd (Ma et al., 2005; Ueno et al., 2005). One example is the accumulation of Cd and phytochelatins (PCs) in the vacuole involving an ATP-binding cassette (ABC) transporter (Hall, 2002). Oat root tonoplast vesicles were found to accumulate Cd^{2+} by a $2H^+$/ion antiport mechanism (Salt and Wagner, 1993).

Several metal-binding ligands have now been recognized in plants and include organic acids, amino acids, peptides, and polypeptides (Rauser, 1999). Among the metal-binding ligands in plant cells the PCs and metallothioneins (MTs) are the best characterized. MTs are cysteine-rich polypeptides encoded by a family of genes whereas PCs are a family of enzymatically synthesized cysteine-rich peptides (Cobbett and Goldsbrough, 2002).

In plants, PC-Cd complexes are sequestered in the vacuole (Cobbett and Goldsbrough, 2002). In mesophyll protoplasts derived from tobacco plants exposed to Cd, almost all of both the Cd and PCs accumulated was confined to the vacuole (Vogeli-Lange and Wagner, 1990). *Lactuca sativa* and *Thlaspi arvense* plants also possess detoxification mechanisms in which PCs play an important role (Ebbs et al., 2002; Maier et al., 2003). *Thlaspi caerulescens* was found to mainly store Cd^{2+} in electron-dense granules inside vacuoles by means of complexation with malate (Ma et al., 2005; Ueno et al., 2005).

Plant metal accumulation and hyperaccumulation

Plants respond to high concentrations of environmental metals in three main ways: metal excluders maintain low and constant metal concentration in their shoots up to a critical soil value; indicator species have internal metal concentrations that reflect the external metal levels, whereas metal accumulators have high accumulation of metal at very low external metal concentration (Greger, 1999). The term hyperaccumulator describes a plant with a highly abnormal capacity for metal accumulation (Reeves and Baker, 2000). Hyperaccumulator plants are found in metalliferous soils, such as calamine (with high levels of Zn, Pb and Cd) and serpentine soils (with high levels of Ni, Cr and Co) (Greger, 1999).

Although Cd is not an essential or beneficial element for plants, they generally exhibit measurable Cd concentrations, particularly in roots, but also in leaves, most probably as a result of inadvertent uptake and translocation (Assunção et al., 2003). A Cd foliar concentration above 100 µg/g DW (0.01%) is considered exceptional and it is used as a threshold value for Cd hyperaccumulation (100 mg/Kg DW) (Reeves and Baker, 2000). The metal hyperaccumulation characteristic is not common in higher terrestrial plants and less than 0.2% of all angiosperms have been identified as metal hyperaccumulators (Reeves and Baker, 2000). The Brassicaceae plant family is well represented among the reported hyperaccumulators. *Thlaspi caerulescens* is the best known hyperaccumulator plant with a capacity to hyperaccumulate Zn, Cd and Ni (Assunção et al., 2003). *Thlaspi caerulescens* plants have been found by Reeves and Baker (2000) to contain more than 100 mg/Kg Cd frequently, and more than 1000 mg/Kg Cd occasionally, with very large variations between sites and populations, and considerable intrasite variability. Several studies have shown that *T. caerulescens* ecotype from metalliferous soils of a Zn/Pb mine spoil in the southern France (Ganges

ecotype) is far superior in Cd accumulation to other ecotypes (e.g. Prayon from Belgium); in hydroponic conditions it was able to accumulate >10,000 mg/kg Cd in the shoots without showing any symptoms of phytotoxicity (Lombi et al., 2000).

Three plants with different patterns of Cd accumulation were the object of study in the present dissertation: lettuce (*Lactuca sativa* L.) is a Cd-accumulating plant and an important human food crop; the alpine pennycress (*Thlaspi caerulescens* J. & C. Presl, Ganges ecotype), which is a hyperaccumulator plant commonly used as a model in metal transport and accumulation studies with a view to their use in phytoremediation (Pence et al., 2000; Assunção et al., 2003; Zhao et al., 2003); and the related non-accumulator, field pennycress (*Thlaspi arvense* L).

Trophic transfer of Cd

A key pathway for metal exposure to animal species, including humans results from the uptake by plants of elements from the soil. However, the study of the trophic transfer of metals from plants to animals is a largely unexplored field. As indicated above, plants have developed mechanisms for sequestering metals in their systems in such a way that the metal is not phytotoxic, but these plants may still pose a threat to the animals that consume them, becoming a risk to ecological and human food chains (McLaughlin, 2002).

Factors affecting trophic transfer of metals

The bioaccumulation of metals is known to differ among species and metals because of differences in uptake and loss rates, exposure pathways and influences of environmental parameters (Fisher and Reinfelder, 1995; Wang and Fisher, 1999). However, less is known about the influence of these factors in the internal storage and detoxification of accumulated metal and subsequent impacts on trophic transfer. Since the ingestion of metal-contaminated food can serve as a source of metals to consumers and can result in sub-lethal toxicity (e.g. Fisher and Hook, 2002), understanding the mechanisms that influence metal trophic transfer is a critical step in the management of metal contaminated ecosystems. In general, to completely understand metal cycling through trophic levels, several factors which control the bioavailability of tissue-bound metals to predators must be considered and understood (e.g. tissue metal distributions and concentrations, duration of exposure, nutritional status and exposure history of

predator). Different species will accumulate and partition metals in varying ways depending on the detoxification mechanisms employed. The subsequent bioavailability of those partitioned metals to a consumer will be dictated by digestive and assimilative mechanisms of its digestive tract and gut passage time (Wang and Fisher, 1999). Added to this complexity is the varying ability of consumers to discriminate between different foods and contaminants, their nutritional status at the time of consumption, the degree of exposure, and the exposure history for the metal in question, all of which can influence the degree of metal assimilation (Wang and Fisher, 1999).

Metal assimilation and assimilation efficiency

One critical parameter in understanding the trophic transfer and accumulation of a metal is its assimilation efficiency (AE) in animals from the ingested food (Wang and Fisher, 1999). Assimilation efficiency has been defined as the fraction of ingested metal that is assimilated across the gut lining into the body tissue (Wang and Fisher, 1999). Assimilation efficiency measurements are difficult to make and often yield variable results (Fisher and Reinfelder, 1995); they can be determined by the mass balance method in which ingested and egested masses are compared to each other or to the mass retained in the animal after an appropriate gut clearance period (Fisher and Reinfelder, 1995).

Determination of AEs is an important endpoint when addressing contaminant bioavailability, it is considered a first-order physiological parameter that can be quantitatively compared among different chemicals, species, and food particles under various environmental conditions (Wang and Fisher, 1999). Furthermore, AE for metals has been shown to be directly proportional to metal bioaccumulation, which highlights the significance of AE in understanding and predicting metal bioaccumulation (Fisher et al., 1996).

The various factors that affect metal assimilation are reflected in the wide variety of Cd AEs that have been reported in organisms of different food chains fed biologically contaminated food. For instance AEs ranging from 1% have been reported in rats fed snail viscera (Hispard et al., 2008), to 4.7% in the lizard *Podarcis carbonelli* fed crickets (Mann et al., 2006), to 52% in the isopod *P. dilatatus* fed lettuce (Calhôa et al., 2006), and up to 76.2 to 94.2% for whelk *Thais clavigera* fed five different species of prey (Cheung and Wang, 2005).

Subcellular partition of metals

The internal distribution and detoxification of metals within an organism can be used to explain trophic transfer of metals but also to predict metal toxicity for the organism itself. The internal metal sequestration strategies of different species are complex and variable and the determination of the metal concentrations in different compartments can be used to understand the complex relationship between metal accumulation and toxicity.

Over the past decades, chemistry-orientated models have been developed to predict the bioavailability and toxicity of metals focusing on identifying which metal forms are present in the aquatic environment, and investigating their interaction with the biological site of action (Paquin et al., 2002). The free ion activity model (FIAM) relied on the free metal ion activity and assumed that uptake from solution was determined by the availability of free metal ions, whereas the biotic ligand model (BLM) which is an extension of the FIAM, assumes that the effect is proportional to the concentration of metal bound to the target site (biotic ligand) and that this site is in direct contact with the external environment. These models perform well in the prediction of metal bioavailability in water-borne exposures of aquatic organisms, but also for plants (Antunes et al., 2006) and soft-bodied organisms (Peijnenburg, 2002). When considering the contribution of the dietary route of metal exposure the gut/instestine can also act as a biotic ligand (Hogstrand et al., 2002) and metal speciation and/or dietary form is likely to be an important factor for metal assimilation.

Metals can be present in various chemical forms in an organism, including the following: (a) free ionic form or complexed ion species (e.g., $CdCl_2$, $CdCl^+$, $CdCl_3^-$); (b) bound in the active center of functional proteins and enzymes; (c) bound to low molecular weight organic acids (e.g., citrate, malate); (d) bound to sequestration proteins (MTs and PCs); (e) bound in vesicles of the lysosomal system, as intracellular granules; (f) precipitated in extracellular granules, mineral deposits, residual bodies, and exoskeletons; (g) bound to cellular constituents potentially causing dysfunction (e.g. DNA) (Vijver et al., 2004).

The various internal metal fractions all have their own binding capacity for metals, which has implications for food-chain transfer to higher trophic levels. A study on the relationship between subcellular Cd distribution in an oligochaete and its trophic transfer to a predatory shrimp showed that only metal present in the soluble fraction (organelles and protein fraction)) of prey is available for the predator (Wallace et al., 1998). Factors influencing the subcellular distribution in the prey will directly alter trophic transfer to

predators. Wallace et al. (1998) showed that differences in subcellular distribution of Cd between resistant and nonresistant worms directly affected Cd availability for the predatory shrimp. When fed resistant worms, shrimp absorbed about 4 times less Cd than when fed non-resistant worms (Wallace et al., 1998). Similar conclusions were found in a study using bivalves as prey, where the metal partitioning to organelles, denaturated proteins, and MTs comprise a subcellular compartment of that was considered as trophically available metal (TAM) to predators (see Figure 1.4).

A subcellular fractionation procedure (Wallace et al., 2003; Wallace and Luoma, 2003) has been successfully applied in several studies of dietary accumulation of metals, particularly in marine food chains, with the purpose of explaining the variability observed in metal accumulation across the different species and food chains. This method has been considered by other authors to be a simple and pragmatic approach in the prediction of trophic transfer of metals and a first step towards a practical tool that could explain most of the variability observed in metal accumulation in organisms (Vijver et al., 2004).

Wallace and Luoma (2003) building on previous studies (Wallace and Lopez, 1997; Wallace et al., 1998), postulated that Cd associated with the subcellular fractions, organelles, heat-denatured proteins (HDP), and heat-stable proteins (HSP) of prey was TAM (Figure 1.4) and was assimilated at an efficiency of approximately 100% by the predator, while Cd bound to metal-rich granules was less bioavailable to predators.
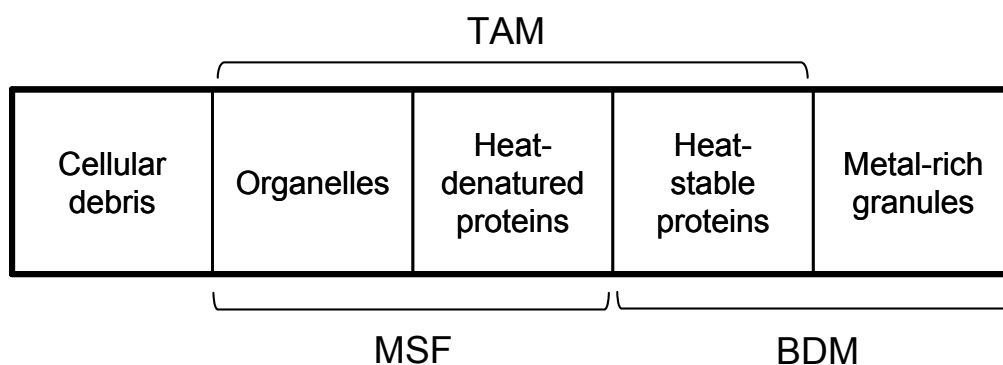
Figure 1.4 - The five subcellular fractions of metals identified in aquatic organisms and their biological significance as attributed by Wallace et al. (2003): TAM – trophically available metal; MSF – metal sensitive fraction; and BDM – Biologically detoxified metal.

Using this procedure the accumulated metals associated with different subcellular compartments were separated into five different fractions by differential centrifugation: cellular debris, granules, organelles, heat-denatured proteins, heat-stable proteins (MTs and PCs). Such subcellular partitioning is dynamic in response to metal exposure and other environmental conditions, and is metal- and organism-specific. The different metal

pools are not equally bioavailable to predators; thus, the determination of the metal concentration in the different subcellular compartments and the differences in its assimilation by consumers can be a useful tool to understand metal transfer to higher trophic levels. In chapter 4 of the present dissertation, a similar procedure of subcellular fractionation to the one developed by Wallace and co-workers (Wallace et al., 2003; Wallace and Luoma, 2003) was adopted as a tool to explain the variability observed in Cd assimilation by isopods fed plants with different patterns of Cd accumulation.

Other applications of this approach have been proposed. Recent studies in aquatic organisms have revealed that the subcellular partitioning model (SPM) may provide an improved method to predict Cd toxicity. As intracellular metal accumulation and the subsequent subcellular distribution of the metal in the cells are directly related to metal toxicity, it is likely that the metal concentration in a particular subcellular fraction will serve as a better toxicity predictor than the activity of the free metal ion in bulk solution (Wang and Rainbow, 2006). In this approach different combinations of the five subcellular fractions have been proposed (Figure 1.4) to represent a metal-sensitive fraction (organelles and HDP) and a biologically detoxified metal fraction (HSP and granules) (Wallace et al., 2003). Rainbow (2002) proposed that when accumulated metal destined for storage in a detoxified form (e.g. by MTs and granules) exceeds the detoxified binding capacity, the metals are subsequently bound with other (metabolically available) forms, with the potential to cause toxicity to the organism. The significance of the subcellular distribution of accumulated metals in toxicity assessments is now receiving increasing attention among aquatic (e.g. Cheung et al., 2006; Perceval et al., 2006; Steen Redeker et al., 2007) and terrestrial organisms (Vijver et al., 2006; Vijver et al., 2007).

Isopods as model species for metal accumulation

Invertebrates are among the major components of soil biomass and play an important role in maintaining the structure and fertility of the soil. Invertebrate-mediated processes such as drainage, aeration, incorporation and degradation of organic matter are important in improving soil quality. Moreover, invertebrates are an important part of the terrestrial food web and can constitute a significant component of the diet of other animals (Peijnenburg, 2002).

The terrestrial isopod *Porcellio dilatatus* (Crustacea) was chosen as a model species in the present dissertation as it is an important representative of the invertebrate soil fauna and a valuable model for the examination of metal assimilation and

accumulation. This species inhabits the upper layer of the soil and surface leaf litter, is quite abundant in southern Europe and is easy to handle under laboratory conditions. Moreover, isopods are strong bioaccumulators of metals; they have an enormous capacity to accumulate large body burdens of toxic metals, predominantly in the hepatopancreas (Donker et al., 1990; Hopkin, 1990; Hames and Hopkin, 1991) with low to negligible depuration rates (Witzel, 2000). As hard-bodied soil invertebrates, the main route for accumulation of metals in isopods was found to be through dietary exposure rather than absorption through the body wall (Vijver et al., 2005). Feeding on leaf litter of metal accumulator plants is thus a potential route of exposure that can enhance metal accumulation by isopods. The effects of Ni hyperaccumulation in the Brassicaceae endemic to serpentine soils in NE Portugal *Alyssum pintodasilvae* to the *P. dilatatus* was recently studied by Gonçalves et al. (2007). These authors suggested that the effects of hyperaccumulator litter on the activity of this important detritivore species may be significantly impaired with potential consequences on the decomposition processes.

Objectives

Plant uptake of Cd from soils is a constant threat not only for the conservation of vegetal biological resources, but also to animal consumers through trophic transfer. Therefore, the main objective of this work is to study the effects of Cd uptake and accumulation in plants and its implications to animal consumers.

The following specific questions are addressed:

i)   What are the main physiological and genotoxic effects of Cd to plants?

ii) How does Cd subcellular distribution in plants affect assimilation of this metal by an animal consumer?

To answer these main questions, the following steps/specific aims were performed:

1) Examination of uptake and toxicity effects of Cd on plants, addressed in Chapter 2.

2) Evaluation of genotoxic effects on plants addressed in Chapter 3.

3) Determination of subcellular distribution of Cd in plants and evaluation of how Cd subcellular distribution in plants affect transfer to animal consumers, addressed in Chapter 4.

In a final section, Chapter 5, the general discussion and concluding remarks of this study are presented, aiming to resume and present the relevant conclusions of this work.

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Assessment of biomarkers of Cd stress in lettuce

Chapter 2

Assessment of biomarkers of cadmium stress in lettuce

Abstract

Laboratory and field studies have provided encouraging insights into the capacity of plants to act as biomonitors of environmental quality through the use of biomarkers. However, a better understanding of the overall process of Cd-induced senescence, describing the cascade of Cd effects in plants is needed for a selection of relevant biomarkers of Cd stress. In order to approach this, five-week-old *Lactuca sativa* L. were exposed for 14 days to 100 µM $Cd(NO_3)_2$ and harvested at days 0, 1, 3, 7 and 14. The parameters measured included classical endpoints (shoot and root growth) and biochemical endpoints related to photosynthesis, nutrients content and oxidative stress. Cadmium-exposed plants displayed nutrient imbalances in leaves and roots. Photosynthetic efficiency was significantly decreased and lipid peroxidation was enhanced. Antioxidant enzymes were significantly altered during exposure - catalase was inhibited by the end of exposure and peroxidase was induced at day 1 in young leaves. These alterations culminated in a decrease in shoot growth after 14-days exposure to Cd. Biochemical alterations could be used in integrative approaches with classical endpoints in ecotoxicological tests for Cd and after further testing in real scenarios conditions, they could form the basis of a plant biomarkers battery for monitoring and predicting early effects of exposure to Cd.

Keywords: chlorophyll content; *Lactuca sativa*; lipid peroxidation; metal; nutrient imbalances; oxidative stress; PSII efficiency

Introduction

Among those environmental pollutants often referred to as 'heavy metals', cadmium (Cd) is of particular concern because of its mobility in the plant-soil system. Although Cd is naturally present in trace amounts in the environment (0.04 to 0.32 µM) (Sanitá di Toppi and Gabbrielli, 1999), fallout from several industrial activities and the agricultural application of phosphate fertilizers and biosolids have enriched soils with this element (Sanitá di Toppi and Gabbrielli, 1999). For instance, it was reported that the use of irrigation with Cd-contaminated river water (65-240 µg/l) downstream a mining area for irrigation in Bolivia has increased the median soil concentration of Cd to 20 mg/kg and median concentration of Cd in soil solutions to 27 µg/l (Oporto et al., 2007). Cadmium is particularly dangerous because plants growing in contaminated soils can absorb and accumulate Cd in edible tissues in large quantities without any visible signs, thereby introducing the metal into the food (Monteiro et al., 2008), including the human diet (McBride, 2003). A recent field study in Europe (Peris et al., 2007) reported contents of Cd in edible parts of vegetables that were above established thresholds. Understanding Cd uptake and physiological responses of plants is thus critical to the long-term safety and conservation of agricultural resources and ecosystems.

Cadmium is particularly damaging to the photosynthetic apparatus. Inhibition of RuBisCO (ribulose 1,5-bisphosphate carboxylase/oxygenase) activity in the Calvin cycle is considered a primary response to Cd stress (Siedlecka et al., 1997), and levels of total chlorophyll and photosystem II (PSII) maximum photochemical efficiency can be reduced in different plant species (Chugh and Sawhney, 1999; Krupa and Moniak, 1998; Lagriffoul et al., 1998; Linger et al., 2005; Mysliwa-Kurdziel and Strzalka, 2002). In general, metal stress in plants can promote the production of reactive oxygen species (ROS, e.g. O_2^-, H_2O_2 and OH^-) that are naturally formed in plant cells, mainly in chloroplasts, peroxisomes and mitochondria, during regular metabolism. ROS can damage cell components such as proteins, polysaccharides, nucleic acids and cause peroxidation of membrane lipids (Apel and Hirt, 2004). Regulation of ROS in the plant cell is mediated through the activity of the antioxidative system, which includes enzymes such as superoxide dismutase (SOD, E.C. 1.15.1.1), catalase (CAT, E.C. 1.11.1.6) and guaiacol peroxidase (POX, E.C. 1.11.1.7) (Apel and Hirt, 2004; Wójcik et al., 2006). Superoxide dismutase converts the strong oxidant O_2^- radicals into H_2O_2 and the accumulation of H_2O_2 is prevented in the cell by reduction to H_2O through the actions of either CAT or POX (Apel and Hirt, 2004; Wójcik et al., 2006). Exposure to Cd also brings about oxidative stress, but it does not seem to act

directly on the production of ROS. On the other hand, Cd ions can inhibit or stimulate the activity of several antioxidant enzymes (Fodor, 2002; Sanitá di Toppi and Gabbrielli, 1999).

Cadmium transport across cell membranes is most likely facilitated by metal transporters that normally act to mobilise essential metals. Also, by substituting for essential divalent cations, Cd limits their uptake (e.g. Cd inhibits the transport of Fe into the shoot). Alternatively, Cd may bind to specific groups of proteins and lipids or channel proteins of membranes, thereby inhibiting transport and disturbing the uptake of many macro and micronutrients. Destruction of the cell membranes can also alter the ratio of essential elements and cause the decrease in their content, thereby inducing nutrient deficiencies (Cseh, 2002).

As sedentary organisms, higher plants offer unique advantages for *in situ* monitoring and screening for the effects of exposure to soil contaminants and are already recognized as excellent indicators of effects of environmental chemicals. However, recommended tests are only focused on non-specific responses of seedling emergence and plant growth (ISO, 1993; ISO, 1995; OECD, 2006). Furthermore, seed germination was found to be a less sensitive parameter or even insensitive to Cd toxicity and is not considered a good indicator to assess Cd toxicity in soils (An, 2004; da Rosa Correa et al., 2006; Wang and Zhou, 2005).

In plants Cd interferes with photosynthesis, respiration and nitrogen metabolism, and induces oxidative stress, all of which can culminate in poor growth and low biomass production (e.g. Sanitá di Toppi and Gabbrielli, 1999; Fodor, 2002; Azevedo et al., 2005a; Azevedo et al., 2005b, c). The biochemical pathways involved in these processes offer a battery of biochemical biomarkers that not only provide mechanistic endpoints of toxicity, but also improve our understanding of toxic mode of action and exposure assessment. Linking these endpoints with more traditional responses (e.g. growth) will improve risk assessment of the pollutant. Understanding the overall process of Cd-induced senescence, describing the cascade of events and the enzymatic protection strategies that plants can adopt against Cd-induced oxidative stress is then critical for the selection of relevant plant biomarkers for ecological risk assessment.

For this study we examined the uptake and toxicity of Cd in lettuce. Lettuce is a plant recommended for standard toxicity tests (*e.g.* ISO/CD 17126) (ISO, 1995). Also, lettuce is a Cd-accumulating plant, and because it is one of the most consumed leafy vegetables, it is of particular concern in human dietary uptake (Cobb et al., 2000; McBride, 2003). We evaluated plant growth, nutritional imbalance, oxidative stress, chlorophyll

content and fluorescence parameters during 14 days of exposure to Cd. The studies were carried out using hydroponically grown lettuce because under these conditions the entire metal pool was accessible to plants, growth conditions are easier to control and the data obtained is more reproducible than in soils. Results from this study can then give important information for selection of useful plant biomarkers for further studies in real scenarios conditions.

Material and Methods

Plant culture and growth conditions

Lactuca sativa L. (cv Reine de Mai) seeds (Oxadis, France) were germinated and grown as described by Monteiro et al. (2007). Briefly, seeds were germinated on perlite saturated with distilled water. Plants were subsequently grown hydroponically in aerated modified Hoagland's medium at 24 ± 2 °C, under light intensity of 200 μmol/m^2/s and photoperiod of 16 h/8h (light/dark). After 5 weeks of culture, lettuce plants were either exposed to the Hoagland's medium supplemented with 100 μM Cd(NO$_3$)$_2$ or maintained as control plants kept on modified Hoagland's medium without Cd.

The concentration of 100 μM of Cd was chosen based on previous works (Azevedo et al., 2005a; Azevedo et al., 2005b; Monteiro et al., 2007), and in order to achieve twice the maximum permitted in irrigation water by Portuguese legislation (0.05 mg/l). Leaves and roots from control and exposed plants were harvested at 0, 1, 3, 7 and 14 days of Cd exposure for the various parameters analysed. Length of shoots and primary roots of control and exposed lettuce (n=6) was recorded at day 14.

Nutritional status and Cd analysis

Cadmium concentration in the hydroponic culture medium of control and Cd-treated plants (n=3) was verified by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Jobin Yvon, JY70 Plus, Longjumeau Cedex, France). Accumulation of Cd and the content of the macro and micronutrients K, Ca, Na, Mg, Mn, Fe, B, Co, Cu and Zn through the exposure time (0, 7 and 14 days) were determined in leaves (n=6) and roots (n=3) dried to constant weight at 60 °C. Prior to drying, roots were washed for 10 min in 0.5 mM CaSO$_4$ to remove (by cation exchange) Cd adsorbed to the

root surface. Dried tissues were treated according to Evans and Bucking (1976) and then analysed by ICP-AES.

Chlorophyll content and photosystem II efficiency

Chlorophyll content and photosystem II efficiency were determined at day 14 of exposure in young and expanded leaves of lettuce plants (n=6). Chlorophyll *a* and *b* content were determined by the method of Arnon (1949). Young and expanded leaves were collected from six individual plants and ground in 10 ml 80% acetone. After centrifugation (2,800 g, 5 min), the absorbance of the supernatant was measured at 645 and 663 nm and chlorophyll *a* and *b* contents and the ratio Chl *a*/ Chl *b* were estimated.

Fluorescence readings were taken using a Plant Efficiency Analyser (Hansatech Instruments Ltd., UK), in order to determine the efficiency of electron transfer in PSII. Chlorophyll fluorescence was monitored in young and expanded leaves of control (n=6) and exposed plants (n=6), with adaxial surface of leaves facing up. Fluorescence measurements were always made between 10 and 12 a.m., and after dark adaptation of leaves for 30 min to open all reaction centres of PSII. The minimum fluorescence (F_0) was measured by applying a weak pulse of light and the maximal fluorescence induction (F_m), which is observed when all PSII reaction centres are closed, was obtained by illuminating the leaves with a beam of saturating light (3000 µmol/m^2/s) (Maxwell and Johnson, 2000). The variable fluorescence ($F_v = F_m - F_0$) and the maximum quantum yield of PSII (F_v/ F_m), were then estimated.

Lipid peroxidation and membrane permeability

Lipid peroxidation of leaves (n=6) was determined by measuring malondialdehyde (MDA) production (Dhindsa et al., 1981). Tissues samples were homogenized in 0.1% trichloroacetic acid, centrifuged (10,000 g, 10 min) and the supernatants were collected. To 1 ml aliquots of supernatant, 4 ml of a solution of 20% trichloroacetic acid and 0.5% thiobarbituric acid was added; the mixture was heated (95 ºC; 30 min), quickly cooled and then centrifuged (10,000 g, 10 min). Supernatants were used to determine MDA content at 532 nm.

Membrane permeability was determined in lettuce leaves (n=6) as described by Lutts et al. (1996). The leaves were washed with deionized water and immersed in 20 ml of deionized water and incubated overnight (25 ºC, 85 rpm). Electrolyte leakage analysis

was performed by measuring conductivity before (L_t) and after (L_0) autoclaving (10 min, 120 ºC).

Antioxidant enzymes and soluble protein content

Tissue samples of young and expanded leaves (n=3) were homogenized and dialysed as described by Santos et al. (2001). The dialysed samples were used for enzymatic and protein content determinations. Activities of CAT, POX, and SOD were determined according to the methods of Aebi (1974), Takahama and Egashira (1991) and Asada et al. (1975), respectively. One unit of CAT and POX is defined as the number of μmoles of H_2O_2 consumed per minute, and one unit of SOD as the enzyme content which gives 50% inhibition of cytochrome *c* reduction. Soluble proteins were determined with Total Protein Kit (Sigma), according to Bradford method (Bradford, 1976).

Statistical analysis

Data were analysed using *t*-test and when necessary data were transformed to achieve normality and equality of variance. When these criteria were not satisfied even with transformed data, the non-parametric Mann-Whitney rank sum test was performed. When justified, Pearson correlations were performed on data satisfying criteria of normality, otherwise non-parametric Spearman correlations were performed and the respective correlation coefficient are presented as *r* or *rs*, respectively. All statistical analysis was performed using SigmaStat for Windows, version 3.1.

Results

Toxicity symptoms and plant growth

Visible manifestations of Cd toxicity were observed in lettuce mainly after 7 days of exposure. Plants developed toxicity symptoms, especially in expanded (older) leaves which exhibited chlorosis. Brown necrotic lesions appeared both on the leaves and stems of exposed plants. Expanded-leaf fall was observed during the second week of exposure. Roots of Cd-treated plants appeared darker than those of control plants.

Exposure to Cd in the nutrient solution produced growth inhibition in lettuce plants (Fig. 2.1). The growth of lettuce shoots was significantly reduced after 14 days of

exposure ($p<0.05$) when compared to the control. Root growth was also lower in Cd-exposed plants, but difference was not significant.
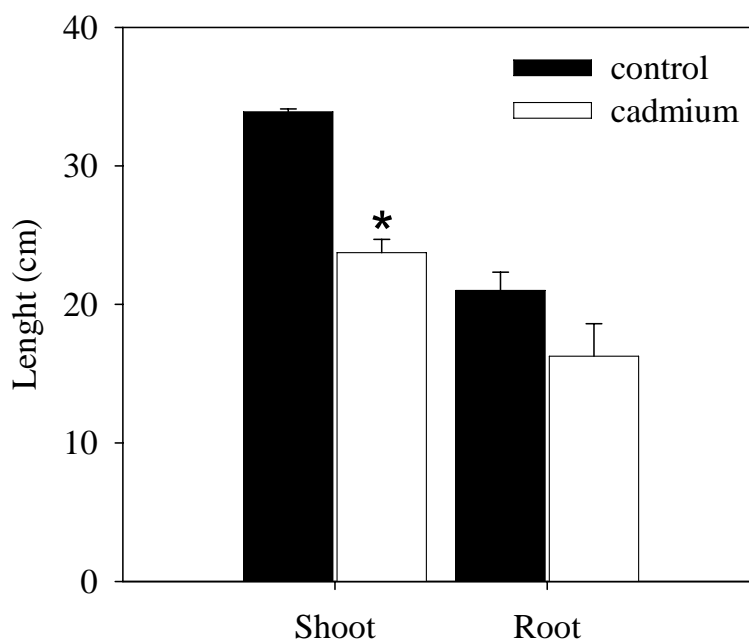

Figure 2.1 – Control and exposed lettuce shoots (a) and roots (b) growth after 14 days of exposure. Results are expressed as mean ± SE of six replicates; (*) significantly different from control at the same day ($p<0.05$).

Cadmium accumulation and nutrient imbalances

Cd concentration was below the ICPS detection limit (<0.01 µM) in the culture medium of control plants and was 104 ± 0.8 µM in the medium with the nominal concentration of 100 µM $Cd(NO_3)_2$. The results of Cd content in lettuce are presented in Figure 2.2. There was an increase with exposure duration in both roots and leaves up to 2.17 ± 0.212 and 0.34 ± 0.062 mg/g DW, respectively. Roots had a higher accumulation of Cd than leaves (8-fold higher at day 14).

Macro and micronutrient imbalances in leaves and roots of lettuce are presented in Table 2.1. At day 14, leaves from Cd-exposed plants displayed significant alterations in nutrients content when compared with control leaves on the same day. With regard to macronutrients, leaves displayed a significant decrease in P ($p<0.05$) and an increase in K ($p<0.01$). Potassium tended to decrease with time in exposed plants, however in control plants this decrease was even more pronounced, which led to the significant difference between control and exposed leaves at day 14. By contrast, at day 7, roots displayed a significant increase in P ($p<0.05$) and a decrease in K ($p<0.05$). Among the micronutrients, Fe and Mn significantly decreased in leaves ($p<0.01$ for Fe and Mn) and B
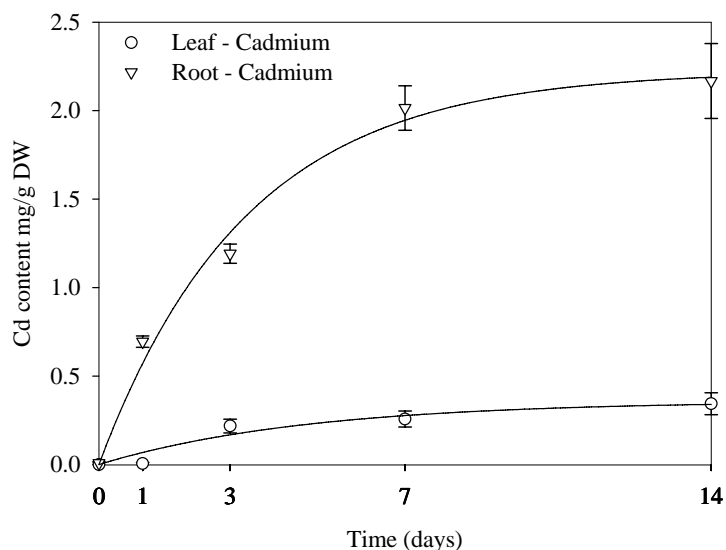
Figure 2.2 – Cadmium content in lettuce roots and leaves of exposed plants during the 14 day exposure. Results are expressed as mean ± SE of three replicates. In control plants Cd levels were below detection limit.

Table 2.1 – Effect of Cd stress on nutrients content in lettuce leaves (a) and roots (b). Results are expressed as mean ± SE (n=5 or 6 for leaves and n=3 in roots); (*) and (**) significantly different from control at the same day $p < 0.05$ and 0.01, respectively.

Element (mg/g DW)	Leaves (a)				
	Day 0	Control - Day 7	Cd - Day 7	Control - Day 14	Cd - Day 14
P	10.3 ± 1.01	7.8 ± 0.55	6.7 ± 0.35	7.6 ± 0.48	5.6 ± 0.54 *
K	68.9 ± 5.66	60.3 ± 6.27	54.5 ± 3.27	43.9 ± 3.43	57.9 ± 2.64 **
Mg	7.9 ± 0.28	7.0 ± 0.70	6.4 ± 0.55	4.6 ± 0.52	5.9 ± 0.47
Na	1.2 ± 0.06	1.3 ± 0.45	2.1 ± 0.49	0.7 ± 0.05	0.8 ± 0.06
Mn	0.3 ± 0.02	0.3 ± 0.03	0.2 ± 0.02	0.2 ± 0.01	0.1 ± 0.01 **
Fe	0.15 ± 0.013	0.13 ± 0.011	0.13 ± 0.021	0.14 ± 0.014	0.07 ± 0.004 **
Zn	0.06 ± 0.004	0.15 ± 0.097	0.06 ± 0.006	0.07 ± 0.013	0.07 ± 0.009
Cu	n.d.	0.11 ± 0.106	0.04 ± 0.015	0.004 ± 0.0030	n.d.
B	0.02 ± 0.010	0.01 ± 0.010	0.03 ± 0.011	n.d.	0.05 ± 0.004 **

Element (mg/g DW)	Roots (b)				
	Day 0	Control - Day 7	Cd - Day 7	Control - Day 14	Cd - Day 14
P	13.5 ± 1.38	11.4 ± 1.03	16.0 ± 0.97 *	14.05 ± 0.70	13.9 ± 0.48
K	64.6 ± 4.06	61.5 ± 4.89	43.6 ± 2.68 *	55.84 ± 8.35	41.0 ± 2.29
Mg	1.4 ± 0.09	1.3 ± 0.05	1.6 ± 0.14	1.12 ± 0.05	1.3 ± 0.09
Na	3.4 ± 0.82	3.1 ± 0.41	3.00 ± 0.42	3.14 ± 0.99	1.2 ± 0.15
Mn	0.9 ± 0.33	0.6 ± 0.11	0.1 ± 0.02 *	0.45 ± 0.13	0.04 ± 0.007 *
Fe	0.38 ± 0.111	0.22 ± 0.033	0.33 ± 0.083	0.18 ± 0.005	0.33 ± 0.061
Zn	0.2 ± 0.03	0.1 ± 0.02	0.1 ± 0.03	0.09 ± 0.01	0.1 ± 0.01
Cu	n.d.	n.d.	0.026 ± 0.0047	0.003 ± 0.0001	0.041 ± 0.0085 *
B	n.d.	n.d.	n.d.	n.d.	0.02 ± 0.008

increased ($p<0.01$). In the roots by contrast, there was a significant decrease in Mn content at days 7 and 14 ($p<0.05$ and $p<0.05$) and an accumulation of Cu ($p<0.05$).

Chlorophyll content and PSII efficiency

After 14 days of exposure, chlorosis was visibly more pronounced in fully expanded leaves than in young leaves of plants exposed to Cd. Furthermore, accentuated necrosis and leaf fall were observed in the oldest plant leaves. These superficial observations were consistent with the chlorophyll contents and the fluorescence parameters at the 14th day of exposure (Table 2.2). Young leaves from exposed plants did not display significant differences in chlorophylls *a* and *b* content. By contrast, expanded leaves contained significantly lower contents of chlorophyll *a* and *b* than control leaves ($p\leq0.001$), displaying a reduction of about 41 % and 43 %, respectively. The ratio of Chl *a*/Chl *b* tends to be slightly higher in exposed leaves but did not present significant changes in neither young nor expanded leaves ($p>0.05$).

Table 2.2 – Effects of Cd stress on Chlorophyll *a* and *b* content, Chl *a*/ Chl *b* ratio and on fluorescence parameters in young and expanded leaves of lettuce after 14 days of exposure. Results are expressed as mean ± SE (n=6); (*) and (***) significantly different from control $p<0.05$ and $p<0.001$, respectively.

Photosynthetic parameters		Young leaves		Expanded leaves	
		Control	Cadmium	Control	Cadmium
Chlorophyll content (μg/g FW)	**Chl *a***	501 ± 40.1	541 ± 29.9	504 ± 19.4	295 ± 28.9 ***
	Chl *b*	183 ± 17.9	192 ± 15.5	169 ± 8.2	96 ± 11.7 ***
Chlorophyll ratio	**Chl *a*/ Chl *b***	2.8 ± 0.11	2.8 ± 0.08	3.0 ± 0.16	3.1 ± 0.28
Fluorescence parameters	F_0	613 ± 34.0	713 ± 29.5	679 ± 31.0	756 ± 81.0
	F_m	3037 ± 397.2	2829 ± 75.2	3632 ± 90.5	2273 ±195.8 ***
	F_v	2423 ± 153.0	2116 ± 67.8	2953 ± 82.9	1510 ± 134.9 ***
	F_v/F_m	0.794 ± 0.0018	0.747 ± 0.0099*	0.812 ± 0.0081	0.665 ± 0.0018 ***

Concerning the effects of Cd upon chlorophyll fluorescence parameters (Table 2.2), young leaves presented non-significant alterations at day 14, exposed leaves displaying a slight trend of increased basal fluorescence, F_0, and decreased F_m and F_v levels. However, the ratio F_v/F_m was affected, registering a significantly lower value (0.75 ± 0.01) in exposed than in control leaves (p= 0.041). With respect to expanded leaves,

exposed leaves presented a slightly higher F_0 level ($p>0.05$), and a significant decrease in both F_m ($p\leq0.001$) and F_v value ($p\leq0.001$) when compared to control. The ratio F_v/F_m also presented a significantly decreased value for Cd expanded leaves (0.665 ± 0.018) when compared to control ($p\leq0.001$).

Concerning the normal values of F_v/F_m obtained for lettuce, control plants presented F_v/F_m medium values of 0.82 ± 0.006 and 0.81 ± 0.008 in young and expanded leaves (media obtained from all the measurements performed during the experiment), respectively.

Lipid peroxidation and membrane permeability

Lipid peroxidation as measured by MDA content in lettuce leaves (Fig. 2.3) increased significantly ($p<0.05$) in the control plants over the 14 days up to 0.18 ± 0.004 nmol/g FW at day 3, then dropped to 0.16 ± 0.004 nmol/g FW at day 7 and reached a maximal content of 0.19 ± 0.007 nmol/g FW at day 14. Cadmium stress enhanced this effect since MDA content was higher in exposed than in control leaves, except at day 14 when MDA content was similar to control. The difference between Cd-exposed and control leaves was statistically significant at day 7. At this time, MDA content in Cd-exposed leaves was sustained (0.20 ± 0.006 nmol/g FW). For Cd-stressed leaves, there was a significant negative correlation between MDA and the photosynthetic parameter F_v ($rs=-0.668$; $p\leq0.001$) and with the content in leaves of the macronutrients K and P (K, $rs=-0.387$; $p<0.05$; P, $r=-0.624$; $p\leq0.001$).

Membrane permeability was assessed by measuring electrolyte leakage (L_t/L_0) (Fig. 2.3). No significant differences in L_t/L_0 were observed between control and Cd-exposed leaves.

Soluble protein content

The content of soluble protein was always higher in young leaves than in expanded leaves of both control and Cd exposed plants (Fig. 2.4). In exposed young leaves, there was a slight decrease in protein content during exposure to Cd, but this trend is not significant when compared to controls on the same days ($p>0.05$), registering at day 14 levels of 8.4 ± 0.42 and 4.6 ± 1.56 mg/g FW for control and Cd treatment, respectively. In expanded leaves the protein content did not differ significantly from control ($p>0.05$).
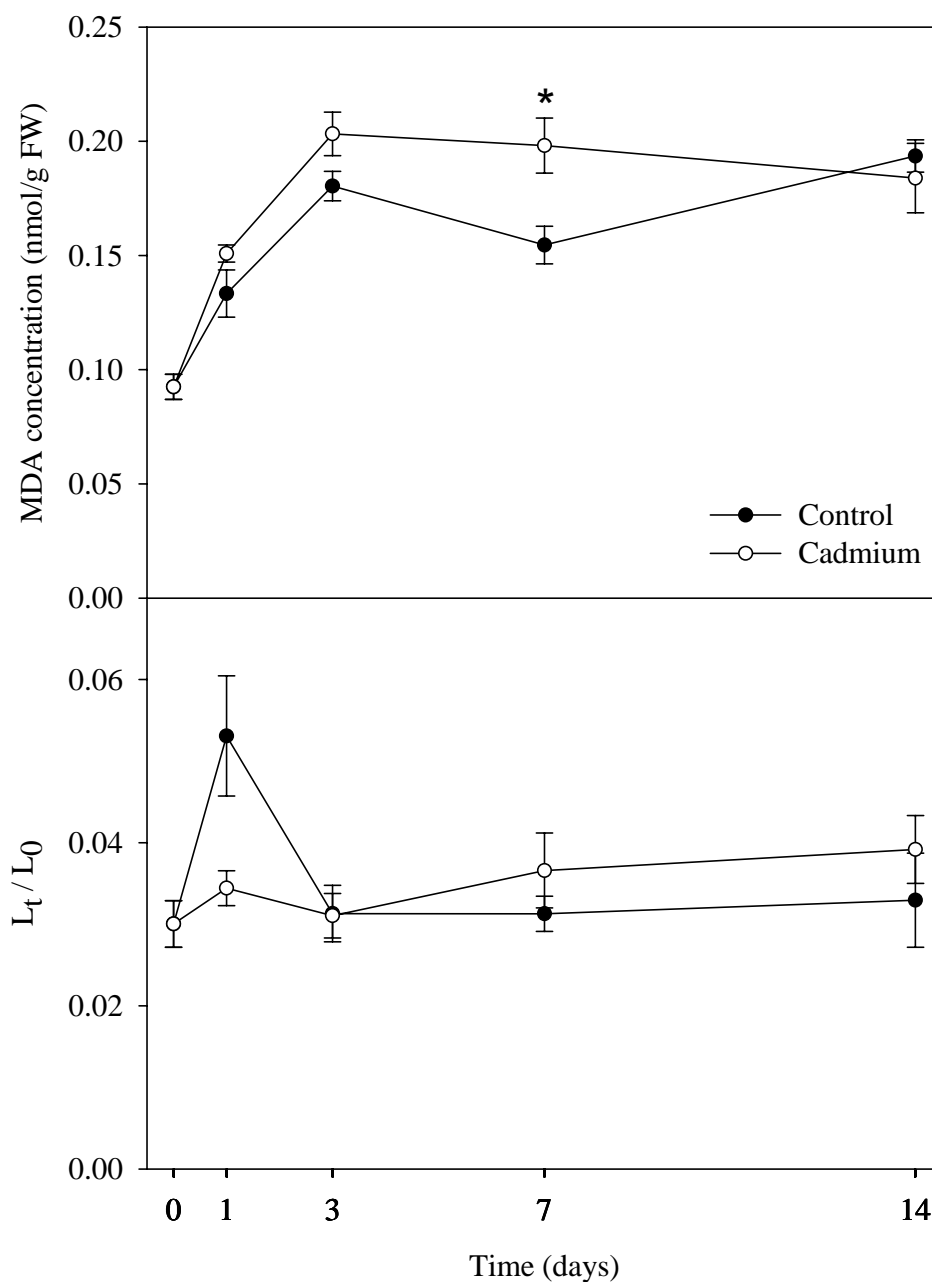
Figure 2.3 – MDA content (a) and electrolyte leakage (b) in lettuce leaves of control and exposed plants during the 14 day exposure. Results are expressed as mean ± SE of six replicates; (*) significantly different from control at the same day ($p<0.05$).

Antioxidant enzymes

The effects of Cd exposure on antioxidant capacities of young and expanded leaves are presented in Figure 2.5. In general, the expanded leaves displayed higher activities of the antioxidant enzymes, CAT, POX and SOD, than young leaves.
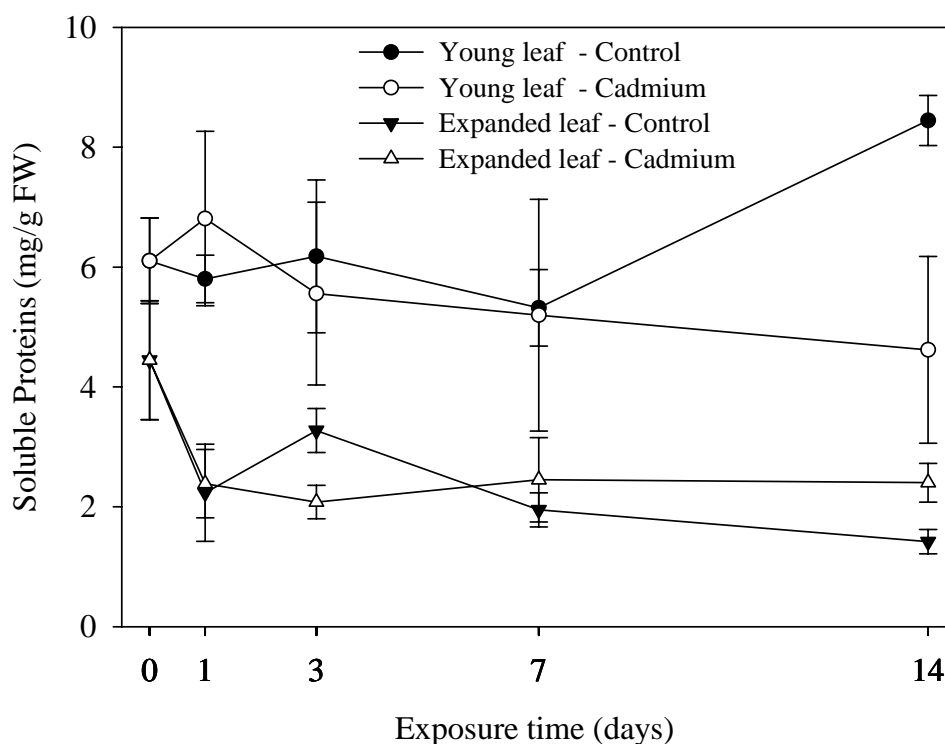
Figure 2.4 – Protein content in young and expanded leaves of lettuce. Results are expressed as mean ± SE (n=3).

As a general response, CAT displayed higher activities at the first days of exposure (days 1 and 3) than at the end of exposure (days 7 and 14), both in young and expanded leaves. At day 14, CAT activity was significantly lower in Cd-treated young and expanded leaves than in the respective control at the same day ($p<0.01$ and $p<0.05$, respectively).

In young leaves POX, as CAT, had relatively high activities in the first three days of exposure. POX displayed significantly higher activity levels than controls at day 1 ($p<0.05$). The response of CAT and POX in young leaves was then followed by an increase of SOD after day 3 up till the end of the experiment.

In expanded leaves, the onset of CAT depression at day 3, is coincident with transient increases in POX and SOD activity in response to Cd stress; the activity of both these enzymes were at their highest at day 3 (38.6 ± 22.02 and 358.8 ± 89.82 U/mg protein, respectively). Both POX and SOD displayed similar trends in activity in expanded leaves during the exposure to Cd (Fig. 2.5), which is underlined by the significant correlation with each other (r=0.534; $p<0.05$).
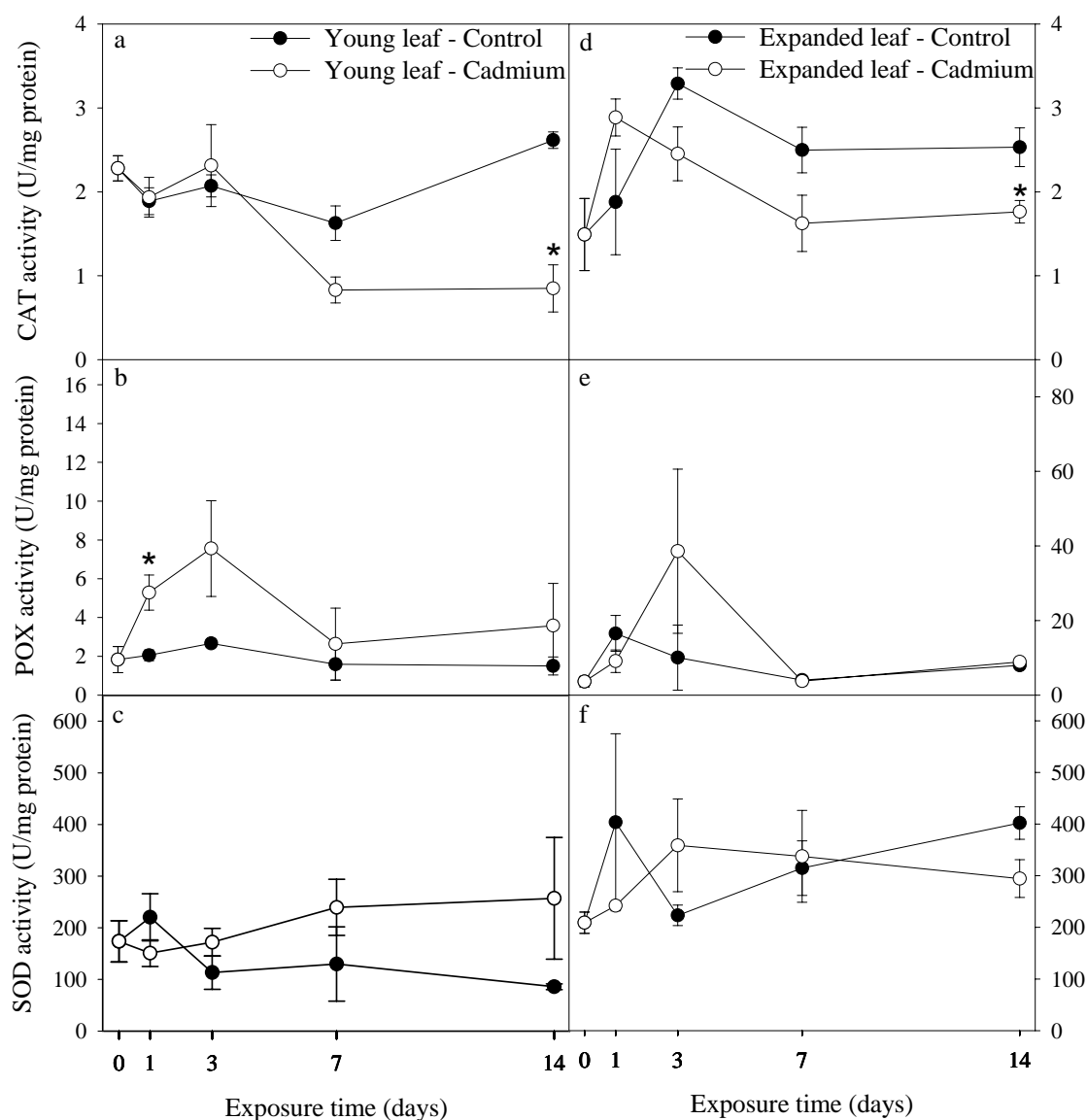
Figure 2.5 – CAT, POX and SOD activities in lettuce young (a, b and c, respectively) and expanded leaves (d, e and f, respectively). Results are expressed as mean ± SE (n=3).

Discussion

Plant growth

Plant growth inhibition is a classical parameter commonly used in the assessment of Cd toxicity to plants (An, 2004; Lagriffoul et al., 1998; Linger et al., 2005) and a recommended endpoint in standard tests for toxicity assessment (ISO, 1993; OECD,

2006). Apart from being an important indicator of toxicity at an individual level, growth inhibition is a non-specific manifestation of alterations at a biochemical level that are produced as a more specific response of plants to the particular stress. Thus, knowing the cascade of events produced by Cd exposure and relating it with growth effects would be an important consideration for risk assessment in plants.

In this work growth inhibition of lettuce shoots was exhibited within 7 days of exposure. No growth inhibition was exhibited in the roots. Costa and Morel (1994) found both root and shoot growth in *L. sativa* L. cv. Reine de Mai exposed to 100 μM of Cd.

Metal accumulation and distribution

Partitioning of metals in different plant parts is a common strategy to avoid toxicity in above-ground parts. The first barrier against Cd stress occurs in the roots where Cd may be immobilized by ligands on cell walls and extracellular carbohydrates (Sanitá di Toppi and Gabbrielli, 1999). In the present study, exposure to 100 µM of Cd resulted in an accumulation of Cd at higher levels in roots than in leaves. This result is consistent with the findings of several studies that demonstrated that Cd ions are mainly retained in the roots and that only small amounts are transported to the shoots; Cd uptake into roots is relatively fast, whereas translocation to shoots is slower (Maier et al., 2003; Sanitá di Toppi and Gabbrielli, 1999; Zhang et al., 2005). In *L. sativa* (cv. Reine de Mai) exposed from 0.01 to 100 µM of Cd, roots always displayed higher Cd contents than shoots and reached 980 μg/g DW at 100 µM Cd (Costa and Morel, 1994).

Chlorophyll content and PSII efficiency

The degeneration of chlorophyll and reduction in photosynthetic rate is a common response in plants exposed to several metals, and particularly Cd (Chugh and Sawhney, 1999; Lagriffoul et al., 1998; Linger et al., 2005; Mysliwa-Kurdziel and Strzalka, 2002). Dark-adapted values of F_v/F_m reflect the potential quantitative efficiency of PSII and are used as a sensitive indicator of plant photosynthetic performance (Maxwell and Johnson, 2000). F_v/F_m mean values obtained for control plants during the 14 days exposure were in the range expected for healthy plants (Maxwell and Johnson, 2000).

In response to Cd treatment, the increase of F_0 together with the decrease of F_m, translated as a decrease of the F_v/F_m ratio in both young and expanded leaves of Cd-exposed lettuce plants, and indicated a reduction in PSII efficiency and thus a reduction in

photosynthetic performance. These results are typical of stressed plants and indicate reduced efficiency in light-harvesting despite the absence of a reduction in chlorophylls in young Cd-treated leaves. Thus, the diminished photosynthetic activity cannot be solely attributed to the effect on chlorophyll content. Similar results were obtained in pea plants exposed to Cd (Chugh and Sawhney, 1999). In that study, the photosynthetic activity was affected to a much greater extent than the reduction in chlorophyll content would have predicted. Like other metals, Cd can substitute for Mg^{2+} ion in the chlorophyll molecule. These could strongly affect photosynthesis since such chlorophylls may have much lower fluorescence quantum yields when compared with Mg-chlorophylls (Krupa et al., 2002). This phenomenon may explain the observed reduction in photosystem II efficiency, with no significant changes of chlorophylls content in young exposed leaves.

In agreement with our results concerning the different responses to Cd stress obtained by young and expanded leaves, Krupa and Moniak (1998) have shown a close relation between the stage of leaf maturity and the efficiency of the photosynthetic apparatus in the monocotyledonous rye plants (*Secale cereale*). In particular, older leaf sections of rye were most heavily affected by Cd with respect to F_v/F_m.

Moreover, the negative correlation obtained in this study with the photosynthetic parameter F_v and the MDA content (the latter indicative of lipidic oxidation, which often correlates with membrane degradation) further suggests that damage to the thylacoidal membranes of chloroplast apparatus might be occurring as a consequence of stress-induced production of ROS, as demonstrated by other authors (Fodor, 2002).

These results are of great importance because inhibition of the light photosynthetic reaction results in a lower photosynthetic capacity and subsequently, in lower biomass production and plant growth.

Macro and micronutrient imbalances

It is known that unfavourable effects of toxic metals on plants are manifested, among other mechanisms, by inhibiting the normal uptake and utilization of mineral nutrients (Fodor, 2002). One of the crucial factors of Cd influence on plant metabolism and physiological processes is its relationship with other mineral nutrients. In this study, the leaf and root content of several essential elements, including Fe, P, K, Mn and B, were significantly affected by the Cd exposure.

Foremost among these was a significant decrease in foliar Fe. Iron-deficiency is a recognised consequence of exposure to other metals, and has implications for various

biological processes (Krupa et al., 2002). Apart from growth and chlorophyll synthesis, Cd induced Fe deficiency affects photosynthetic electron transport (Krupa et al., 2002). In agreement with this, the reduction in leaf-Fe was indeed correlated with a reduction in photosynthetic efficiency.

Potassium content in both leaves and roots was also affected in this study. Considering that one of the main roles of K is as an osmoticum, the decrease of its content both in control and exposed leaves suggests that both age and Cd influence osmotic regulation.

Manganese is a micronutrient essential for several important metabolic processes, such as the photolysis of H_2O by PSII or for the assimilation of NO_2^- in chloroplasts (Fodor, 2002). The deficiency of Mn in both lettuce roots and leaves (and underlined by the strong negative correlation between Mn and Cd content in leaves) might cause the impairment of such processes. Our results are consistent with reports by Hernandez et al. (1998) and Lagriffoul et al. (1998). These authors demonstrated that pea plants challenged with 10 and 100 µM Cd for 10 days almost completely inhibited Mn uptake; both roots and shoots presented a large decrease in Mn content (Hernandez et al., 1998). Similarly, in maize cultivated with Cd concentrations up to 25 µM, Mn contents were lower in both leaves and roots (Lagriffoul et al., 1998). In contrast, Ramos et al. (2002) found that concentrations of 0.1 and 1 mg/l Cd (about 0.9 µM and 9.0 µM, respectively) caused an increase in Mn uptake and translocation to the shoots of lettuce plants, more specifically an increase in Mn content in the chloroplasts.

The interaction between Cd and Mn obtained in the present study with lettuce requires further investigation, since Mn is known to be involved in some Mn-metalloproteins, for example, the mitochondrial Mn-superoxide dismutase (Apel and Hirt, 2004). Moreover, Mn is an important co-factor of a class of plant peroxidases, essential to complete the catalytic cycle of H_2O_2 scavenging (Apel and Hirt, 2004).

It is well known that rates of photosynthesis depend on external inorganic phosphate supply, and that in general, metals may decrease intracellular levels of this essential element. In the present work, exposure to Cd significantly decreased P content in leaves. Krupa et al. (1999) also reported that Cd affected levels of this macronutrient in rye leaves, which lead to a disturbance of photosynthetic electron transport and to affect the crucial enzyme RuBisCO.

Accumulation of micronutrients in Cd stressed plants is not fully understood and needs further study. The behaviour of lettuce leaves and roots was different with respect to micronutrient accumulation. Boron increased in leaves, while Cu accumulated in roots.

This fact may be due to different cellular organization of these organs. Copper might be accumulated in roots as consequence of an interaction with Cd^{2+} that might impair the translocation of Cu^{2+} from roots to leaves. Boron is known to have a role in the inhibition of the synthesis of phenolic compounds, thus protecting leaves from being damaged by such compounds.

Lipid peroxidation and membrane permeability

Malondialdehyde is a cytotoxic product of lipid peroxidation and its formation is routinely used as a general indicator of the extent of lipid peroxidation resulting from oxidative stress. The elevated MDA content obtained in lettuce leaves suggests that Cd, by the indirect production of ROS or by the inhibition of oxidative stress enzymes, induces oxidative damage in lettuce as evidenced by increased lipid peroxidation. Furthermore, Zhang et al. (2007) found MDA content increase in leaves of *Bruguiera gymnorrhiza* exposed to multiple metals, and recommended lipid peroxidation as a biomarker of heavy metal stress in this mangrove plant for pollution monitoring purposes.

In general, Cd-induced senescence was not significantly reflected in solute leakage (L_t/L_0) as it was expected by the observed increase in MDA content, which is an indicator of injury to biological membranes. Leaf water content, osmolality and electrolyte leakage (quantified by relative leakage ratio, RLR) also presented no significant alterations (data not shown). Other authors have previously demonstrated decreases in L_t/L_0 and RLR due to salt-stress, which were indeed correlated with MDA increase (e.g. Lutts et al., 1996).

Soluble protein content

Soluble protein decrease is a potential indicator of proteolysis, a senescence parameter. The slight decrease of protein content in young lettuce leaves exposed to Cd may have been consequent to an increase in degradation and/or to a decrease in synthesis of proteins. These findings are supported by various authors; Cd stress could be manifested as protein degradation, via amino acid catabolism resulting from a general reduction of plant development (Costa and Spitz, 1997), and also by the inhibition of RuBisCO activity, the major soluble protein in leaf and the primary site of CO_2 fixation in the Calvin cycle (Siedlecka et al., 1997; Muthuchelian et al., 2001).

Antioxidant response to Cd

Metals, including Cd, are known to cause molecular damage to plant cells either directly or indirectly through the burst of ROS, which can react with fatty acids leading to the peroxidation of lipids, destroying biological membranes (Apel and Hirt, 2004; Fodor, 2002). One of the plant responses to ROS production is the increase in antioxidant enzyme activities providing protection from oxidative damage induced by several environmental stresses (Apel and Hirt, 2004). The tolerance of some plants to heavy metal stress has been associated with higher activities of antioxidant enzymes (Dixit et al., 2001; Lagriffoul et al., 1998; Singh et al., 2006; Zhang et al., 2005). At the concentration used, Cd sequentially induced some enzymes over the 14 day exposure, suggesting that this complex of antioxidative enzymes (predominantly POX and SOD) act in combination to reduce the impact of Cd toxicity, especially in young leaves. However, the ability of plants to increase antioxidant protection to combat negative consequences of heavy metal-induced oxidative stress appears to be limited. Many studies showed that exposure to high concentrations of Cd resulted in a decreased antioxidant capacity (Sanitá di Toppi and Gabbrielli, 1999; Fodor, 2002). In our study, CAT seemed to be inhibited with extended exposure to Cd, in both young and exposed leaves. Catalase activity has been shown to be suppressed in several plants exposed to Cd, such as bean, *Phaseolus vulgaris* (Chaoui et al., 1997), and more recently in pea, *Pisum sativum* (Chaoui and El Ferjani, 2005), and in the aquatic plant *Bacopa monnieri* (Singh et al., 2006).

Conclusions

In this work we have demonstrated that Cd stress induced senescence in lettuce, as measured in general as photosynthetic efficiency reduction, nutrient imbalances, MDA production, and a decrease in the overall antioxidant capacities of lettuce plants. These alterations were accompanied by an inhibition in the classical endpoint, shoot growth, at the end of exposure. These biomarkers, in particular photosynthetic efficiency, MDA production and oxidative stress enzymes, could be used in integrative approaches with classical endpoints in ecotoxicological tests with Cd and after further studies in real scenarios conditions they could form the basis for monitoring and be predictive of early effects of this pollutant before they give rise to significant changes in natural community structures.

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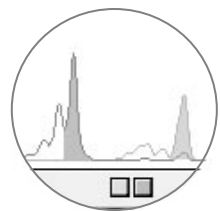
Cd genotoxic effects in plants

Chapter 3

Evaluation of Cd genotoxicity in
Lactuca sativa L. using nuclear microsatellites

Chapter 3.1

Evaluation of cadmium genotoxicity in *Lactuca sativa* L. using nuclear microsatellites

Abstract

Cadmium (Cd) is a non-essential element and is a widespread environmental pollutant. Exposure to Cd can result in cytotoxic, carcinogenic and mutagenic effects. Mutagenesis is indicative of genetic instability and can be assayed using microsatellites. Microsatellites or simple sequence repeats (SSRs) are composed of tandem repeats of short sequence motifs (1–6 bp) that are polymorphic, mainly in the number of tandem repeated units. Therefore, chromosomic mutations like inversion, deletion or translocation and point mutations can be detected by this type of molecular marker. In this study we have evaluated the mutagenic/genotoxic effects of Cd in lettuce (*Lactuca sativa* L.). Five-week-old lettuce plants grown in a modified Hoagland's medium were exposed for a further 14 days to a medium containing 100 μM Cd(NO$_3$)$_2$. Genomic DNA was extracted from lettuce leaves and roots, harvested at days 0, 1, 3, 7 and 14, and nine SSRs were tested, amplified and analysed to evaluate microsatellite instability (MSI). Mutagenic effects of Cd on microsatellite DNA loci were assessed and no MSI was observed in the used markers.

Keywords: Cadmium; Genotoxic effects; *Lactuca sativa*; Microsatellite instability (MSI); Simple sequence repeats (SSRs)

Introduction

Cadmium (Cd) is a non-essential metal that has an outstanding importance among toxic metals because of its mobility in the plant–soil system and subsequent movement through the food chain (Rojas et al., 1999). Plants growing in contaminated soils can absorb and accumulate Cd in edible tissues, thereby introducing the metal into the food chain by trophic transfer, including the human diet (McBride, 2003). Understanding Cd uptake and its effects on plants is thus critical to the long-term safety and conservation of agricultural resources.

Cadmium is a cytotoxic, carcinogenic and mutagenic metal with no known biological role. The genotoxic effects of Cd have been extensively studied in mammals and humans, and it has been shown that Cd is implicated in the induction of tumours in experimental animals and exposed human cell lines (Dalton et al., 2000; Jin et al., 2003). However, the molecular mechanism responsible for the genotoxicity of Cd remains unclear. It has been suggested that it may involve direct interaction of Cd^{2+} with DNA through the binding of Cd at G, A and T bases (Valverde et al., 2001; Hossain and Huq, 2002). Furthermore, recent studies indicate that Cd acts as a mutagen primarily by direct inhibition of an essential DNA mismatch repair, resulting in a high level of genetic instability (Jin et al., 2003). Cadmium cellular toxicity and genotoxicity may also be mediated indirectly; cells under oxidative stress display various dysfunctions due to lesions caused by reactive oxygen species (ROS, e.g. O^{2-}, H_2O_2 and OH^-) to lipids, proteins and DNA (Sanità di Toppi and Gabbrielli, 1999). In plants, Cd produces a wide range of biochemical effects, interacting with photosynthetic, respiratory and nitrogen metabolism resulting in growth retardation, low biomass production, leaf chlorosis, water and nutrient imbalances and also promoting the production of ROS (Sanità di Toppi and Gabbrielli, 1999).

As sedentary organisms, higher plant systems are excellent indicators of genotoxic effects of complex mixtures and provide unique advantages for *in situ* monitoring and screening for the detection of possible genetic damage resulting from exposure to chemicals in their environment (Grant, 1999). Several plant bioassays have used the Comet assay, micronucleus (MCN) induction or RAPDs (random-amplified polymorphic DNAs) for the detection of environmental genotoxins (Steinkellner et al., 1998; Angelis et al., 2000; Arkhipchuk et al., 2000; De Wolf et al., 2004; Liu et al., 2005). Furthermore, transgenic tests with plants carrying a recombination- or mutation-reporter transgene (β-glucuronidase gene, GUS) allowed direct scoring of DNA damage and have been applied

to study the genetic effects of heavy metals in plants (Kovalchuk et al., 2001; Kovalchuk et al., 2005).

Techniques that allow direct measurement of genotoxicity are advantageous, mainly due to their high sensitivity and short response time. The recent advances in molecular biology led to the development of several PCR-based techniques, which can be used for DNA analysis in the field of genotoxicology. RAPD, RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism) and SSR (simple sequence repeats, microsatellite) markers are among those techniques.

SSRs are tandemly repeated tracts of DNA composed of 1–6 base pair (bp) long units spread throughout the genome and, in comparison to the other markers, they are more abundant, ubiquitous in presence and highly polymorphic, and thus extremely useful for fine-scale genetic analysis (Gupta et al., 1996; Tóth et al., 2000). Furthermore, SSRs are likely to be one of the most reproducible techniques, especially when compared to RAPDs (Powell et al., 1996; Jones et al., 1997). Because of these advantages, SSRs have already been used to study genotoxic effects in several species (Angelis et al., 2000; Zienolddiny et al., 2000; Speit and Merk, 2002; Jin et al., 2003; Ohshima, 2003; Berckmoes et al., 2005). In plant research, SSRs have been extensively used in taxonomy studies (Prasad et al., 2000), genetic mapping (Ma et al., 2004) and also in the emerging scientific discipline of environmental population genetics (D'Surney et al., 2001, for a review), focusing on the relationships between environmental selective agents (stressors) and genotypic variability of plant natural populations (Mengoni et al., 2001; van Rossum et al., 2004). Furthermore, a study performed by Kovalchuk et al. (2000) uses SSRs as a methodology to assess genetic instability in the offspring of wheat plants exposed to radiation near the Chernobyl nuclear power plant. However, the application of SSRs in higher plant bioassays for the detection of genomic DNA damage and/or mutational events (e.g. deletions, insertions, point mutations) as a consequence of exposure to environmental pollutants remains unexplored.

To test the hypothesis that *in vivo* exposure to Cd will induce DNA damage in lettuce, hydroponic cultures of lettuce were exposed to a high concentration of Cd and DNA damage was assessed in leaves and roots using microsatellite analysis as a new tool for plant genotoxicity assessment. Lettuce is a Cd-accumulating plant and an important human food crop (McBride, 2003). Hydroponic culture of *L. sativa* was chosen as the most suitable culturing method because it avoids complications associated with the adsorption of the contaminant to organic and inorganic soil components, and therefore provides the most consistent and reproducible levels of contamination in lettuce.

Material and Methods

Plant culture and growth conditions

Lactuca sativa L. (Reine de Mai de Pleine Terre) seeds (Oxadis, France) were germinated using a perlite support media in polystyrene seedling trays (n = 5) floating on distilled water under dark conditions. After germination, the trays were maintained under a light intensity of 200 $\mu mol/m^2/s$ and photoperiod of 16 h/8 h (light/dark), in a culture room maintained at 25 ± 1 °C. After 1 week of culture, distilled water was replaced by modified Hoagland's hydroponic medium (concentration in mg/l: 605 KNO_3, 945 $Ca(NO_3)_2 \cdot 4H_2O$, 230 $NH_4H_2PO_4$, 490 $MgSO_4 \cdot 7H_2O$, 2.86 H_3BO_3, 0.22 $ZnSO_4 \cdot 7H_2O$, 0.09 $CuSO_4 \cdot 5H_2O$, 0.09 $NaMoO_4 \cdot 2H_2O$, 1.82 $MnCl_2 \cdot 4H_2O$, 4.78 Na_2-EDTA titriplex, 1.21 $FeCl_3$ 10%, w/v) and plants were grown hydroponically for 5 weeks. The nutrient solution was continuously aerated and changed twice a week.

After 5 weeks of culture, lettuce plants were either exposed to Hoagland's solution augmented with 100 μM $Cd(NO_3)_2$ or maintained as controls (no Cd). A previous study demonstrated that >90% of the Cd in the growth medium remained in solution, and was therefore, available for uptake (Mann et al., 2005). Five-week-old plants presented a shoot and root height of 25 ± 2.1 and 22 ± 3.8 cm, respectively. The nutrient solution was changed on alternate days to avoid depletion of nutrients and changes in Cd concentration during the course of the exposure to the metal (Mann et al., 2005). Five control and five Cd-exposed plants were harvested at days 0, 1, 3, 7 and 14 for young leaves and at day 14 for root tips. Samples of about 80 mg each were stored at −30 °C until DNA extraction.

Cadmium analysis

Cadmium concentration in the hydroponic culture medium of control and Cd-treated plants (n = 3) was verified by inductively coupled plasma spectroscopy (ICPS, Jobin Ivon, JY70 Plus, France). Accumulation of Cd in leaves (n=6) and roots (n=3; roots were previously washed in 0.5 mM $CaSO_4$ and in deionised water to remove adsorbed Cd^{2+} ions) of lettuce during the time of exposure was also determined by ICPS.

Microsatellite analysis

Total genomic DNA was extracted from 80 mg of *L. sativa* leaves and roots with the DNeasy® Plant Mini Kit (QIAGEN, Germany), according to the manufacturer's instructions. Concentration and purity of the extracted DNA were estimated using 0.8% agarose gel electrophoresis with ethidium bromide (EB) staining, comparing with a standard molecular weight marker (lambda *Hind*III, NEB) and spectrophotometrically in a Beckman DU®-68 Spectrophotometer ($1A_{260}$ unit of dsDNA= 50 μg/mL H_2O; pure DNA: A_{260}/A_{280} ≥1.8).

From the available nuclear SSRs in *L. sativa* developed by van de Wiel et al. (1999), nine were chosen on the basis of their level of polymorphism information content (PIC, see Table 3.1.1) and their PCR product quality index. Details of the studied SSRs are presented in Table 3.1.1. For the amplification of the abovementioned microsatellites, the primers designed by the authors were used. The forward primers for SSRs 7, 8 and 9 were synthesized by MWG-Biotech (Germany) and the remaining primers were supplied by Plant Research International (Wageningen, The Netherlands).

Table 3.1.1 - Description of the lettuce SSRs used: locus, repeat structure, allele size, number of alleles and PIC value described in the original publication and the PCR conditions (van de Wiel et al., 1999) and ABI dyes used. Polymorphism Information Content, PIC = 1 - Σ p_i^2, where p_i is the frequency of the ith allele.

Key	SSR	Repeat Structure	Allele size (pb)	No. Alleles / PIC values	PCR Conditions	ABI dye
1	LsA004a	$(GA)_{19}(GT)_7(GAGT)_4(GA)_{10}$	200	2 / 0.52	55ºC / 30	FAM
2	LsB101	$(GT)_{12}(AT)_5(GT)_{17}$	184	3 / 0.56	55ºC / 30	NED
3	LsB104	$(GA)_5(GT)_7TATT(GT)_{12}$-$(T)_4(GT)_8(GA)_{11}$	164	4 / 0.64	55ºC / 30	FAM
4	LsD106G	$(TCT)_{17}(T)_5(TCT)_2$	190	3 / 0.56	55ºC / 30	HEX
5	LsD109	$(TCT)_{22}$	155	4 / 0.80	55ºC / 30	HEX
6	LsE003a	$(TGT)_{24}(TA)(TGT)_{10}(TAT)_2$	208	2 / 0.32	55ºC / 30	HEX
7	LsB108a	$(GT)_8(AT)_7$ $GT)_{25}(GA)_2(GT)_5$	197	3 / 0.56	55ºC / 30	FAM
8	LsD110a	$(TCT)_{21}(TCC)_2(TCT)_7$	234	-	55ºC / 30	JOE
9	LsG001G	$(GATA)_{31}(GA)_{17}$	299	3 / 0.72	50ºC / 30	FAM

SSRs were then tested and amplified in a gradient Thermal Cycler (Thermo, Germany) using PCR conditions according to van de Wiel et al. (1999) (see Table 3.1.1).

As certain primer pairs were mixed in duos, duplex PCR amplifications were performed following the QIAGEN Multiplex PCR protocol.

After PCR amplification, PCR products were electrophoresed in 2% agarose gels stained with EB, visualized on a UV transilluminator and the image digitized using KODAK 1D 2.0 software package (Life-Technologies). Then, 1 μL of water-diluted PCR product was mixed with 0.5 μL of GeneScan internal size standard labelled with ROX and 25 μL of formamide. The mixture was briefly vortexed and visualized by Capillary Electrophoresis (CE) on an ABI Prism 310 Genetic Analyser (PE Biosystems).

The fluorescent label attached to the 5' end of the forward primers allowed the detection of the PCR products by CE and then their correspondent fragment sizes were automatically calculated to two decimal places using the Local Southern Method option of the GeneScan v.3.1 software.

Results

Cd concentration was below the ICPS detection limit (<0.01 μM) in the culture medium of control plants and was 104.0 ± 0.8 μM in the medium with the nominal concentration of 100 μM $Cd(NO_3)_2$. The accumulation of Cd in roots and leaves of lettuce during the 14 days of exposure is presented in Figure 3.1.1. The results of Cd
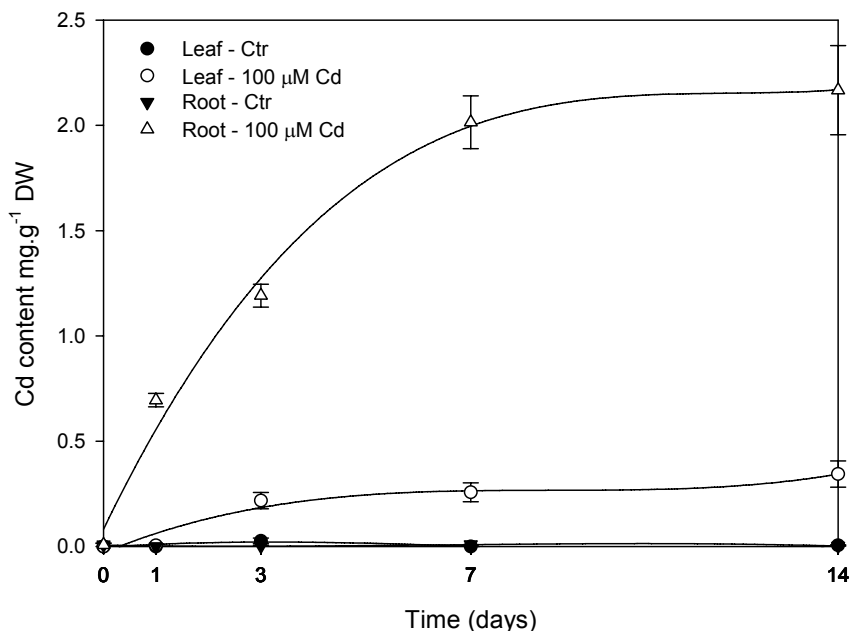

Figure 3.1.1 - Cd accumulation in *Lactuca sativa* roots and leaves of control and exposed plants during a 14 day exposure period. Results are expressed as mean ± standard error.

accumulation showed an increase with exposure duration in both roots and leaves up to 2.167 ± 0.2115 and 0.344 ± 0.0621 mg/g DW, respectively. Roots had a higher accumulation of Cd than leaves (eight-fold higher at day 14).

Exposure to Cd in the nutrient solution produced growth inhibition in lettuce plants (Fig. 3.1.2). The growth of lettuce shoot was significantly reduced at days 7 and 14 of exposure (p<0.05). Roots also presented a reduction of growth during the Cd-exposure, but this reduction was not significant when compared to the control at the same day.

The integrity of the lettuce genomic DNA extracted by the QIAGEN® (Germany) method was evaluated. The electrophoresis of the extracted DNA showed clear bands of about 23 kb, therefore, this method is suitable for DNA extraction in lettuce. Furthermore, all the nine pairs of oligonucleotide primers tested produced amplification products as observed in the agarose gel electrophoresis (data not shown).
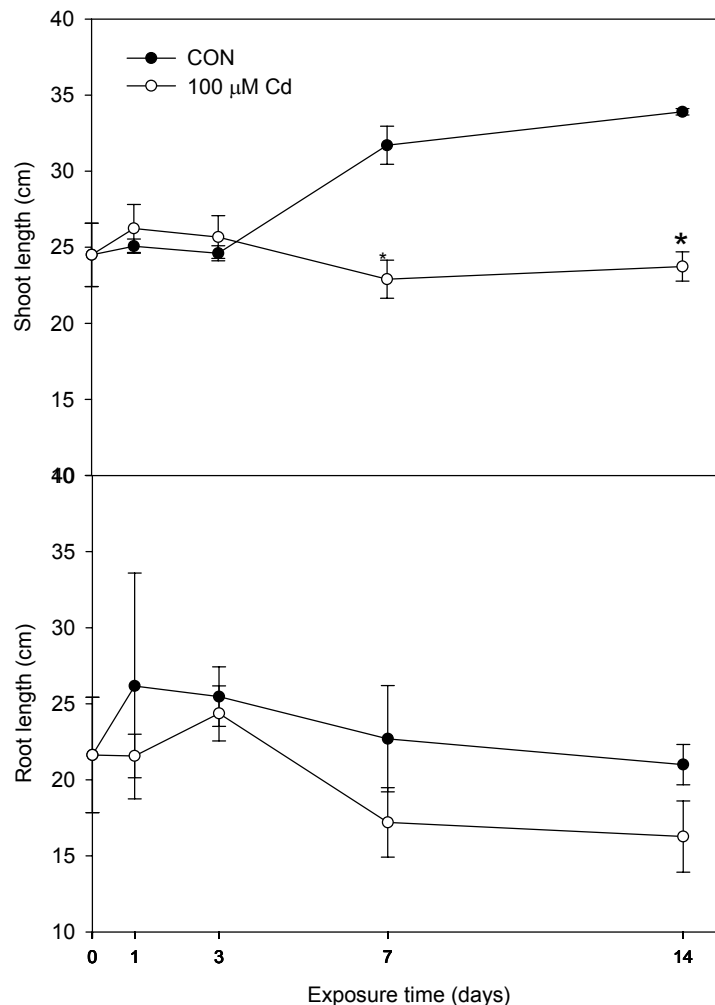

Figure 3.1.2 – Shoot and root length in *Lactuca sativa* plants during the 14 days exposure to Cd. Results are expressed as mean ± standard error; (*) significantly different from control at the same day (p<0.05).

After the analysis of the PCR products in the CE apparatus, all fragment sizes were scored and the results from days 1, 3, 7 and 14 of exposed lettuce plants were compared both to day 0 from the same plant and to control plants. A summary of the allele size of the SSRs analysed in control and Cd-exposed plants is presented in Table 3.1.2. All the SSRs scored presented similar sizes for the amplification products (alleles) that were monomorphic across all the treatments.

Table 3.1.2 – Summary of the allele sizes obtained for the SSRs analysed in control and Cd-exposed lettuce plants.

SSR	Allele size (pb)	
	Control	Cd exposed
1	198	198
2	206	206
3	191	191
4	193	193
5	171	171
6	206	206
7	203	203
8	230	230
9	255	255

As an example of the results obtained with the CE apparatus, a set of electropherograms is presented in Figure 3.1.3, showing the amplification products of a duplex PCR of SSRs 3 and 6. In the six electropherograms, both SSRs presented the same pattern in the Cd-treated plant harvested at different days. SSRs 3 and 6 presented homozygous individuals with one allele each, with sizes of 191 and 206 bp, respectively. The allele sizes obtained for the remaining SSRs analysed were 198, 206, 193, 203, 203, 230, 255, respectively, for SSRs 1, 2, 4, 5, 7, 8 and 9, and they were similar to those obtained by the group who developed lettuce SSRs (van de Wiel et al., 1999, see Table 1). According to these results, no MSI was detected on the exposed lettuce leaves or roots.
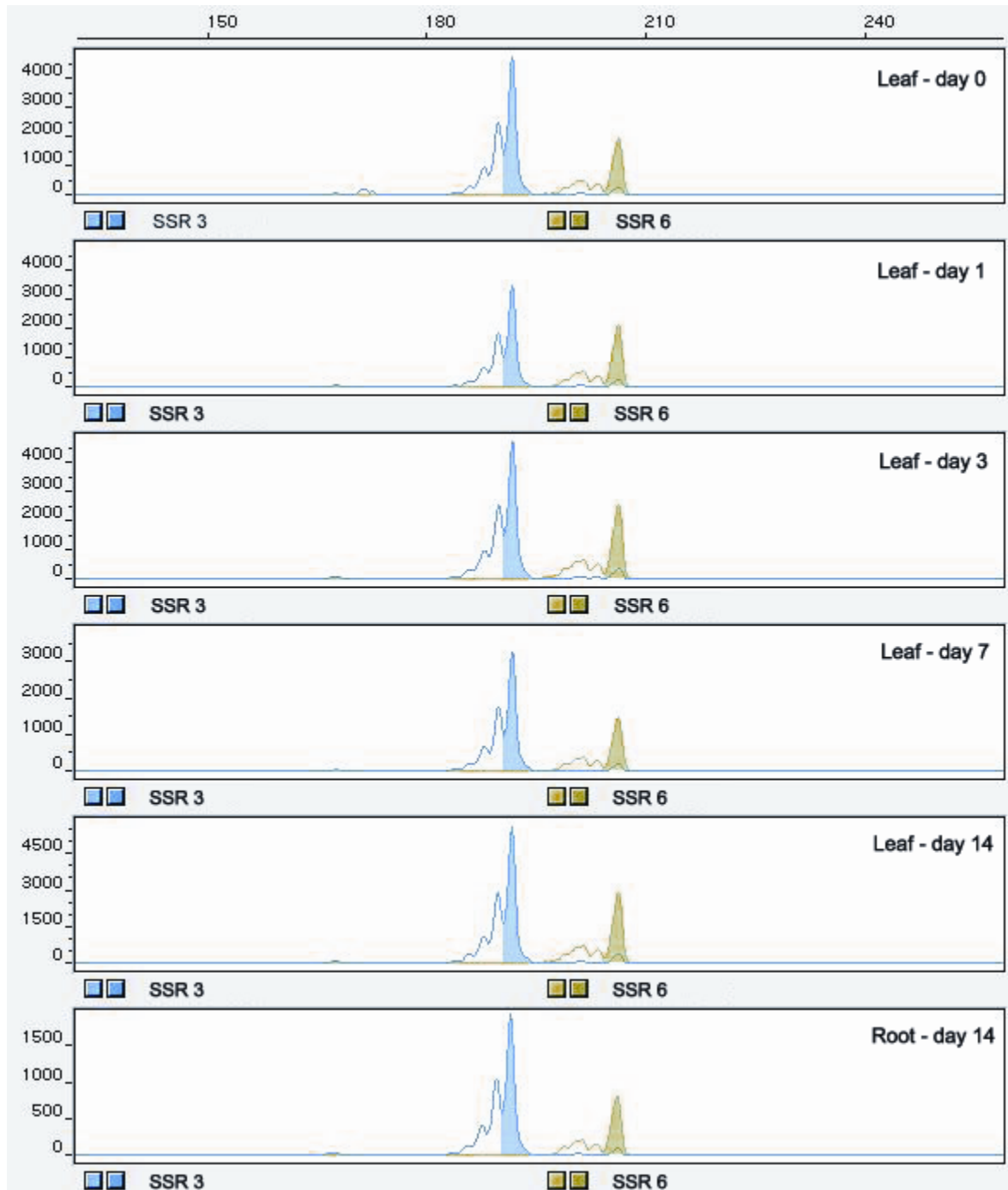
Figure 3.1.3 - Amplification products of a duplex PCR: SSR 3 (FAM dye – on the left) and SSR 6 (HEX dye – on the right). The electropherograms correspond to the same Cd-treated plant harvested for leaves or roots at different days. All electropherograms present the same pattern for both microsatellites: SSRs 3 and 6 correspond to homozygous individuals with one allele of ca. 191 and 206 bp, respectively. Top scale indicates fragment size in nucleotides. Left scale indicates fluorescence intensity measured in relative fluorescence units.

Discussion

In the present study the application of SSR marker analysis is reported for an assessment of chemical genotoxicity to plants. This technique allowed the determination of genetic stability of the SSRs analysed in 100 μM Cd-exposed lettuce plants.

The concentration of Cd used in the present work produced an accumulation of Cd in lettuce leaves and root tissues at levels of 0.344 ± 0.0621 and 2.167 ± 0.2115 mg/g DW, respectively, and significantly inhibited growth on lettuce shoots. Furthermore, Cd induced senescence in lettuce plants as observed by the chlorosis of the young leaves and the necrotic spots in several leaves. Fluctuations were also observed on antioxidant enzymes levels (catalase, peroxidase and superoxide-dismutase) and lipid peroxidation (malondialdehyde production) in response to Cd (Monteiro et al., 2004a, Chapter 2 of this thesis). Besides the physiological effects observed and the levels of Cd accumulation, particularly in roots, it does not seem to be interfering with DNA integrity. Despite being chosen as the most polymorphic SSRs available for lettuce (van de Wiel et al., 1999), all the SSRs scored presented alleles that were monomorphic across all the treatments. Hence, the severe toxicity observed in lettuce plants was reflected by MSI neither in leaves nor in roots, as measured by these specific SSRs under the conditions of this in vivo toxicity test.

As for the genetic stability, similar results were obtained using flow cytometry to quantify nuclear DNA content, a technique that could in theory detect ploidy differences, cell cycle changes and chromosome aberrations. No major ploidy changes were detected by flow cytometry, neither in lettuce plants exposed under the same experimental conditions as those used in the present work (Monteiro et al., 2004b) nor during the 2-month exposure of lettuce seedlings to 10 μM Cd(NO$_3$)$_2$ (Monteiro et al., 2005).

Plant cells can resort to a number of defense systems to prevent Cd toxicity, including, immobilization of Cd at the level of the cell wall or at extracellular carbohydrates, synthesis of phytochelatins, which chelate Cd preventing it from circulating as free Cd^{2+} inside the cytosol, and vacuolar compartmentalization. Induction of phytochelatins by Cd has been demonstrated in lettuce (Sanità di Toppi and Gabbrielli, 1999). Notwithstanding the presumed induction of this detoxification pathway, access to the cytosol by a toxic species of Cd must also be assumed, because exposure to Cd under the same conditions resulted in various biochemical and morphological disruptions (Monteiro et al., 2004a, Chapter I of this thesis). However, access to the nucleus and subsequent DNA damage in lettuce has not been demonstrated in this study.

Other authors obtained similar results for Cd using minisatellites. In a field approach, Rogstad et al. (2003) examined whether mutation rates at minisatellite DNA loci in dandelions (*Taraxacum officinale*) increased with increasing exposure to metal pollution in 16 sites (Colorado to Pennsylvania, USA). Across sites, mutation rates were significantly and positively correlated to increasing leaf-tissue concentrations of Cr, Fe, Mn, and Ni. However, no such correlation was observed for Cd and other metals (Cu, Pb, and Zn).

In a somewhat different approach, SSRs have been successfully used as a methodology in environmental population genetics to detect (or not) relationships between stressors and genotypic variability of plant natural populations. For instance, in the study of van Rossum et al. (2004), five SSR markers were used to investigate the spatial genetic structure at a microgeographical scale (ranging from 10 cm to 500 m) in a metallicolous population of *Arabidopsis halleri*, located in a metal impacted site, in which the soil presented a gradient of metal concentrations (mainly Zn, Pb, Cd). No evidence of genetic divergence due to spatial metal heterogeneity was found between zones with low or high pollution levels. In another study, using chloroplast microsatellite loci (cpSSR) analysis, Mengoni et al. (2001) have characterized tolerant and non-tolerant natural populations of *Silene paradoxa* growing in copper mine deposits, in serpentine outcrops or in uncontaminated soil, with respect to their genetic variation and relationships. Their findings indicated a reduction of genetic diversity in copper tolerant populations, the results from cpSSR markers gave statistical significance to the grouping of populations according to their geographical location.

Several higher plant bioassays, such as Comet assay and micronucleus induction have been tested and recommended for use in mutation screening and monitoring of genotoxicity induced by environmental chemicals (Grant, 1999). In plants exposed to Cd, genetic instability has already been verified with these and other techniques (e.g. RAPDs), however all these studies have used either exceedingly high concentrations of Cd and/or isolated tissues *in vitro.* For example, in the work of Gichner et al. (Gichner et al., 2004) Cd was applied on tobacco seedlings in the form of cadmium chloride (0.02–0.1 mM), and induced significant DNA damage as measured by cellular Comet assay, but only in roots. In the same study, Cd did not induce DNA damage, neither in treated isolated root nuclei, analysed by use of the acellular Comet assay, nor in leaves.

Similarly, micronucleus induction has been observed in several plant studies with Cd. Steinkellner et al. (1998) reported genotoxic effects of four metals, including Cd, in MCN assays with pollen mother cells of *Tradescantia* sp. and with isolated meristematic

root tip cells of *Allium cepa* and *Vicia faba*. In experiments with *Tradescantia*, induction of MCN was observed in a concentration range between 1 and 10 mM, whereas in tests with root tip cells, higher concentrations (10–1000 mM) were required to show significant effects (Steinkellner et al., 1998). A significant increase in micronucleus formation was also observed on *Vicia faba* roots exposed to the concentrations of 20, 200 and 2000 μM CdCl$_2$ in solution (Cordova Rosa et al., 2003). Furthermore, significant changes occurred in RAPD profiles of 1.5 cm-root tips of barley seedlings exposed to Cd in the range of 30–120 mg/L (about 136–547 mM) for 6 days (Liu et al., 2005).

In the study presented here, although the exposure is still acutely toxic, using a relatively high concentration of Cd (*cf.* Westfall et al., 2005), we have used an *in vivo* exposure with whole plants, which may more accurately represent agricultural practice. Detoxification systems (e.g. induction of phytochelatins and anti-oxidant systems) were therefore available to the whole plants to ameliorate metal toxicity and may be adequate to explain the absence of observable genotoxic effects.

However, in a different approach, Kovalchuk et al. (2001) when using transgenic tests in the assessment of genetic effects of heavy metals in *Arabidopsis thaliana* plants grown *in vitro* on liquid and agar medium, showed that several heavy metals strongly influenced the rate of homologous recombination and point mutation in a concentration-dependent manner. Point mutations were induced by Cd, even at the low concentration of 0.001 mg/L (Kovalchuk et al., 2001). Kovalchuk et al. (2005) have also found that homologous recombination frequency increased about 2.7-fold in transgenic *A. thaliana* plants grown *in vitro* on agar medium supplemented with 100 μM Cd. Furthermore, in an application of this approach to monitor chemically polluted environments, these authors noted a 4–7-fold increase in the frequency of homologous recombination and a 5–10-fold induction of point mutations in plants grown in metal contaminated soils compared with those grown in clean control soil (Kovalchuk et al., 2001).

In conclusion, and considering the microsatellite loci analysed and the experimental conditions used, the uniform microsatellite patterns observed for the SSRs seem to suggest that Cd generated no genetic instability. However, a combination of methods for analysing structural aberrations of genetic apparatus, namely MCN test and Comet assay, should be performed to complement and/or confirm these results. Despite the apparent absence of Cd effects in this study, SSR analyses were successfully applied in this work and seem to be a useful and cost-effective method (once the primers are readily available) to be implemented as a plant bioassay in the screening for genotoxins,

which could be used as a complementary tool for elucidating the different genotoxicity effects of compounds.

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Microsatellite instability in *Lactuca sativa* chronically exposed to cadmium

Abstract

Cadmium (Cd) is a cytotoxic, mutagenic and carcinogenic metal. Mutagenesis is indicative of genetic instability and can be assayed by use of microsatellite markers (simple sequence repeats, SSRs). These are tandem-repeated tracts of DNA composed of units that are 1-6 base pairs (bp) long, spread throughout the genome and highly polymorphic. SSRs can be used in the detection of genomic DNA damage and/or mutational events (e.g. deletions, insertions, point mutations). In order to study chronic exposure to Cd, *Lactuca sativa* L. seeds were germinated in distilled water and grown on modified Hoagland's medium, both supplemented with 0, 10 and 100 μM $Cd(NO_3)_2$. After 28 days of exposure, the plants were harvested to assess shoot and root length and accumulation of Cd. DNA was extracted from young and expanded leaves and roots in order to analyse microsatellite instability (MSI). Mutagenic effects of Cd were evaluated on nine microsatellite loci. No MSI was found in leaves, but a 2-bp deletion in one lettuce root SSR was detected among the SSRs that were analysed. Thus, SSR analyses may provide a complementary tool in the assessment of different genotoxic effects of compounds on plants.

Keywords: Genotoxicity; Lettuce; Metal; Simple sequence repeats

Introduction

Environmental contamination with metals has increased drastically over the past decades. Soils have been contaminated with cadmium (Cd) mainly through atmospheric deposition, urban–industrial activities and agricultural practices (e.g. use of phosphate fertilizers and application of sewage sludge). Jung and Thornton (1996) have found Cd concentrations up to 40 mg/kg in surface soils taken from a mining area in Korea.

Among other biological effects (e.g. Azevedo et al., 2005a; Azevedo et al., 2005b, c), Cd exposure may cause genotoxic effects, namely mutagenesis. Cadmium has been shown to induce large deletions in mammalian cells (Filipic and Hei, 2004). The molecular mechanism of Cd genotoxicity in organisms is still relatively poorly understood, but it has been suggested that it may involve direct binding of Cd^{2+} to DNA, possibly at guanine, adenine and thymine centres (Hossain and Huq, 2002), or direct inhibition of DNA mismatch repair (Jin et al., 2003). Genotoxicity of Cd may also be indirect, through promotion of the production of reactive oxygen species (ROS), which may then damage nucleic acids (Valverde et al., 2001; Apel and Hirt, 2004). Plants are ideal assay systems for genotoxicity, for screening as well as for monitoring environmental mutagens, and they provide vital information from the standpoint of safeguarding biodiversity and ecological resources (Grant, 1994; Panda and Panda, 2002). Therefore, the assessment of genotoxic effects of metals is an important topic in environmental research and increasing attention has been paid to this field in the last years (Steinkellner et al., 1998; Grant, 1999; Kleinjans and van Schooten, 2002).

The development of molecular biology has led to several PCR-based techniques that can be used to evaluate DNA damage in toxicological studies. Analysis of microsatellite markers, also called simple sequence repeats (SSRs), is among those techniques. SSRs are tandem repeats of DNA sequences of 1–6 base pair (bp) long. They are an important class of DNA markers because of their abundance, random occurrence and high degree of polymorphism (Gupta et al., 1996). These markers can be used in the detection of genomic DNA damage and/or mutational events (e.g. deletions, insertions, point mutations) (Tóth et al., 2000). Metals have been found to induce microsatellite instability (MSI); nickel (Ni) has been reported to promote SSR mutations in human cell lines (Zienolddiny et al., 2000) and Cd to induce MSI by increasing the frequency of mutant alleles (Jin et al., 2003). In plant research, SSRs are already a powerful tool in taxonomy, genetic mapping and environmental population genetics (Gupta and Varshney, 2000; D'Surney et al., 2001; Dimsoski and Tóth, 2001). Additionally,

SSRs can be used to screen plant genomic DNA for evidence of mutational events as in a genotoxicity test for the detection of DNA damage induced by environmental contaminants.

In a previous study performed by our group on the assessment of the genotoxicity of Cd on lettuce, no MSI was observed in 5-week-old lettuce plants exposed *in vivo* to 100 μM Cd (Monteiro et al., 2007b). The effects of metals are strongly dependent on the age of the plant at the time of exposure: the older the plant the larger the amount of metal that can be tolerated, because metals accumulate at metabolically inactive sites such as cell walls and vacuoles (Fodor, 2002). This may explain the absence of observable genotoxic effects in the previous study (Monteiro et al., 2007b). In the same study the plants were already severely affected by the toxic effects of Cd by the end of the 14-day exposure period, becoming necrotic after that. Therefore, it was decided to assess the genotoxic effects in young plants, over a longer period of time using a lower but still environmentally relevant concentration of 10 μM Cd. Soil solutions having a Cd concentration from 0.32 to about 1 μM are considered as polluted to a moderate level and an environmentally relevant Cd level is about 10-fold higher than 1 μM, which may ensure the survival of plants in the 'field situation' up to reproduction (Sanità di Toppi and Gabbrielli, 1999). Under hydroponic conditions, plants exposed to 10 μM Cd will survive for a longer period and allow the assessment of DNA damage as a consequence of chronic Cd stress.

The aim of the present study was to assess if *in vivo* exposure to Cd from the time of seed germination will induce MSI in lettuce (*L. sativa* L.). In order to assess this, lettuce seeds were germinated and grown for 28 days in 0, 10 and 100 μM Cd and nine SSRs were analysed in roots and in young and expanded leaves.

Material and methods

Plant culture and growth conditions

Lettuce seeds (*L. sativa* L. cv. Reine de Mai; Oxadis, France) were germinated in the dark in distilled water containing 0, 10 or 100 μM Cd(NO$_3$)$_2$ (pH 5.8), with perlite as support medium in polystyrene seedling trays. After 6 days the distilled water was replaced by modified Hoagland's nutrient solution (pH 5.8) as described by Monteiro et al. (2007b), also containing 0, 10 or 100 μM Cd(NO$_3$)$_2$. Plants were maintained at 25 ± 2ºC, under a light intensity of 200 μmol/m^2/s with a light/dark period of 16h/8h. The nutrient solution was continuously aerated and changed twice each week to avoid nutrient depletion and large changes in Cd concentration (Mann et al., 2005).

After 28 days of exposure, shoot and root length of the plants (18 controls and 17 Cd-exposed) were measured and plants were harvested to measure Cd accumulation and analyse microsatellites. Roots and leaves were stored at −80 ºC until DNA extraction.

Cadmium analysis

The nominal concentration of Cd in the hydroponic culture medium of control and Cd-treated plants was verified by inductively coupled plasma spectroscopy (ICPS, Jobin Yvon, JY70 Plus, Longjumeau, France).

Accumulation of Cd was determined in leaves (n=3) and roots (n=3) of control and Cd-treated plants that had been dried to constant weight at 60ºC. Prior to drying, roots were washed for 10 min in 0.5 mM $CaSO_4$ to remove Cd adsorbed onto the root surface, according to Santos et al. (2002). Dried tissues were treated according to Evans and Bucking (1976) and then analysed by ICPS.

Microsatellite analysis

Total genomic DNA was extracted with the DNeasy® Plant Mini Kit (QIAGEN, Germany) from roots and young and expanded leaves (80 mg each) of control and Cd-exposed (10 μM) plants (n=3), following instructions of the manufacturer. Plants exposed to 100 μM Cd presented less than 40 mg of either leaf or root material and the amount of genomic DNA was not enough for DNA extraction and/or MSI analysis. Pooling of material was avoided since this would not preserve the individuality of each plant replicate. Therefore, this treatment was not analysed for MSI.

In order to assess the occurrence of MSI, the same nine SSR loci used in the previous study of Monteiro et al. (2007b) were assessed in the present work. The primers used (see Table 3.2.1) were designed and supplied by Plant Research International (Wageningen, The Netherlands), with the exception of the forward primers for SSRs 7, 8 and 9, which were synthesized by MWG-Biotech (Germany).

SSRs were amplified in a gradient Thermal Cycler (Thermo, Germany). The PCR conditions used were according to van de Wiel et al. (1999). Duplex PCR amplifications were performed for SSRs 1 and 6, and 2 and 4, according to the QIAGEN Multiplex PCR protocol and mixing the respective primers in pairs. Amplification of PCR products was first assessed in 2% agarose gels stained with ethidium bromide, visualized on the UV

transilluminator G:Box (Syngene, Cambridge, UK). The respective software (GeneSnap) was used for image acquisition and analysis.

PCR product (1 μL) was mixed with formamide (25 μL) and GeneScan internal size-standard labelled with ROX (0.5 μL), vortexed and visualized by Capillary Electrophoresis (CE) on an ABI Prism 310 Genetic Analyser (PE Biosystems, USA). The fluorescent label attached to the 5'-end of the forward primers allowed the detection of the PCR products by CE and their correspondent fragment sizes were automatically calculated to two decimal places using the Local Southern Method option of the GeneScan v.3.1 software.

Table 3.2.1 – Description of SSRs used: locus, repeat structure and allele size obtained in the original publication of *L. sativa* SSRs (van de Wiel, 1999).

Key	SSR	Repeat structure	Allele size (pb)
1	LsA004a	$(GA)_{19}(GT)_7(GAGT)_4(GA)_{10}$	200
2	LsB101	$(GT)_{12}(AT)_5(GT)_{17}$	184
3	LsB104	$(GA)_5(GT)_7TATT(GT)_{12}(T)_4(GT)_8(GA)_{11}$	164
4	LsD106G	$(TCT)_{17}(T)_5(TCT)_2$	190
5	LsD109	$(TCT)_{22}$	155
6	LsE003a	$(TGT)_{24}(TA)(TGT)_{10}(TAT)_2$	208
7	LsB108a	$(GT)_8(AT)_7(GT)_{25}(GA)_2(GT)_5$	197
8	LsD110a	$(TCT)_{21}(TCC)_2(TCT)_7$	234
9	LsG001G	$(GATA)_{31}(GA)_{17}$	299

Statistical analysis

Statistical analysis of differences in shoot length between control and exposed plants was performed using Kruskal-Wallis one-way ANOVA (since even with transformed data no normality and/or equality of variance were achieved) and Dunn's post-hoc test. Root length was analysed using one-way ANOVA and Dunnett's method. SigmaStat (version 3.01, SPSS, Chicago, IL, USA) was used to perform all statistical tests.

Results

Cd accumulation and plant growth

Nominal concentrations of Cd in the nutrient solutions were verified by ICPS and were below the detection limit for controls (<0.01 μM) and 10.50 and 96.97 μM for 10 and 100 μM Cd treatments, respectively. Figure 3.2.1A presents the accumulation of Cd in leaves and roots of lettuce after 28 days of exposure. The contents in plants treated with 10 μM were 0.058 ± 0.0024 and 0.512 ± 0.2630 μg Cd per mg dry weight (mean ± SE) in leaves and roots, respectively. In plants exposed to 100 μM Cd accumulation was 0.804 ± 0.0851 and 1.774 ± 0.1060 μg per mg dry weight (mean ± SE) in leaves and roots, respectively. Roots of plants exposed to 10 μM Cd showed about 9-fold higher accumulation of Cd than leaves, but this difference was not statistically significant (p=0.160). Roots of plants treated wih 100 μM Cd showed about 2-fold higher accumulation than leaves (p<0.05).
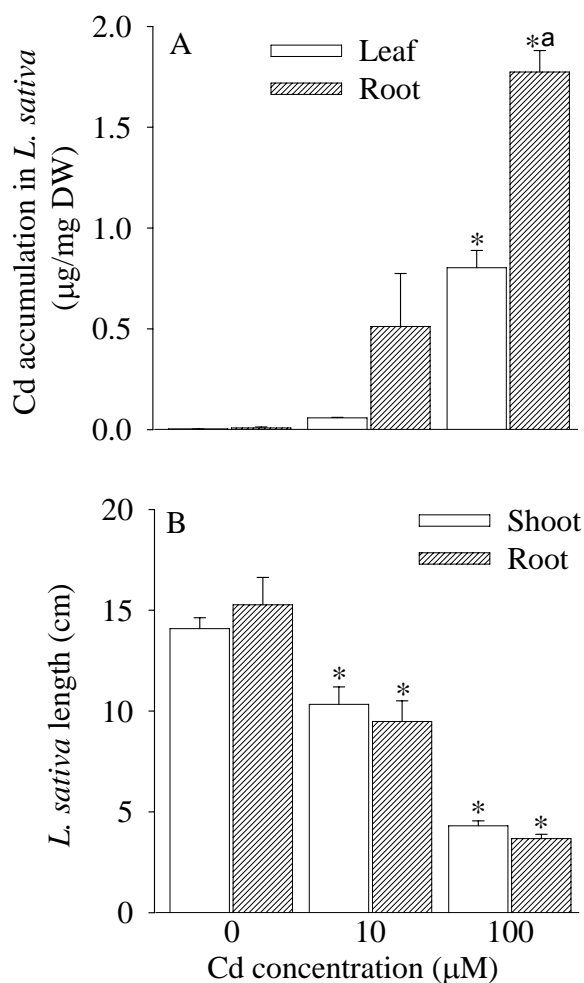

Figure 3.2.1 – (A) Cd accumulation in leaves and roots (n=3) and (B) shoot and root length of lettuce plants after 28 days of exposure (n=17-18). Results are expressed as mean ± standard error; (*) significantly different from control p<0.05; (a) significantly different from leaves that received the same Cd-treatment.

Figure 3.2.1B represents lettuce shoot and root length after 28 days of exposure. Lettuce exposed to 10 and 100 μM Cd showed significant growth inhibition in both shoot and roots (p<0.05) when compared with the respective control. Moreover, Cd treatment affected plant survival: in each treatment group one plant was completely necrosed.

Microsatellite analysis

PCR products were analysed by CE and fragment sizes from control and exposed plants were scored and compared. A summary of the allele size of the SSRs analysed in control and exposed plants harvested after 28 days of Cd-exposure is presented in Table 3.2.2. The amplification products (alleles) retrieved from control and exposed plants (both young and expanded leaves) for each of the SSRs were of similar size.

Table 3.2.2 – Lettuce SSRs with the allele size obtained in leaves and roots of control and Cd-treated plants, and in previous studies of Monteiro et al. (2007b).

SSR	Control 28 days exposure	10 μM Cd 28 days exposure	Monteiro *et al.* (2007b) (5-week old plants, 14 days exposure to 100 μM Cd)
1	197	197	198
2	206	206	206
3	192	190*, 192	191
4	194	194	193
5	170	170	171
6	206	206	206
7	203	203	203
8	232	232	230
9	256	256	255

* One sample of a 10 μM Cd-treated lettuce root presented an allele of 190 bp (see Figure 3.2.3).

As an example of the results obtained with the CE apparatus, a set of electrophoretograms is presented in Figures 3.2.2 and 3.2.3, showing the amplification products of a duplex PCR of SSRs 5 and 7 and of SSR 3, respectively. For both young and expanded leaves the pattern of amplification products of SSRs 5 and 7 and SSR 3 is the same in control and exposed plant leaves, revealing homozygous individuals with

alleles of 170, 203 and 192 bp, respectively. The allele sizes obtained for the remaining SSRs analysed were 197, 206, 194, 206, 232, 256 bp for SSRs 1, 2, 4, 6, 8 and 9, respectively, in both control and exposed young and expanded leaves. No MSI was detected in young or expanded leaves of exposed plants.
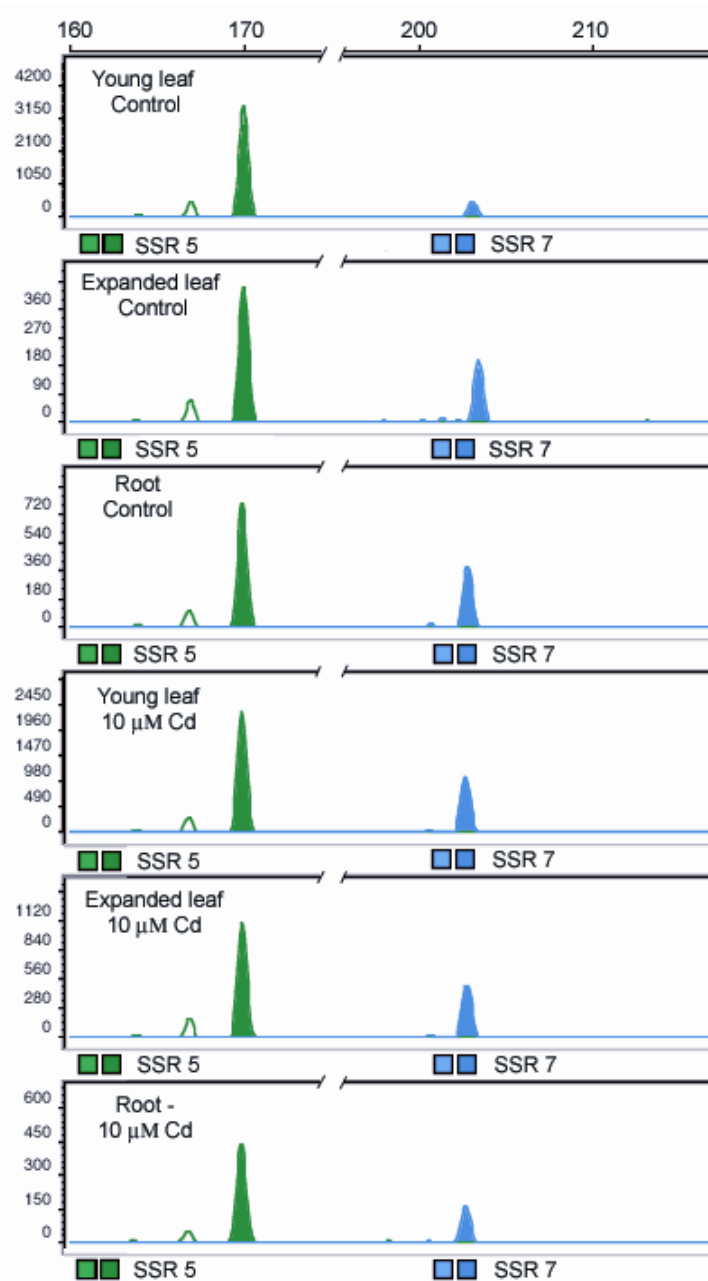

Figure 3.2.2 – Amplification products of a duplex PCR: SSR 5 (HEX dye – green colour) and SSR 7 (FAM dye – blue colour). The electrophoretograms correspond to control and Cd-treated plants harvested for young or expanded leaves or root at the 28th day of exposure (from top to bottom, respectively). Top scale indicates fragment size in nucleotides. Left scale indicates fluorescence intensity.

In roots, the PCR products were also of similar size in both control and exposed plants for all SSRs (197, 206, 194, 170, 206, 203, 232, 256, respectively, for SSRs 1, 2, 4, 5, 6, 7, 8 and 9), except for SSR 3. As shown in the electrophoretograms of exposed roots presented in Figure 3.2.3, a Cd-treated (10 μM) lettuce root showed an allele of 190 bp instead of 192 bp for SSR 3 retrieved from the remaining samples of lettuce roots and leaves. These results are indicative of MSI in roots of Cd-exposed lettuce plants, which presented a mutation frequency of 3.7% ((1 root plant/3 plants analysed)*(1 SSR / 9 SSR analysed)).
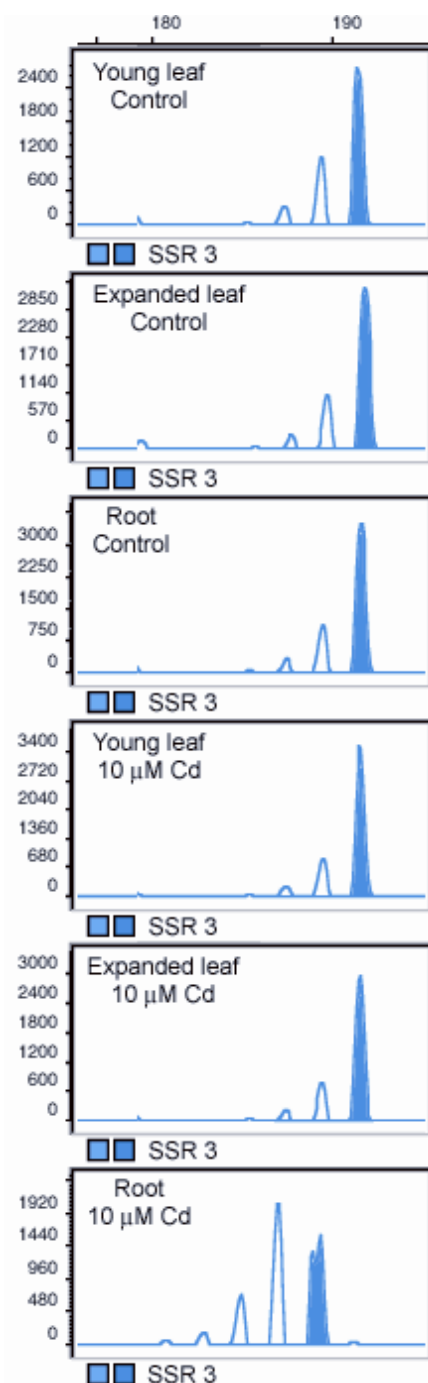

Figure 3.2.3 – Amplification products of SRR 3 (FAM dye – blue colour). The electrophoretograms correspond to control and Cd-treated plants harvested for young or expanded leaves or root at the 28th day of exposure (from top to bottom, respectively). Top scale indicates fragment size in nucleotides. Left scale indicates fluorescence intensity.

To test the reproducibility of the technique, duplicates were run for five of the samples. The sample where the MSI was found was also run five times to verify that it was no artefact. These assays were complete re-runs, i.e., all procedures from PCR amplification to fragment analysis on the CE apparatus took place on different occasions for each of the replicates. All sets of replicates yielded exactly the same banding patterns.

Discussion

The assessment of genotoxic effects as a consequence of exposure to metals is an important issue in environmental research with plants and can be improved with the application of new PCR-based methods to detect toxicant-induced alterations in the plant genomes. As far as known, SSRs were first adopted by Kovalchuk et al. (2000) as tools to assess genetic instability in the offspring of wheat plants exposed to radiation near the Chernobyl nuclear power plant. More recently, the application of SSRs in higher plant bioassays for the detection of genomic DNA damage and/or mutational events such as deletions, insertions and point mutations as a consequence of exposure to environmental pollutants has been reported by Monteiro et al. (2007b, chapter 3.1 of this thesis). That work comprised an assessment of MSI in root and leaf tissue of 5-week-old lettuce exposed to 100 μM Cd for 14 days. The reported absence of MSI in these plants due to Cd stress may be related with the age of the plants, since the effects of metals are known to be highly dependent on the age of plants at time of exposure (Fodor, 2002). Exposure of young cucumber plants to Cd caused more effects than exposure of older plants, even though they accumulated a smaller amount of Cd (Láng et al., 1998).

In the present study, the lettuce plants were exposed to Cd stress throughout germination and growth, and although the plants manifested symptoms of Cd toxicity within the leaves (senescence of expanded leaves and inhibition of shoot growth), Cd did not seem to interfere with DNA integrity. In this case, MSI was observed in lettuce roots exposed *in vivo* to 10 μM of Cd, with a mutation rate of 3.7% in the SSRs analysed.

The MSI results presented here are complemented by data collected in a parallel study that employed flow cytometry to assess clastogenic damage. Monteiro et al. (2007a, chapter 3.3 of this thesis) presented data indicating that a 28-day exposure of *L. sativa* to 100 μM Cd may lead to clastogenic damage in root tissues, since nuclear DNA content significantly decreased and FPCV (full peak coefficient variation of the G_0/G_1 peak) levels were significantly higher than in control plants. However, exposure to 10 μM Cd did not have the same effect in root tissue: neither nuclear DNA content nor FPCV were affected.

In the present work, exposure to Cd resulted in greater accumulations of Cd in roots than in leaves. In previous studies by Costa and Morel (1994), roots of *L. sativa* plants (cv. Reine de Mai) exposed to hydroponic Cd solutions ranging from 0.01 to 100 µM always displayed higher Cd contents than shoots. These results are in agreement with the findings of several authors who demonstrated that Cd ions are mainly retained in the roots and that only small amounts are transported to the shoots: Cd uptake into roots is relatively fast, whereas translocation to shoots is slower (Sanità di Toppi and Gabbrielli, 1999; Maier et al., 2003; Zhang et al., 2005). Partitioning of metals in different parts of the plant is a common strategy to avoid toxicity in above-ground parts. The first barrier against Cd stress occurs in the roots where Cd may be immobilized by ligands on cell walls and extracellular carbohydrates (Sanità di Toppi and Gabbrielli, 1999).

The higher accumulation of Cd in roots may explain the observed genotoxicity in this tissue, whereas the absence of MSI and no/low clastogenicity found in leaves (Monteiro et al., 2007a) may be related with lower Cd accumulation in leaves. Similar results were obtained by Gichner et al. (2004; 2008); in both of these studies the authors related the absence of genotoxic effects of Cd in plant leaves with the lower accumulation of Cd in this tissue and also to the better antioxidant defence system of leaves in comparison to roots, which may protect the nuclear DNA in leaf cells from Cd-induced oxidative stress. Gichner et al. (2004) found that activity of the antioxidant enzyme catalase was about 30 times higher in tobacco leaves than in roots.

Considering the allele size of SSRs obtained in lettuce leaves and roots, results were similar (except for the mutation found - see Table 3.2.2) to those obtained by Monteiro et al. (2007b). The difference in allele size of 1 or 2 bp obtained between both studies in some SSRs is attributed to the use of different lots of plant seeds and to the different devices of CE used to assess fragment sizes. Also, it is known that identical alleles can generally migrate within 0.5 bp of each other on the same sequencing run and larger variations can be observed when comparing data from the same sample on different sequencing runs (Santos et al., 2007).

Conclusions

The uniform microsatellite patterns found for the nine SSRs analysed seem to suggest that the Cd treatment performed generated no microsatellite instability on lettuce leaves. The 2-base pair deletion in one lettuce root SSR suggests the occurrence of MSI in this organ. Because Cd content is greater in roots than in leaves, roots may be more

exposed to internal Cd^{2+} than leaves. However, additional chronic toxicological trials should be performed in order to assess genotoxic effects of chronic exposure to Cd and to test the sensitivity of the method under more realistic exposure scenarios.

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Flow cytometric assessment of Cd
genotoxicity in three plants with different
metal accumulation and detoxification capacities

Chapter 3.3

Flow cytometric assessment of cadmium genotoxicity in three plants with different metal accumulation and detoxification capacities

Chapter section submitted as original article:

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Abstract

Cadmium (Cd) is a widespread environmental contaminant, strongly mutagenic and known to cause DNA damage in plants. In this work, flow cytometry (FCM) was applied to determine if chronic exposure to Cd would induce genotoxic effects at the genome level. The hyper-accumulator *Thlaspi caerulescens* (J. & C. Presl), the related non-accumulator *Thlaspi arvense* L. and the accumulator crop species *Lactuca sativa* L. were germinated in distilled water and grown in modified Hoagland's medium with increasing concentrations of $Cd(NO_3)_2$ (0, 1, 10 and 100 μM). After 28 days of exposure, shoot and root growth was recorded and the tissues were harvested for Cd and FCM analysis. In general, roots from treated plants contained higher content of Cd than leaves and it was observed growth inhibition in the treated plants. Nuclear DNA content was estimated and the G_0/G_1 full peak coefficient of variation (FPCV), as an indicator of clastogenic damage, was recorded. In *T. arvense* and *T. caerulescens* no significant differences were detected between control and exposed plants. Leaves of *L. sativa* exposed to 10 μM Cd presented a statistically significant increase in FPCV values in comparison with the control group. Furthermore, roots exposed to 100 μM Cd presented a reduction in nuclear DNA content and an increase in FPCV when compared to the control. FCM data suggests that no major DNA damage was induced on both Cd-exposed *Thlaspi* species. On the contrary, chronic exposure to 10 μM and 100 μM of Cd seemed to cause genotoxic effects in *L. sativa*, with more severe effects being detected in the roots.

Keywords: DNA, *Lactuca sativa*, metal, *Thlaspi arvense*, *Thlaspi caerulescens*

Introduction

Cadmium (Cd) is a cytotoxic and mutagenic metal that can affect plant growth and development (Fodor, 2002). Atmospheric deposition, urban–industrial activities, coal ash and agricultural practices (e.g. use of agrochemical products and addition of sewage sludge) are the main anthropogenic sources of Cd in soils. The molecular mechanisms of Cd genotoxicity in organisms are not well understood, but it has been suggested that it may involve direct binding of Cd to the nucleotides guanine, adenine and thymine (Valverde et al., 2001; Hossain and Huq, 2002), direct inhibition of DNA mismatch repair (Hartwig, 1994; Jin et al., 2003), or may be processed indirectly by promoting the production of reactive oxygen species (ROS) that may then damage nucleic acids (Fodor, 2002). Cadmium is known to induce genotoxicity in plants (Panda and Panda, 2002); Borboa and de La Torre (1996) have shown clastogenicity and aneugenicity in *Allium cepa* as a consequence of Cd exposure.

Flow cytometry (FCM) is a technique that theoretically has the potential to detect minute differences in nuclear DNA (nDNA) content, as well as chromosomal damage, in exposed organisms. Otto and Oldiges (1980) were able to assess chromosomal damage induced by clastogenic agents and irradiation on Chinese hamster cell lines and mice through the analysis of the coefficient of variation (CV) of the G_0/G_1 peak. The increase in CV was positively correlated with the clastogenic effects observed by microscopic examination. Flow cytometry measurement of the dispersion in the nDNA content as induced by the interactions of DNA with environmental agents, emerged then as a powerful tool in cytogenetic investigations and in mutagenicity testing (Otto et al., 1981).

Although FCM is routinely used in animal toxicological studies (Biradar and Rayburn, 1995; Easton et al., 1997; Bickham et al., 1998; Whittier and McBee, 1999), the use of similar approaches in plant genotoxicity assessment remains much less common. Significant changes in nDNA content have been detected in maize plants (*Zea mays*) exposed to coal fly ash (McMurphy and Rayburn, 1993) and to the fungicides captan (Rayburn et al., 1993) and triticonazole (Biradar et al., 1994). Furthermore, the mean CV of the G_0/G_1 peaks also increased in *Z. mays* individuals subjected to coal fly ash treatments (McMurphy and Rayburn, 1993). More recently, Rayburn and Wetzel (2002) reported that an increase in the CV values of the G_0/G_1 peak in both a maize mutant and in wheat grown in soil with high levels of aluminium was correlated with the number of abnormal anaphase figures.

In combination with amplified fragment length polymorphism (AFLP) analysis, FCM was also successfully introduced as a new biomonitoring tool to assess soil genotoxicity (Citterio et al., 2002). These authors demonstrated that exposure of *Trifolium repens* to Cd and Cr resulted in a decrease in the DNA index with increasing concentrations of Cr, and to an increase of debris background at the highest concentrations of Cd and Cr. More recently, Aina et al. (2006) using the same method and the same species did not find any differences in nDNA content between plants exposed to different polycyclic aromatic hydrocarbons and the control.

The aim of the present study was to evaluate by FCM the dose-response relationship on nDNA content and CV of the G_0/G_1 peak after chronic exposure to Cd. For this, plants with different patterns of Cd accumulation were chosen: the hyper-accumulator alpine pennycress (*Thlaspi caerulescens* J. & C. Presl) that accumulates high levels of Cd in shoot tissues (up to 10.000 mg.Kg-1 in the Ganges ecotype, Lombi et al., 2000); the related field pennycress (*Thlaspi arvense* L.), which is a non-accumulator plant; and lettuce (*Lactuca sativa* L.), a Cd-accumulating plant and important human food crop recommended in several standard tests (e.g. ISO/CD 17126) (ISO, 1995).

Material and Methods

Plant culture and growth conditions

Seeds of *L. sativa* (cv. Reine de Mai, Oxadis, France), *T. arvense* (Amsterdam) and *T. caerulescens* (Saint-Félix-de-Pallières, Ganges, France) were germinated under dark conditions in distilled water, using perlite as support media in polystyrene seedling trays. After germination, lettuce plants were grown on modified Hoagland's nutrient solution (Monteiro et al., 2007), and *Thlaspi* sp. were grown on modified Rorison's nutrient solution (Monteiro et al., in press). Both distilled water and nutrient solutions were supplemented with 0, 1, 10 and 100 μM Cd(NO$_3$)$_2$. Plants were grown at 24 ± 2 ºC, under light intensity of 200 µmol/m^2/s and photoperiod of 16h/8h (light/dark). Nutrient solution was continuously aerated and changed twice a week to avoid nutrient depletion and changes in Cd concentration (Mann et al., 2005). After 28 days of exposure, plants were harvested. Morphological symptoms of Cd toxicity were noted, and shoot and root length was recorded (n=15-17). Also, plant material was collected for Cd accumulation assessment and FCM analysis.

Cadmium accumulation assessment

Cadmium concentration in the hydroponic culture medium of control and Cd-treated plants was verified by inductively coupled plasma spectroscopy (ICPS, Jobin Ivon, JY70 Plus, Longjumeau Cedex, France). Accumulation of Cd was determined in leaves and roots (n=3) dried to constant weight at 60 ºC. Roots were previously washed for 10 min in 0.5 mM $CaSO_4$ to remove, by cation exchange, the Cd adsorbed to the root surface. Dried tissues were treated as described by Santos et al. (2001) and subsequently analysed by ICPS.

Flow cytometric analysis

Nuclear suspensions from plant leaves and roots were prepared according to Galbraith et al. (1983). In brief, to release nuclei from the cells, leaf sections (1 to 2 cm^2) and root apices (1 to 2 cm from the tip of 3 to 4 roots), were chopped with a sharp razor blade together with a young leaf (2 cm^2) of the internal reference standard *Pisum sativum* cv. Ctirad (for *L. sativa*; 2C = 9.09 pg DNA (Doležel et al., 1998)) or *Solanum lycopersicum* cv. Stupicke (for the two *Thlaspi* spp.; 2C = 1.96 pg DNA (Doležel et al., 1992)), in LB01 buffer (Doležel et al., 1989) (15 mM Tris, 2 mM Na_2EDTA, 0.5 mM spermine.4HCl, 80 mM KCl, 20 mM NaCl, 0.1% (v/v) Triton X-100, pH 8.0). The suspension of nuclei was then filtered through an 80 μm nylon filter to remove large tissue fragments. Afterwards, 50 μg/ml of propidium iodide (PI; Fluka, Buchs, Switzerland) and 50 μg/ml of RNase (Sigma, St. Louis, MO, USA) were added to the samples to stain nDNA and prevent the binding of PI to double stranded RNA, respectively. At least 5,000 nuclei per sample were analysed in a Coulter EPICS-XL (Coulter Electronics, Hialeah, Florida, USA) flow cytometer. The instrument was equipped with an air-cooled argon-ion laser (15 mW operating at 488 nm). Before starting the analysis, the instrument was checked for linearity with fluorescent check beads (Coulter Electronics, Hialeah, FL) and the amplification was adjusted to position the G_0/G_1 peak of sample nuclei at channel 200. This setting was kept constant throughout the analysis. The results were acquired using the SYSTEM II software (v. 3.0, Beckman Coulter[®]) in the form of three histograms: linear-fluorescence light scatter (FL); FL pulse integral *versus* FL pulse height and forward angle (FS) *versus* side angle (SS)-light scatter in logarithmic scale. In the last two cytograms, "interest zones" were defined to separate intact nuclei from doublets and debris.

Three replicates per condition and tissue were analyzed. In order to assess putative genotoxic effects on exposed plants, two different parameters were determined in each histogram: nuclear DNA content and full peak coefficient of variation (FPCV) of the G_0/G_1 nuclei. The nuclear DNA content was given by the ratio between the mean channel position of the sample and the internal standard multiplied by the nuclear DNA content of the reference standard. The FPCV was chosen instead of the more usual half peak coefficient of variation as it was our intention to analyse the whole dispersion of nDNA content, as diagnostic for clastogenic damage (as recommended for toxicological studies by Misra and Easton (1999). Since the control samples of *Thlaspi* spp. roots presented very high FPCV values (>10 %) and debris background, the samples corresponding to exposed plants of these species were not analysed and only the results concerning the leaves are presented.

Statistical analysis

Statistical differences between control and exposed leaves and roots were analysed using a one-way ANOVA, followed by the appropriate post-hoc tests (Dunnett's method). Where necessary, data were transformed to achieve normality and equality of variance. If these criteria were not satisfied even after data transformation, non-parametric tests were performed (Kruskal-Wallis one-way ANOVA). SigmaStat (version 3.01, SPSS, Chicago, IL, USA) was used to perform all statistical tests.

Results

Cd accumulation assessment

The concentration of Cd in the nutrient solution of control plants was below the ICPS detection limit (<0.01 μM). In the nutrient solutions with the nominal concentrations of 1, 10 and 100 μM $Cd(NO_3)_2$ the concentrations of Cd were 1.88, 10.50 and 96.97 μM for lettuce and 1.25, 11.56 and 100.52 μM for *Thlaspi* spp., respectively.

The accumulation of Cd in roots and leaves of lettuce and *Thlaspi* spp. plants at the 28th day of exposure is presented in Figure 3.3.1. In general, roots from treated plants contained higher content of Cd than leaves. Specifically, plants exposed to 100 μM Cd presented about 2, 8 and 1.4-fold higher content of Cd in roots than in leaves of *L. sativa*, *T. arvense* and *T. caerulescens*, respectively. In contrast, among *T. caerulescens* plants

exposed to 10 µM Cd, the content of Cd in the leaves was more than 3-fold higher than in roots.
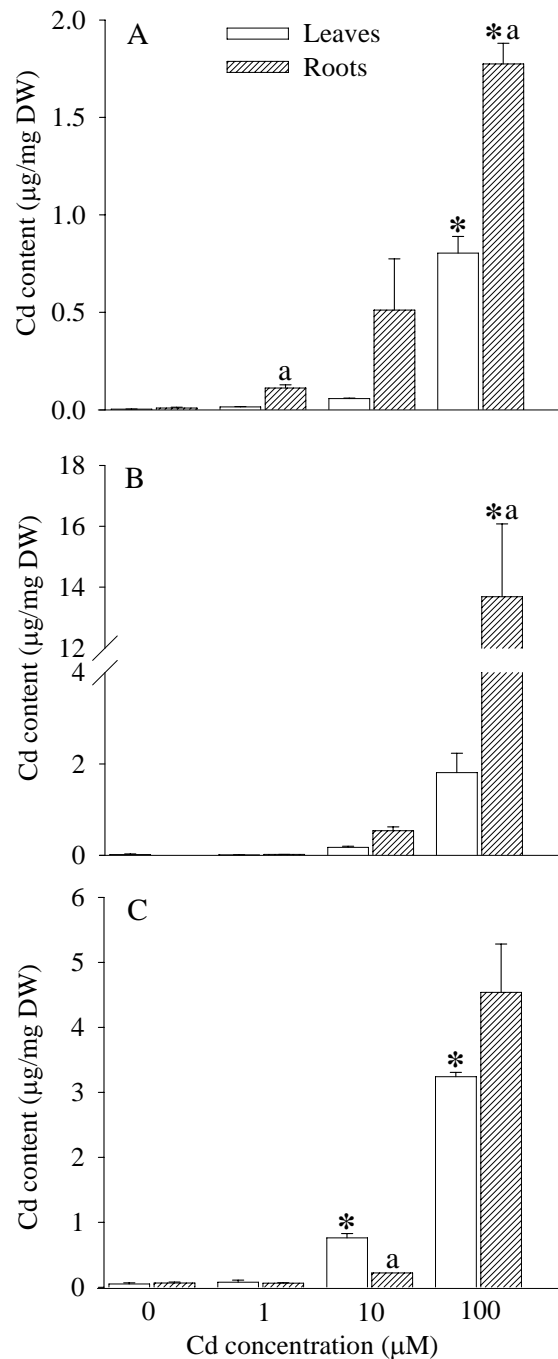

Figure 3.3.1 - Cadmium content in leaves and roots of *Lactuca sativa* (A), *Thlaspi arvense* (B) and *Thlaspi caerulescens* (C) plants after 28 days of exposure to 0, 1, 10 and 100 µM of Cd. Results are expressed as mean ± standard error. Significantly different values (*) when compared with the control group at $p<0.05$ and (a) when compared with leaf group at the same concentration of Cd.

Toxicity symptoms and plant growth

Physical manifestations of Cd toxicity were observed in plants within the 28 days of culture. Plants developed toxicity symptoms in the form of chlorotic lesions, especially in expanded leaves of *L. sativa* and *T. arvense*, (3.3.2). Expanded leaf-fall was among Cd-treated plants during exposure, and the roots of Cd-treated plants appeared darker than those of control plants.
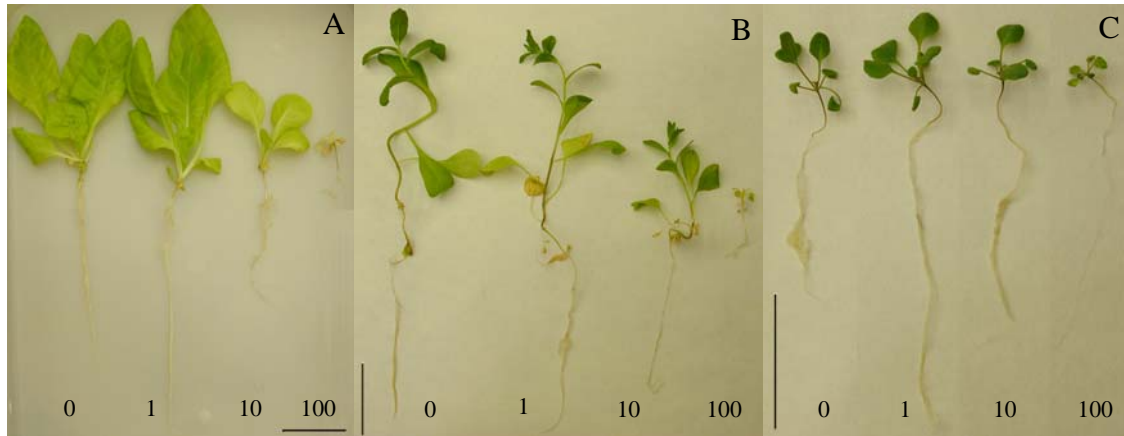

Figure 3.3.2 – Morphological aspect of the plants of *Lactuca sativa* (A), *Thlaspi arvense* (B) and *Thlaspi caerulescens* (C) after 28 days of exposure to 0, 1, 10 and 100 μM of Cd. Bars represent 5 cm.

In general, the exposure to Cd in the nutrient solution led to growth inhibition in the studied plants (Fig. 3.3.3). The growth of lettuce shoots and roots was significantly reduced at Cd concentrations of 10 and 100 μM ($p<0.05$). In the case of *T. arvense* growth inhibition was evident, with statistically significant differences ($p<0.05$) being observed in leaves after an exposure to 10 and 100 μM of Cd and on roots at a Cd concentration of 100 μM ($p<0.05$). Finally, growth in *T. caerulescens* was significantly reduced ($p<0.05$) only after exposure to 100 μM Cd.

Flow cytometric analysis

Cadmium treated plants of *T. caerulescens* and *T. arvense* presented similar nDNA content and FPCV values to those obtained for the control group (see Table 3.3.1). The small differences that were observed were not statistically significant ($p>0.05$). The nDNA contents of *T. caerulescens* and *T. arvense* were estimated to be 0.63 ± 0.006 pg/2C and 1.19 ± 0.005 pg/2C, respectively. To our knowledge, this is the first estimation of nDNA content for the species *T. caerulescens*. FPCV values were much higher in *T.*

caerulescens than in *T. arvense*, which is likely associated with the higher impact that autofluorescent particles (such as chloroplasts) may have in the nuclei with lower fluorescence (those of *T. caerulescens*).
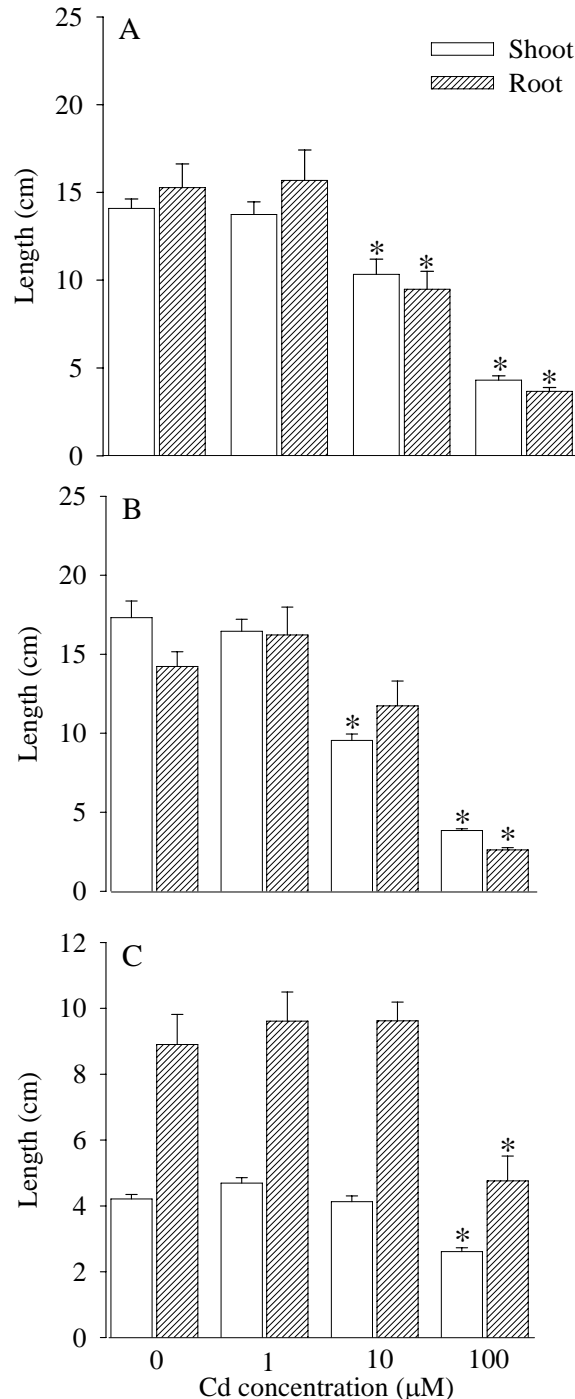

Figure 3.3.3 - Shoot and root length of *Lactuca sativa* (A), *Thlaspi arvense* (B) and *Thlaspi caerulescens* (C) plants after 28 days of exposure to 0, 1, 10 and 100 μM of Cd. Results are expressed as mean ± standard error. (*) Significantly different values when compared with the control group at $p < 0.05$.

In *L. sativa* (Table 3.3.1, Figure 3.3.4), roots exposed to 100 μM Cd presented a statistically significant reduction (p<0.05) in nDNA content (5.89 ± 0.056 pg/2C) when compared to the estimation obtained for the control plants (6.13 ± 0.055 pg/2C). On the other hand, in leaves, no statistically significant differences in nDNA content were obtained between control and exposed plants. There was a statistically significant increase in FPCV after the exposition of leaves to 10 μM Cd and of roots to 100 μM Cd.

Table 3.3.1 - Nuclear DNA content and FPCV of plants exposed to 0, 1, 10 and 100 μM Cd. Results are expressed as mean ± standard deviation (n=3).

Sample Plant species & Organ	Cd (μM)	DNA content (pg/2C)	FPCV (%)
Thlaspi caerulescens Leaf	0	0.63 ± 0.006	8.60 ± 0.441
	1	0.64 ± 0.012	8.71 ± 0.232
	10	0.66 ± 0.002	8.35 ± 0.930
	100	0.65 ± 0.017	9.83 ± 0.990
Thlaspi arvense Leaf	0	1.19 ± 0.005	4.53 ± 0.456
	1	1.17 ± 0.008	4.66 ± 0.269
	10	1.17 ± 0.022	4.91 ± 0.335
	100	1.18 ± 0.027	4.90 ± 0.689
Lactuca sativa Leaf	0	6.41 ± 0.044	3.65 ± 0.287
	1	6.36 ± 0.073	4.26 ± 0.590
	10	6.32 ± 0.077	**4.90 ± 0.512**[*]
	100	6.25 ± 0.194	4.64 ± 0.43
Lactuca sativa Root	0	6.13 ± 0.055	4.77 ± 0.643
	1	6.27 ± 0.045	4.63 ± 0.358
	10	6.23 ± 0.081	4.94 ± 0.283
	100	**5.89 ± 0.056**[*]	**6.63 ± 0.619**[*]

Discussion

Exposure of the three plants to Cd resulted in an array of dose dependent morphological effects that are symptomatic of Cd toxicity in plants, including a strong inhibition of leaf and root growth, chlorosis in the leaves and browning of the roots. This is in agreement with the current knowledge on the effect of this heavy metal in *Thlaspi*

species (Ozturk et al., 2003; Wójcik et al., 2005) and lettuce (Michalska and Asp, 2001) as well as other plant species (Prasad, 1995).

Flow cytometric analyses of the leaves of *T. arvense* and *T. caerulescens* revealed homogeneity in nDNA content and FPCV values, suggesting that no clastogenic damage occurred due to Cd exposure. These results seem to indicate that besides the severe inhibition of growth and the accumulation of Cd in roots and subsequent translocation to the leaves, both *Thlaspi* species possess mechanisms to cope with the Cd toxicity at the cellular level. These mechanisms involve the chelation of Cd by phytochelatins and/or
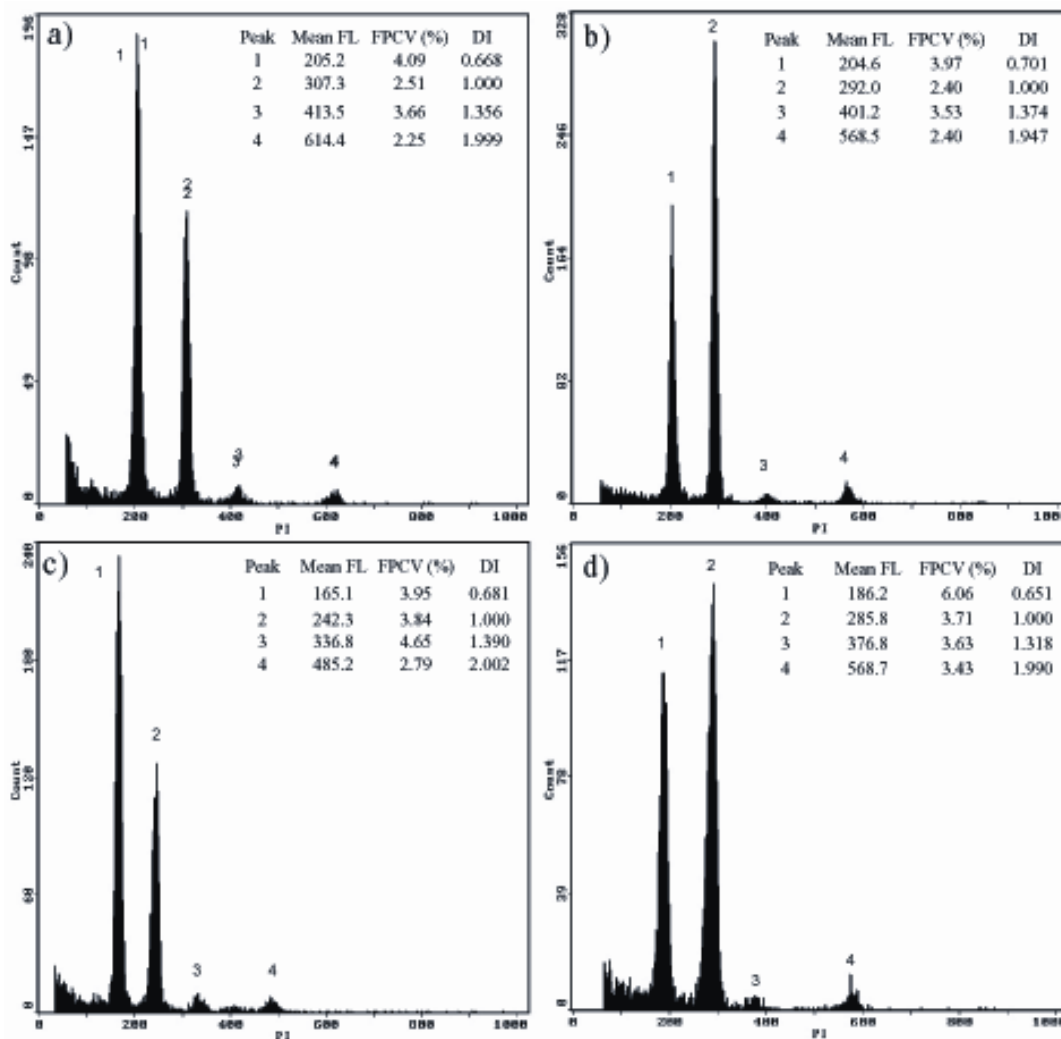
Figure 3.3.4 - Histograms of relative fluorescence intensity obtained after simultaneous analysis of nuclei isolated from *Pisum sativum* cv. Ctirad (as reference standard) and *Lactuca sativa* leaves (a and c) and roots (b and d) exposed to 0 (a and b) and 100 μM Cd (c and d). In all FL histograms four peaks were observed: 1 – nuclei at G_0/G_1 phase of *L. sativa*; 2 – nuclei at G_0/G_1 phase of *P. sativum*; 3 - nuclei at G_2 phase of *L. sativa*; 4 – nuclei at G_2 phase of *P. sativum*. For each peak the following information is given: mean fluorescence in arbitrary units (Mean FL), full peak coefficient of variation (FPCV) and the DNA index (ratio between the mean FL of each peak and the mean FL of the G_0/G_1 peak of the internal standard, DI).

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organic acids and storage in vacuoles (Ueno et al., 2005; Wójcik et al., 2005) and may prevent the accumulation of ionic Cd^{2+} in the cells where it could cause severe genotoxicity either by direct binding to DNA, by indirect inhibition of DNA mismatch repair, or by causing oxidative stress.

Lactuca sativa has been for some time a "model" species for Cd genotoxicity tests in our laboratory. In preliminary trials using the same methodology, no changes in nDNA content and in CV (HPCV) values were detected neither in five-week-old lettuce plants exposed to 100 μM Cd for 14 days (Monteiro et al., 2004) nor in lettuce plants germinated and grown for 2 months in 10 μM Cd and analysed every 15 days (Monteiro et al., 2005). However in the present work, statistically significant changes in nDNA content and FPCV values have been detected. These results suggest that Cd stress may be leading to clastogenic DNA damage as a consequence of loss of chromosome portions, because nDNA content is depressed. Still, caution should be taken in the interpretation of these results as other factors may be governing the observed differences: i) higher condensation of nDNA in exposed tissues, which could lead to a lower binding of PI (a chromatin state sensitive fluorochrome) to DNA structure and thus to a lower estimation of nDNA content (Doležel and Bartos, 2005); ii) higher amounts of secondary metabolites (e.g. phenolic compounds) in the cytosol of cells exposed to Cd. It was previously demonstrated that phenolic compounds affect the fluorescence and light scatter properties of plant nuclei by interfering with the stoichiometric binding of PI to DNA and by aggregating other particles to plant nuclei, which leads to higher CV values (Loureiro et al., 2006). Several authors have already demonstrated that part of the mechanism for metal tolerance in plants involves the production of organic acids and the release of phenolic compounds to the cytosol (Delhaize et al., 1993a; Delhaize et al., 1993b; Mullet et al., 2002). In the particular case of Cd, (Irtelli and Navari-Izzo, 2006) revealed an increase of phenolic compounds in leaves of *Brassica juncea* that were under Cd exposure.

In support of the hypothesis that Cd stress has indeed resulted in DNA damage, previous work by Monteiro et al. (2007) using microsatellite markers demonstrated a mutation rate of 3.7% in roots of lettuce exposed to 10 μM Cd. Moreover, in the present study and in the work of Monteiro et al. (2007), the genotoxic effects occurred mainly in the roots and not in the leaves (except the increase in FPCV registered in leaves of 10 μM Cd-exposed plants). Actually, Cd was accumulated in far greater concentrations (2-fold) in the roots than in the leaves of *L. sativa* exposed to 100 μM Cd, which may well be related with the genotoxicity patterns that were detected. Gichner and co-workers obtained similar results, with Cd exposure inducing DNA damage only in the roots of *Nicotiana tabacum* L.,

but not on the leaves (Gichner et al., 2004), and with the absence of Cd genotoxicity in the leaves of *Solanum tuberosum* L. subjected to short-term treatments with Cd (Gichner et al., 2008). In both studies the authors related the absence of genotoxic effects of Cd in plant leaves, to the lower accumulation of this metal in this organ and to the presence of a better antioxidant defence system that might protect the nuclear DNA in leaf cells from Cd-induced oxidative stress (Gichner et al., 2004; Gichner et al., 2008). Gichner et al. (2004) found that the activity of catalase (an anti-oxidant enzyme) was about 30 times higher in tobacco leaves than in roots, which underscores the differences in sensitivity to Cd exposure in leaves and roots.

Conclusions

The results suggest that on the basis of FCM analysis, a long-term exposure to Cd induced cyto/genotoxicity in *L. sativa* but not in *Thlaspi* spp., highlighting the usefulness of this technique to screen and monitor *in vivo* effects of environmental pollutants. These data have been complemented with other molecular assays (e.g. SSR) which, together with physiological and cytological data, will surely help to give further insights to the results obtained using flow cytometry.

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Trophic transfer of Cd from plants to isopods

Chapter 4

Does subcellular distribution in plants dictate the trophic bioavailability of Cd to *Porcellio dilatatus* (Crustacea, Isopoda)?

Abstract

The present study examined how subcellular partitioning of Cd in plants with different strategies to store and detoxify Cd may affect trophic transfer of Cd to the isopod *Porcellio dilatatus*. The plant species used were *Lactuca sativa*, a horticultural metal accumulator species; *Thlaspi caerulescens*, a herbaceous hyperaccumulator species; and the nonaccumulator, *T. arvense*. Taking into account that differences in subcellular distribution of Cd in plants might have an important role in the bioavailability of Cd to a consumer, a differential centrifugation technique was adopted to separate plant leaf tissues into four different fractions: cell debris, organelles, heat-denatured proteins, and heat-stable proteins (metallothionein-like proteins). Plants were grown in replicate hydroponic systems and were exposed for 7 d to 100 μM Cd spiked with [109]Cd. After a 14-d feeding trial, net assimilation of Cd in isopods following consumption of *T. caerulescens* and *T. arvense* leaves reached 16.0 ± 2.33 and 21.9 ± 1.94 μg/g animal, respectively. Cadmium assimilation efficiencies were significantly lower in isopods fed *T. caerulescens* (10.0 ± 0.92%) than in those fed *T. arvense* (15.0 ± 1.03%). In further experiments, Cd assimilation efficiencies were determined among isopods provided with purified subcellular fractions of the three plants. On the basis of our results, Cd bound to heat-stable proteins was the least bioavailable to isopods (14.4–19.6%), while Cd bound to heat-denatured proteins was the most trophically available to isopods (34.4–52.8%). Assimilation efficiencies were comparable in isopods fed purified subcellular fractions from different plants, further indicating the importance of subcellular Cd distribution in the assimilation. These results point to the ecological relevance of the subcellular Cd distribution in plants, which directly influence the trophic transfer of Cd to the animal consumer.

Keywords: Assimilation efficiency; Centrifugal fractionation; Dietary metal; *Porcellio dilatatus*; Trophically available metal

Introduction

Cadmium (Cd) is a toxic metal that is able to accumulate in soils. It reaches soils mainly from mining (e.g., zinc mining), several industrial activities, and agricultural use of phosphate fertilizers and sewage sludge. Cadmium can be strongly cytotoxic and mutagenic to plants and animals, interfering in a wide variety of metabolic processes in plants and animals (Prasad, 1995). The toxic action of Cd is facilitated through its propensity to bind to the sulfhydryl groups of proteins and substitute for essential metals such as Zn in metalloenzymes and through the inhibition of DNA mismatch repair and production of reactive oxygen species (Prasad, 1995; Jin et al., 2003).

However, some plants are able to accumulate Cd in edible tissues at high concentrations without showing symptoms of toxicity, thereby introducing the metal into the food chain by trophic transfer. Plants use sequestration mechanisms to detoxify metals and prevent interaction with important biomolecules. These mechanisms include binding to proteins and other ligands (e.g., metallothionein (MT)-like proteins) and storage of metals into metabolically inactive cellular sites, such as granules inside vacuoles (Prasad, 1995; Clemens et al., 2002). These two major detoxification pathways in plants may have implications for the trophic transfer to animal consumers.

Extensive studies have been made on Cd bioaccumulation and toxicity to organisms, and models have been developed to predict the bioavailability and toxicity of metals (Paquin et al., 2002; Wang and Rainbow, 2006). However, they do not yet consider the contribution of the dietary route of metal exposure and the relevance of the complexity of internal metal subcellular partitioning in prey, which may significantly affect the subsequent trophic transfer of metals to predators (Wang and Rainbow, 2006). In an attempt to develop a predictive model for the dietary accumulation of metals in marine food chains, a subcellular fractionation procedure has gained popularity (Wallace et al., 1998; Wallace et al., 2003; Wallace and Luoma, 2003; Cheung et al., 2006; Seebaugh et al., 2006; Zhang and Wang, 2006; Steen Redeker et al., 2007). Wallace and Luoma (2003) postulated that Cd associated with the subcellular fractions organelles, heat-denatured proteins (HDP), and heat-stable proteins (HSP) of prey was trophically available metal (TAM) and was assimilated at an efficiency of approximately 100% by the predator, while Cd bound to metal-rich granules was less bioavailable to predators (Wallace et al., 1998; Wallace and Luoma, 2003). This is considered a simple and pragmatic approach in the prediction of trophic transfer of metals and a first step toward a practical tool that could explain most of the variability observed in metal accumulation and

toxicity in organisms (Vijver et al., 2004). However, there is a need to apply this approach to other food chains in order to verify its utility.

To the best of our knowledge, trophic transfer of metals from plants through terrestrial food chains has not received much attention, despite being of great relevance. However, there have been some studies focusing on the cellular and subcellular distribution of metals in plants. Subcellular localization of Cd in plants has been assessed through subcellular fractionation (Weigel and Jäger, 1980; Ramos et al., 2002), and other techniques such as autoradiography (Cosio et al., 2005), energy dispersive X-ray microanalysis (Kupper et al., 2000; Cosio et al., 2005; Wójcik et al., 2005a), and electron energy loss spectroscopy (Liu and Kottke, 2003). In lettuce 64% of accumulated Cd is partitioned to cell walls (Ramos et al., 2002), and both *Lactuca sativa* L. and *Thlaspi arvense* L. possess detoxification mechanisms in which phytochelatins (PC) play an important role (Ebbs et al., 2002; Maier et al., 2003). *Thlaspi caerulescens* was found to mainly store Cd in electron-dense granules inside vacuoles by means of complexation with malate (Ma et al., 2005; Ueno et al., 2005). Cadmium distribution reflects internal processing that occurs during Cd uptake and accumulation in plants and can be used to interpret metal toxicity and tolerance. In addition, knowledge of how organisms handle their accumulated metal may allow more accurate predictions of the eventual transfer of metals to higher trophic levels (Wallace and Luoma, 2003).

In the present study we tested the hypothesis that subcellular distribution in plants will dictate the trophic bioavailability of Cd to isopods. To achieve this, the transfer of Cd in a food chain comprised of plant leaves and a detritivorous animal, the isopod *Porcellio dilatatus*, was examined with the following specific aims: to investigate the relevance of different subcellular distribution in plants in the assimilation efficiency (AE) of the isopod, and to determine the assimilation of Cd from each subcellular fraction of plants, to directly assess the role of each fraction in the assimilation of Cd by isopods. For this, three plants with different patterns of Cd accumulation were studied: *T. caerulescens* J. & C. Presl is a hyperaccumulator of several metals, including Cd, and is a plant commonly used as a model in metal transport and accumulation studies with a view to use in phytoremediation (Pence et al., 2000; Assunção et al., 2003; Zhao et al., 2003); the related nonaccumulator *T. arvense*; and lettuce (*L. sativa*), which is a Cd-accumulating plant and an important human food crop. The terrestrial isopod *P. dilatatus*, inhabiting the upper layer of the soil and surface leaf litter, is quite abundant in southern Europe and an important representative of the detritivorous soil fauna. Moreover, isopods have an enormous

capacity to accumulate large body burdens of toxic metals (Donker et al., 1990) making them a valuable model for the examination of metal assimilation and accumulation.

Material and methods

Cadmium trophic transfer from plants to isopods was assessed in three different and complementary experiments: assessment of Cd subcellular distribution in the plants *L. sativa*,

T. caerulescens, and *T. arvense*; feeding experiment 1, an assessment of isopod (*P. dilatatus*) Cd AEs from plant leaves of *T. caerulescens* and *T. arvense*; feeding experiment 2, an assessment of isopod Cd AEs from individual subcellular fractions of *L. sativa*, *T. caerulescens*, and *T. arvense* plant leaves.

Plant culture and growth conditions

Seeds from *L. sativa* (Reine de Mai de Pleine Terre) (Oxadis, Saint Quentin Fallavier, France), *T. caerulescens* (Saint-Féix-de-Pallièes, Ganges, France), and *T. arvense* (Amsterdam, The Netherlands) were germinated under dark conditions on filter paper moistened with distilled water. After germination, seedlings were transferred to perlite support media in polystyrene seedling trays floating on nutrient solution. The trays were maintained in a plant growth chamber (APT.line® KBWF, Binder, Tuttlingen, Germany) with controlled temperature (20 ± 1°C), 16:8 h light:dark photoperiod, 80% humidity, and 200 μmol/m^2/s light intensity. Lettuce was grown in modified Hoagland's nutrient solution according to Monteiro et al. (2007). *Thlaspi* plants were grown on modified Rorison nutrient solution with the following basic composition (μM): 1500 KNO_3, 1000 $Ca(NO_3)_2 \cdot 4H_2O$, 500 $NH_4H_2PO_4$, 500 $MgSO_4.7H_2O$, 46.25 H_3BO_3, 0.77 $ZnSO_4.7H_2O$, 0.36 $CuSO_4.5H_2O$, 0.37 $NaMoO_4.2H_2O$, 10.12 $MnCl_2.4H_2O$, 17.91 $FeCl_3$. Before Cd exposure, *L. sativa* and *T. arvense* were grown for five weeks. Since *T. caerulescens* grows more slowly, to achieve a similar plant biomass, this plant was grown for nine weeks before Cd exposure. Plants were exposed to Cd for 7 d; the respective nutrient solution was supplemented with $Cd(NO_3)_2$ at 100 μM and 77 nCi/ml ^{109}Cd (Amersham Biosciences, London, England). Control plants were maintained in nutrient solution with no addition of Cd. The nutrient solution was continuously aerated and changed on alternate days to avoid depletion of nutrients and changes in Cd concentration during the course of the exposure to the metal (Mann et al., 2005). After 7 d of exposure, plant

leaves were frozen in liquid nitrogen and stored at -80ºC until subcellular fractionation analysis or dried at 60ºC for subsequent use in isopod feeding experiment.

Subcellular Cd distribution in plant leaves

Differences in plant subcellular Cd distribution were investigated by subjecting plant leaves to the following procedure. Replicated (n = 6–10) 1-g samples of leaves from each species were each reduced to powder with liquid nitrogen using a mortar and pestle and homogenized in 4 ml of buffer containing 0.25 M sucrose, 1 mM dithioerythritol, and 50 mM Tris-HCl (pH 7.5) (Weigel and Jäger, 1980). All steps were performed at 4ºC and according to Weigel and Jäger (1980) with some modifications based on Wallace et al. (2003). The resulting homogenate was filtered through nylon cloth (50 μm) and washed twice with homogenization buffer. The filtrate was then centrifuged at 500 g for 5 min. The resulting pellet combined with the residue of the filtration contained mainly cell walls, tissue fragments, and other cellular debris and was designated as cell debris. The supernatant of the first centrifugation step, containing the cytosol, was then centrifuged at 100,000 g for 30 min to sediment organelle components (i.e., chloroplasts, mitochondria). The pellet was designated as the organelle fraction. The 100,000 g supernatant containing the cytosol fraction was then heat denatured at 80ºC for 10 min and cooled on ice for 15 min. Heat-denatured proteins were separated from the HSPs (MT-like proteins) by centrifugation at 50,000 g for 10 min. All fractions were assayed for Cd by radiospectrometry and metal contents were used to calculate distributions of Cd within plant leaves based on summation of Cd content of the four subcellular fractions.

Isopod culture and feeding test conditions

Cadmium AEs in isopods fed *T. arvense* and *T caerulescens* leaves was assessed in this experiment. A similar experiment has already been performed in *L. sativa* leaves by Calhôa et al. (2006). In the current experiment we used *P. dilatatus* from laboratory cultures derived from individuals collected in a secondary coastal dune system in central Portugal. Isopods were maintained in plastic containers with sand substrate and kept at 20ºC with a 16:8 h light:dark photoperiod. Alder leaves were provided as a source of food and distilled water was added to maintain moisture. Twenty juvenile isopods (weight range: 14–19 mg) per treatment were selected and isolated individually in test boxes for 24 h before the test without food to purge the gut. No distinction was made between sexes.

Polyethylene terephthalate boxes (diameter 85 mm × 43 mm; Termoformagen, Leiria, Portugal), containing a thin layer of plaster of Paris mixed with activated charcoal (8:1 v/v) for the retention of added moisture were used as individual test boxes. Food was replaced every week to prevent consumption of food that had become inoculated with fungi; fungi growth may alter Cd bioavailability. Fecal pellets were collected every day to prevent coprophagy.

Feeding experiment 1

Leaves from control and Cd-exposed plants of *T. arvense* and *T. caerulescens* were cut into individual portions weighing approximately 10 mg (range 8.1–10.9 mg dry wt), assayed for ^{109}Cd (438.9 ± 88.76 and 236.2 ± 53.30 μg Cd/g dry weight (mean ± standard deviation) in Cd-exposed *T. arvense* and *T. caerulescens*, respectively) and moistened before being placed in test boxes. Animals were fed for a period of 14 d exclusively on leaves according to treatment. Food was replaced every week with fresh leaves and the remains of food were dried (2 d at 60ºC), weighed and analyzed for Cd by radiospectrometry. After 14 d, isopods were left for 24 h without food to purge their guts and were then weighed and analyzed for Cd. Fecal pellets were collected and dried (2 d at 60ºC) to be weighed. Data on isopod, fecal pellet, and leaf mass were used to determine indices of isopod growth, food consumption, and AE. Plant AE by isopods was calculated as:

$$AE_{plant} = (C_{plant} - F)/C_{plant} \times 100 \tag{1}$$

where C_{plant} is the mass of plant leaf consumed by isopods, and F is the mass of fecal material produced. Radiospectrometry data obtained from the isopods and food were used to determine indices of Cd AE. Cadmium AE was calculated as:

$$AE_{Cd} = I_{Cd}/C_{Cd} \times 100 \tag{2}$$

where I_{Cd} is the amount of Cd within the isopod at the termination of the feeding trial, and C_{Cd} is the amount of Cd consumed.

Feeding experiment 2

Leaves of *L. sativa*, *T. arvense*, and *T. caerulescens* exposed to Cd and were subjected to subcellular fractionation as described above. The four different fractions obtained (cell debris, organelles, HDP, and HSP) were mixed (1:2) with a gelatine solution prepared from 2.5 g gelatine powder (VWR Prolabo, Fontenay Sous Bois, France) and 12.5 ml ultra pure water and were then mixed by vortexing (Wallace and Lopez, 1996). As a supernatant, HSP fraction presented higher volume than the other pellet fractions, therefore these fractions were concentrated by evaporation under a stream of nitrogen before being mixed with gelatine. Aliquots of 7 μl of the mixture (fraction and gelatine) were pipetted onto Parafilm® (Pechiney Plastic Packaging, Menasha, WI, USA), forming gelatine discs that were stored frozen at -20ºC until required (Wallace and Lopez, 1997). Additionally, gelatine discs were prepared containing either gelatine alone or a mixture of homogenate of control plant leaves and gelatine solution (1:2) to be used as a control foods (control 1 and control 2, respectively).

Isopods were fed gelatine discs for a period of 28 d. Because some of the fractions were likely to contain very small quantities of Cd, a longer duration was chosen for this feeding experiment to ensure that accumulated Cd was above the detection limits of analysis. This longer period of exposure will not influence Cd AE since elimination of Cd is negligible in isopods (Witzel, 1998) and therefore allows for comparisons with the above experiment. Gelatine discs were previously assayed for Cd by radiospectrometry before being fed to isopods and were replaced every week; the remains of food were also assayed for Cd. After 28 d isopods were left in test boxes without food to purge their guts, and after 24 h were weighed and analyzed for Cd. The Cd content and mass data obtained for isopods and the gelatine discs were used to determine isopod growth and Cd AE as described above.

Estimation of AE of Cd from whole plant leaves (AE_{whole}) based on AE for individual fractions was calculated by the following mass balance equation:

$$AE_{whole} = \sum AE_i \times Cd_i \tag{3}$$

where AE_i is the Cd AE of isopod fed a purified fraction *i* and Cd_i is the percentage of Cd in the subcellular fraction *i* (Wallace and Lopez, 1997; Zhang and Wang, 2006).

Cadmium analysis

All samples of subcellular Cd distribution were placed in 10.4-ml polycarbonate tubes (Beckman instruments, Fullerton, CA, USA) and analyzed for [109]Cd in a Genesis Gamma-1 bench-top gamma counter (Laboratory Technologies, Maple Park, IL, USA). Sections of dry plant leaf and gelatine discs (before feeding and remains after feeding), isopods, and fecal material were placed in 3.5-ml Röhren tubes (Sarstedt, Newtown, NC, USA) and were analyzed for Cd by radiospectometry. Data on Cd content of leaves, isopods, and fecal material were used to determine indices of Cd consumption and AE.

Cadmium concentration in the hydroponic culture medium was verified by inductively coupled plasma spectroscopy (Horiba Jobin Yvon, 70 Plus, Longjumeau, France) compared with radiospectometry measurements and used as a reference for calculations of total Cd content.

Statistical analysis

Statistical analysis was carried out by *t* tests or one-way analysis of variance and Tukey post hoc tests as appropriate. When necessary, data were transformed to achieve normality and equality of variance. When these criteria were not satisfied even with transformed data, nonparametric tests were performed, namely Kruskal–Wallis one-way analysis of variance followed by Dunn's method post hoc test. SigmaStat[®] (Ver 3.01, SPSS, Chicago, IL, USA) was used to perform all statistical tests.

Results

Cadmium subcellular distribution in plant leaves

Cadmium subcellular distribution in *L. sativa*, *T. arvense*, and *T. caerulescens* in relation to the total Cd accumulated in leaves is shown in Figure 4.1. The cell debris fraction represents an important pool of the total accumulated Cd (28.0–43.8%) in all the plants analyzed. In lettuce leaves the cell debris fraction was the dominant pool for Cd storage, displaying the highest percentage of the total Cd present in leaves, which is significantly different from the other fractions ($p<0.05$).
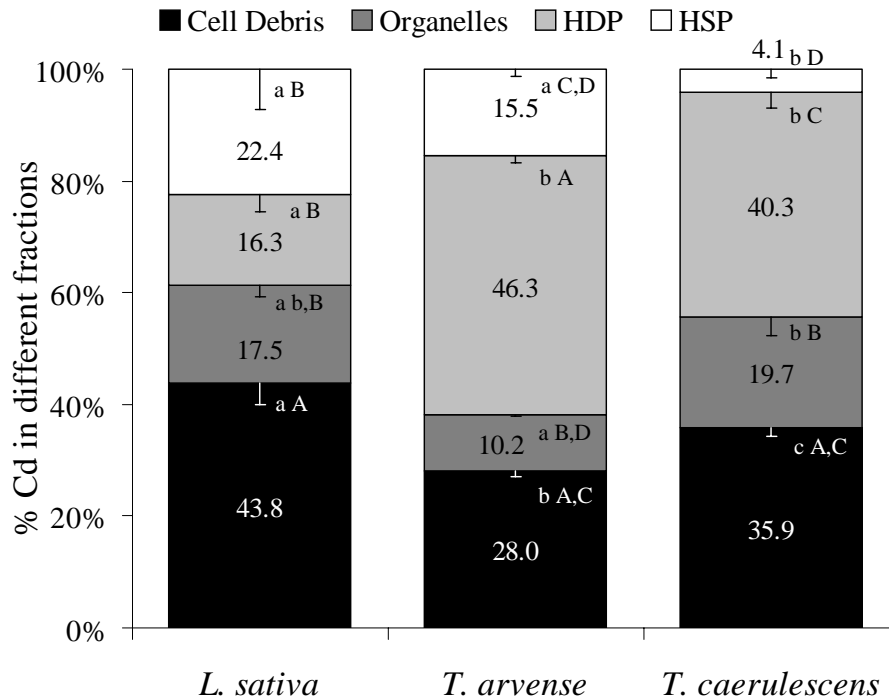
Figure 4.1 - Subcellular Cd distribution in *Lactuca sativa*, *Thlaspi arvense*, and *Thlaspi caerulescens* in relation to the total accumulated Cd. In each fraction numbers represent mean (%), error bars represent standard error for each fraction (n=6–10).

The organelle fraction accounted for 10.2 and 19.7% of the Cd in *T. arvense* and *T. caerulescens*, respectively. In *T. arvense* the organelles fraction accounted for the lowest percentage of Cd in the leaves of this plant, being significantly lower than the percentage of Cd in the cell debris and HDP fractions (p<0.05).

The HDP fraction was the dominant fraction for Cd binding in *T. arvense* (46.3%) and *T. caerulescens* (40.3%), but accounted for a significantly lower percentage of Cd (16.3%) in lettuce leaves (p<0.001).

The HSP fraction contained the lowest percentage of Cd in *T. caerulescens* leaves (4.1%), when compared to the other fractions in the same plant (p<0.001) and also, when compared with the same fraction in the leaves of *L. sativa* and *T. arvense* (p< 0.05).

Feeding experiment 1

Isopod growth

During the 14 d of the feeding experiment with *Thlaspi* leaves isopods all increased in weight, except those provided with control leaves of *T. caerulescens* (Fig.

4.2A). Growth of isopods fed *T. caerulescens* control leaves was significantly different from that of isopods fed *T. arvense* control leaves (p<0.05). No significant difference was found between growth of isopods fed control and treated leaves of both plants. Mortality was below 10% in all treatments.
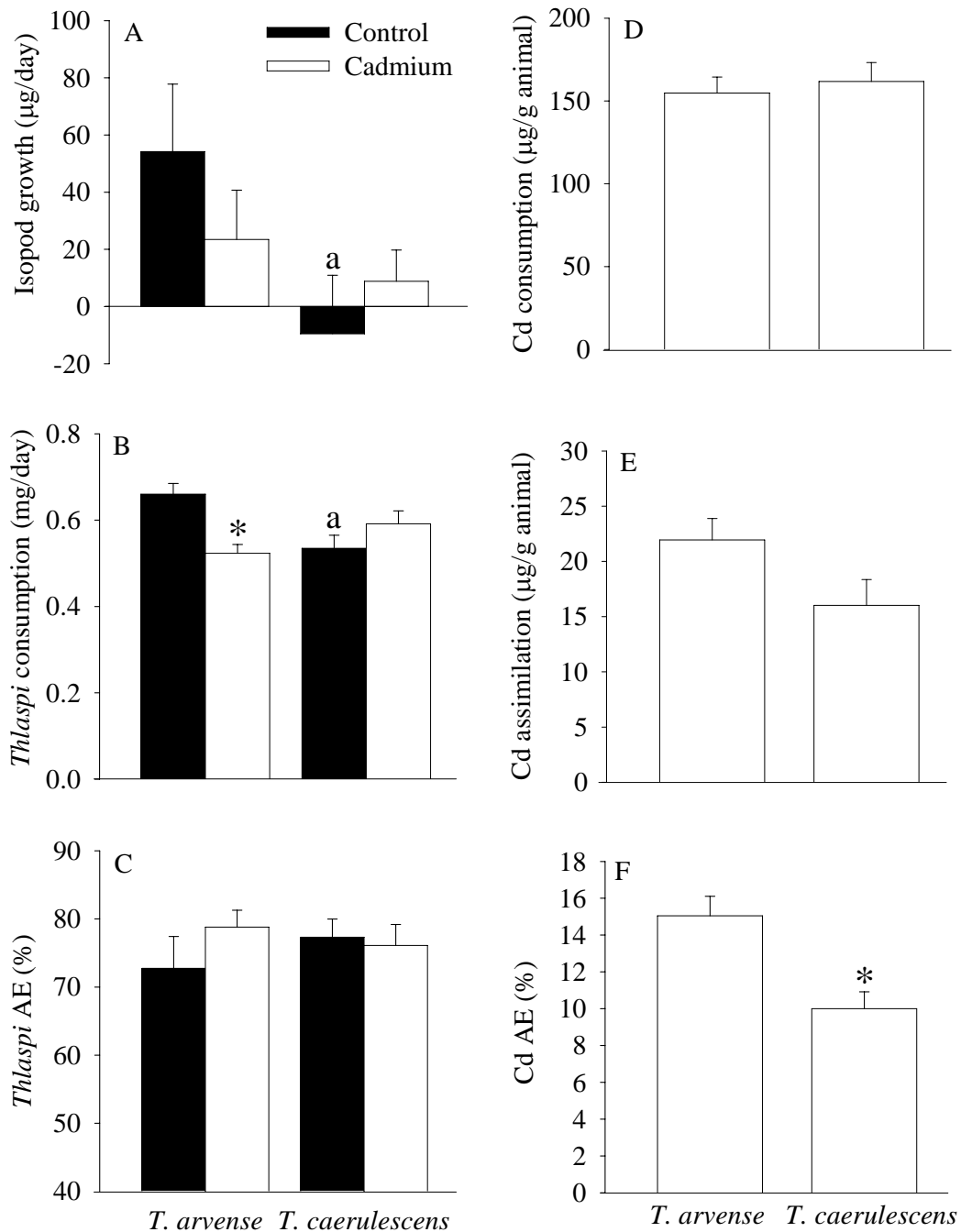
Figure 4.2 - Growth (A), *Thlaspi* plant leaf consumption (B), plant assimilation efficiencies (C), Cd consumption (D), Cd assimilation (E), and Cd assimilation efficiencies (AEs) (F) of isopods fed exclusively control or Cd-treated *T. arvense* and *T. caerulescens* leaves for 14 d. Bars represent mean ± standard error (n=20). (*) Significantly different from control (B) or between treatments (F), p<0.001. (a) Significant difference between plant controls, p<0.05.

Plant consumption and assimilation efficiency

Plant consumption by isopods was significantly different between the *Thlaspi* species (p<0.01). Isopods fed *T. caerulescens* consumed less than isopods fed *T. arvense* leaves (Fig. 4.2B). Treatment with Cd significantly reduced plant consumption for *T. arvense* (p<0.001) but not for *T. caerulescens* (p>0.05). Plant AE was not significantly different between isopods of the different treatments (p<0.05). Isopods fed controls leaves displayed plant AEs of 73 ± 4.6% for *T. arvense* and 77 ± 2.7% for *T. caerulescens* (Fig. 4.2C).

Cadmium consumption, assimilation, and AE

Cadmium consumption by isopods (Fig. 4.2D) was similar between the plant species studied. Isopods fed *T. arvense* and *T. caerulescens* consumed (mean ± standard error) 155 ± 9.6 and 162 ± 11.5 μg/g animal, respectively. Assimilation of Cd was lower in isopods fed *T. caerulescens* (Fig. 4.2E) than in those fed *T. arvense*, however a *t* test indicated a marginally nonsignificant difference (p=0.058) between treatments. The AE of Cd by isopods fed *T. caerulescens* leaves, 10.0 ± 0.92%, was significantly lower than the AE of Cd by isopods fed T. arvense leaves, 15.0 ± 1.03% (p<0.001) (Fig. 4.2F).

Feeding experiment 2

Growth

The effects of subcellular Cd distribution in plants on Cd AE were further determined in the experiments when isopods were fed pure subcellular fractions (cell debris, organelles, HDP, and HSP) derived from the leaves of *L. sativa*, *T. arvense*, and *T. caerulescens*. Isopods displayed positive growth in all treatments with the three different plant species studied (Fig. 4.3A). Isopods fed pure gelatine discs (control 1) grew less than isopods fed gelatine added with the respective plant leaf homogenate (control 2) or subcellular fractions. However this difference was not always significant. Growth among isopods in the control 2 treatment was significantly higher than in control 1 for isopods fed *T. caerulescens* leaves. Isopods fed the cell debris fraction of the three plants displayed significantly higher growth (p<0.05) when compared to control 1, as was the case with the organelles fraction of *T. arvense* (p<0.05). Isopods fed the organelle fraction of *T. caerulescens* displayed significantly lower growth when compared to the respective C2

(p<0.05). Furthermore, when comparing growth in isopods fed different subcellular fractions of *L. sativa*, HDP and HSP fractions presented a significant reduction in isopod growth when compared to the cell debris fraction (p<0.05).
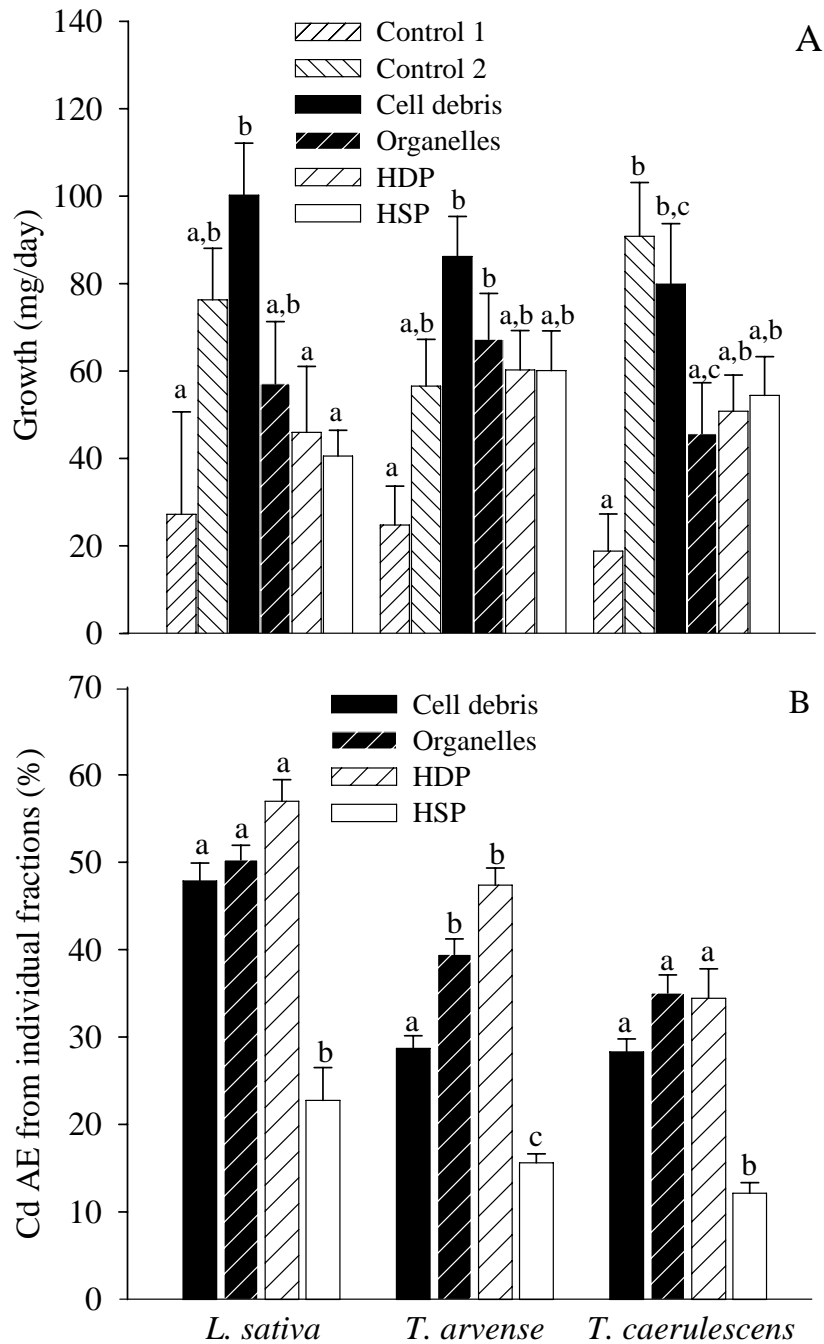
Figure 4.3 – Isopods fed exclusively separated subcellular fractions (heat-denatured proteins (HDP) or heat-stable proteins (HSP)) of *Lactuca sativa*, *Thlaspi arvense*, and *Thlaspi caerulescens* leaves for 28 d. (A) Isopod growth, among control isopods fed pure gelatine discs (Control 1) or gelatine with control leaf homogenate (Control 2), or isopods fed subcellular fractions. Statistical differences: (a) different from Control 1 (p<0.05), (b) different from Control 2 (p<0.05), (c) different from D (p<0.05). (B) Isopods Cd assimilation efficiencies (AE), bars with different letters are significantly different (p<0.05) for the same plant. Bars represent mean ± standard error.

Cadmium AE from purified subcellular fractions

As a general pattern across plant species, isopods fed the HSP fraction displayed significantly lower AE compared to the AEs for the other fractions (p<0.001) (Fig. 4.3B). Isopods fed the cell debris fraction displayed similar AEs to those fed the organelles and HDP fractions for *L. sativa* and *T. caerulescens*, but for *T. arvense*, AE for the cell debris fraction was significantly lower than that for the organelles and HSP fractions (p<0.001). The order of Cd AE from each subcellular fraction was similar across the three species analyzed, HSP (22.8%) < cell debris = organelles = HDP (57.0%) for *L. sativa*; HSP (15.6%) < cell debris < organelles = HDP (47.4%) for *T. arvense*; and HSP (12.1%) < cell debris = HDP = organelles (35.0%) for *T. caerulescens*.

Estimation of Cd AEs

Figure 4.4 presents a comparison between the predicted and the actual Cd AEs in isopods. Estimation of Cd AEs through mass balance equation (Eqn. 3) using data obtained in feeding experiment 2 indicated that isopods would be expected to assimilate about 44.1, 36.4, and 26.4% of Cd from *L. sativa*, *T. arvense*, and *T. caerulescens*, respectively. The trend displayed by actual AEs (data from feeding experiment 1 and Calhôa et al. (2006)) was similar to the estimated AEs; *L. sativa* was the plant species displaying higher Cd AEs in isopods; whereas, Cd in *T. caerulescens* presents the lowest AEs. However, in both *Thlaspi* species AEs seem to be overestimated, whereas in *L. sativa* the estimation of Cd AE is slightly lower than the observed in the study performed by Calhôa et al. (2006).

Figure 4.4 also displays the individual contribution of each subcellular fraction to the total estimated AEs. It is apparent from Figure 4.4 that Cd sequestered in HSP fraction constitutes only a minor source of the Cd assimilated by isopods from the three plant species studied. In *L. sativa*, the cell debris fraction, which includes cell walls and granules, is the major source of Cd, accounting for about half the estimated Cd AE (21% out of the total estimated 44.1%). In *Thlaspi* species, HDP displayed the highest contribution to the estimated AEs, accounting for about half of the estimated Cd AE by isopods (21.9% out of the total 36.4% for *T. arvense* and 13.0% out of the total 26.4% for *T. caerulescens*).

Figure 4.4 – Estimated and whole-leaf Cd assimilation efficiencies (AEs) in the isopod *Porcellio dilatatus* fed *Lactuca sativa*, *Thlaspi arvense*, and *Thlaspi caer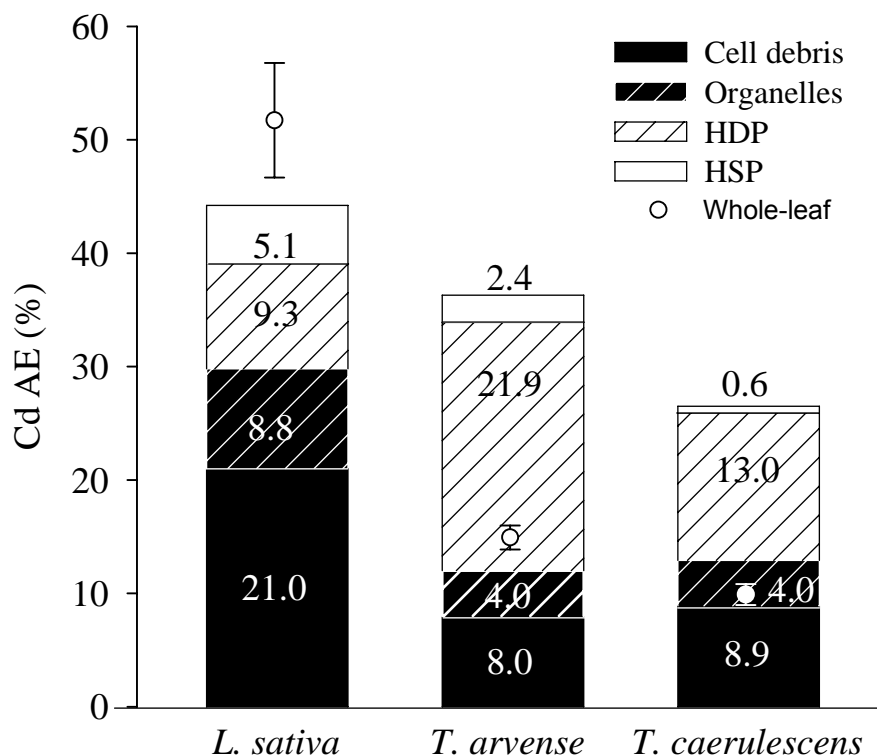ulescens*. Error bars represent standard error (n=20). The estimated Cd (AEs) were calculated from the isopod Cd AEs from individual fractions (heat-denatured proteins (HDP] or heat-stable proteins (HSP)) and the subcellular distributions of cadmium in the plant (see mass balance equation, Eqn. 3). Whole-leaf Cd AEs from *L. sativa* leaves is from the work of Calhôa et al. (2006).

Discussion

Predicting the bioavailability of tissue-bound metals to a consumer or predator is fraught with difficulty, as several important aspects of trophic transfer must be considered. Different prey species will accumulate and partition metals in varying ways depending on the detoxification mechanisms employed. The subsequent bioavailability of those partitioned metals to a consumer will be dictated by digestive and assimilative mechanisms of the digestive tract. Added to this complexity is the varying ability of consumers to discriminate between different foods and contaminants, their nutritional status at the time of consumption, the degree of exposure, and exposure history for the metal in question, all of which can influence the degree of metal assimilation. These various factors are reflected in the wide variety of Cd AEs that have been reported in organisms fed biologically contaminated food, ranging from 1% in rats fed snail viscera

(Hispard et al., 2008), to 52% in the isopod *P. dilatatus* fed lettuce (Calhôa et al., 2006), and up to 76.2 to 94.2% for whelk *Thais clavigera* fed five different species of prey (Cheung and Wang, 2005). In the present study we have shown that the centrifugal fractionation techniques previously advocated by Wallace et al. (2003), can be used to explain the pattern of Cd assimilation in isopods fed different plant species contaminated with Cd.

Subcellular distribution of Cd in plants

Lactuca sativa

In our work, Cd was found mainly in the cell debris fraction of *L. sativa* leaves, which was obtained from the residue of filtration and the 500 g pellet of the filtrate and includes cell walls and cell debris. These results are in good agreement with those obtained by Ramos et al. (2002) for *L. sativa* (cv. Grandes Lagos). Using a subcellular fractionation method similar to the one used in the present study, they found 64% of accumulated Cd associated with the fraction named cell wall fraction, which is equivalent to the cell debris fraction in the present study. Ramos et al. (2002) also found approximately 12% of Cd associated with chloroplasts, which also is consistent with the proportion of Cd found in the organelles fraction in the present study. Lettuce also had a substantial proportion of Cd associated with the HSP fraction, and this is consistent with reports that have shown a strong induction of PCs following Cd exposure (Maier et al., 2003).

Thlaspi caerulescens

In *T. caerulescens* (ecotype Ganges), Ma et al. (2005) found that mesophyll was a major storage site of Cd in leaves (65–70% of total Cd) and through the isolation of protoplasts and vacuoles they showed that most of the Cd in the mesophyll cells was localized in the protoplast (91%), and within this partition, 100% was inside the vacuoles. Accordingly, Wójcik et al. (2005a), using energy dispersive X-ray microanalysis, found Cd only in electron-dense deposits inside vacuoles of *T. caerulescens* leaves, and suggested that vacuoles are the main compartment of Cd storage and detoxification in these plant organs. The form of vacuolar storage was determined to be as a complex with malate (Ueno et al., 2005). In the present study, only a relatively small proportion of the Cd was

partitioned to the organelles fraction; however, it seems likely that large vacuoles would not survive the homogenization procedures used to prepare our fractions. Therefore, any Cd bound to small molecules like malate, is likely to remain suspended within the cytosol and ultimately appear in the HSP fraction, or, if Cd-malate remains as insoluble electron-dense deposits, which seems likely at the pH used in the fractionation buffer (pH 7.5), it will remain in cell debris fraction rather than in HSP. In the present study, Cd bound to HSP, which is presumed to contain predominantly MT-like proteins such as PCs, represents a very low percentage of total accumulated Cd (4.1%). This result is supported by previous studies that indicated that PCs have no role in the mechanism of *T. caerulescens* tolerance to Cd, since total PC concentration were generally low compared to Cd concentrations (Ebbs et al., 2002; Wójcik et al., 2005b). Indeed the Cd found within HSP may be at least partially explained by the presence of small Cd bound molecules of vacuolar origin (Clemens et al., 2002; Ueno et al., 2005). Apart from the cell debris fraction which contained 31.5 to 34.9% of accumulated Cd, HDP seems to be the major site for Cd binding in *T. caerulescens*, suggesting an important role for non–heat-stable proteins in Cd accumulation in this plant. Identification of the Cd-binding components in this fraction might explain the role of HDP in *T. caerulescens* Cd accumulation.

Thlaspi arvense

In contrast to *T. caerulescens*, PCs are known to be an important component in the response to Cd by *T. arvense*. Results obtained by Ebbs et al. (2002) for *T. arvense* were consistent with a PC-mediated response; PC concentrations in *T. arvense* (15.5–42.5%) being 2 to 3-fold higher in this species than in *T. caerulescens*. This difference is reflected in our data that shows a relatively high proportion of Cd within the HSP fraction.

Cadmium assimilation from plants

Other than determining Cd subcellular distribution in plant leaves, this study also demonstrated how this distribution can influence Cd assimilation in isopods. Several studies have investigated the effects of Cd distribution within prey on Cd assimilation by predators in aquatic food chains (Wallace and Lopez, 1996, 1997; Wallace et al., 2003; Wallace and Luoma, 2003; Cheung et al., 2006; Wang and Rainbow, 2006; Zhang and Wang, 2006). A shift in subcellular distribution can have an important impact on the trophic transfer of metals. To the best of our knowledge, this is the first study applying this

approach to a plant–animal food chain. It is important to note that metal assimilation is often assessed through pulse-chase techniques that attempt to assess metal assimilation prior to elimination. In both of the feeding experiments of the present study, the assimilation phase continued for an extended period and accumulation of Cd occurred as a consequence of net assimilation. Elimination of Cd in a closely related species, *Porcellio scaber* (and other terrestrial isopods), is known to be negligible, and the error in the calculation of AE is expected to be small (Hames and Hopkin, 1991; Witzel, 1998).

Confounding effects

Nutritional quality of the food is known to affect assimilation of contaminants (Wang and Fisher, 1999). Although consumption and assimilation of both *Thlaspi* species were similar, isopods fed uncontaminated *T. arvense* displayed significantly higher growth than those fed uncontaminated *T. caerulescens*. However, no significant difference in growth was found among isopods fed contaminated leaves of either species. Therefore, food nutritive quality was unlikely to be an important confounding factor in this study. Results of consumption by isopods of *Thlaspi* plants and the respective AEs are in the range of those obtained for lettuce by Calhôa et al. (2006) in a similar feeding study with the same isopod species.

Cadmium assimilation efficiencies

In the present study, plant species with different detoxifying mechanisms and accumulation patterns were studied in order to examine if there were particular relationships between subcellular Cd distributions in plants and Cd AE by isopods. Wallace and Luoma (2003) recently introduced the concept of TAM and defined it as a combination of fractions organelles, HDP, and HSP. They deduced a 1:1 relationship between the percentage of Cd in TAM of several invertebrate prey items (bivalve and resistant and nonresistant oligochaetes) and Cd AE by the predator shrimps *Palaemonetes pugio* and *Palaemon macrodactylus*. In further experiments, Seebaugh et al. (2005) found a weaker relation between Cd deposition in the proposed TAM fraction of prey *Artemia franciscana* (about 63%) and Cd AE by the predator *Palaemonetes pugio* (37%). Cheung et al. (2006) have also found a significant positive correlation between the Cd subcellular distribution in HSP and Cd body concentration in the welk *Thais clavigera* fed the snail *Monodonta labio*. However, this type of relationship between the proposed

TAM fraction or individual fractions and Cd AE is not universal. In the present study no statistically significant correlation was found between Cd AE by isopods and Cd present in individual or combined subcellular fractions (data not shown). Performing a similar approach for three metals, Zhang and Wang (2006) found a positive correlation for Se and Zn from the combination of HDP and HSP fractions, but for Cd no relationship was found between AEs and any of the subcellular fractions or a combination of fractions. These authors suggest that this result for Cd was due to the low AE of Cd in the marine fish *Terapon jarbua* fed different prey types (copepods, barnacles, clams, mussels, and fish viscera).

Assimilation efficiencies were reasonably well predicted on the basis of metal subcellular distribution and from AE of each subcellular fraction in studies with Cd (Wallace and Lopez, 1997) and other metals (Zhang and Wang, 2006). Two feeding studies were conducted in the present study; one with whole leaves (*T. arvense* and *T. caerulescens*) contaminated with Cd, and a second with individual fractions (*T. arvense*, *T. caerulescens*, and *L. sativa*). Figure 4.4 presents for each of the three species both the Cd AE for whole leaves and the estimated Cd AEs obtained through mass balance equation (Eqn. 3), using Cd AE of isopods fed purified fractions and the percentage of Cd in the each subcellular fraction. For the purpose of comparing whole leaf AEs and estimated Cd AEs, we included in Figure 4.4 data from a previous study by Calhôa et al. (2006) that generated AEs for whole lettuce leaf contaminated with Cd. The data from that study are comparable to those collected in the present study because there is a great deal of consistency between the studies, including the nutritional status and source of the isopods, the physical conditions under which the trials were conducted, and the concentrations of Cd in the respective leaves. The concentration of Cd in the food is of particular importance as it is known to affect Cd AE in isopods. Specifically, AE is reduced with increasing Cd concentration in food (Zidar et al., 2003). The Cd concentration in *T. arvense* and *T. caerulescens* leaves were in the same range (438.9 ± 88.76 and 236.2 ± 53.30 μg Cd/g dry wt [mean ± standard deviation], respectively) as lettuce leaves (300–600 μg Cd/g dry wt (Calhôa et al., 2006)).

The Cd AE obtained in the present study by isopods fed *T. arvense* and *T. caerulescens* (whole leaf) were lower than the predicted AEs (Fig. 4.4) obtained from Cd AE from individual fractions. In contrast, the Cd AE among isopods fed lettuce (whole leaf) reported by Calhôa et al. (2006) was very close to the predicted AEs. In order to demonstrate the direct influence of Cd subcellular distribution in plants on Cd assimilation by the isopod, individual subcellular fractions were embedded in gelatine to produce

discrete packets of food in feeding experiments. Wallace and Lopez (1997) have demonstrated that embedding homogenized preys in gelatine did not alter Cd bioavailability to the predator, since its Cd AE was similar to the AE obtained for predators fed entire preys. Therefore, it was assumed in the present study that this method could be used in feeding experiments with subcellular fractions without affecting Cd bioavailability to the isopod. However Zhang and Wang (2006), using the same method, have also obtained overestimation of Cd AE and suggested that the homogenization step for subcellular fractionation may have facilitated the digestion of Cd by breaking the prey tissues into smaller portions, and addition of buffer may have increased the Cd bioavailability. Furthermore, gelatine obviously increases nutritional quality of food to isopods, and this is a biological factor known to directly influence assimilation of contaminants (Wang and Fisher, 1999). The inclusion of a homogenate of Cd-contaminated leaves embedded in gelatine in feeding experiments as an additional control treatment would have allowed a direct comparison to the Cd AE of whole leaves and might help to clarify the overestimations of Cd AE observed in the present study.

Figure 4.4 also displays the individual contribution of each subcellular fraction to the total estimated AEs. It is apparent from Figure 4.4 that Cd sequestered in the HSP fraction constitutes only a minor source of the Cd assimilated by isopods from the three plant species studied, in part because only 12 to 23% of this fraction is trophically available (Fig. 4.3). This result contrasts with those obtained for animals, since HSP fraction is part of the proposed TAM fraction, contributing significantly to the trophic transfer of Cd from several invertebrate animals to shrimp (Wallace et al., 1998; Wallace and Luoma, 2003) and marine fish (Zhang and Wang, 2006). It was shown that metal partitioned to a subcellular compartment containing TAM is readily available to predators and may be enhanced by increased binding of metal to HSP (Wallace and Luoma, 2003). This fraction is considered to be dominated by MT-like proteins, a family of low-molecular-weight, cysteine-rich proteins that can bind to essential metals and sequester toxic metals, and therefore, can also be considered as biologically detoxified metal (Wallace et al., 2003). As indicated above, HSP may contain other Cd-bound molecules, and a closer examination (or a further fractionation) of the HSP fraction might clarify this apparent difference between the AE of Cd from HSP fraction of prey animals and plants. Beyond differences in HSP content, differences in AE of HSP-bound Cd might also be related to the different capacity of isopods to assimilated Cd bound to HSP, due to digestive physiology, in comparison to other predators, such as marine animals.

In all three species, Cd in the cell debris fraction contributes more to isopod assimilation of Cd than would be expected according to the TAM model proposed by Wallace and Luoma (2003). The composition of the cell debris fraction includes tissue fragments and cell walls and might contain metal-rich granules. This fraction was originally considered as trophically unavailable in marine invertebrate food chains (Wallace and Luoma, 2003). However, in a previous study, these same authors reported the bioavailability to predators of Cd bound to the cellular debris fraction at 19.0% (Wallace and Lopez, 1997). Furthermore, direct evidence on the bioavailability of metal-rich granules to a marine predator has been demonstrated (Cheung and Wang, 2005). In the present study, among isopods fed the cell debris fraction of lettuce, the AE of Cd was 47.8 ± 2.05%, and this fraction accounts for approximately half the estimated Cd AE (21% out of the total estimated 44.1%); thus, the cell debris fraction can be considered at least partially trophically available. The bioavailability of Cd bound to tissue fragments, cell walls and metal rich granules must to a certain extent be dictated by gut physiology. For example, gut pH may play a role in dissolution of metal-rich granules; the terrestrial isopod *P. scaber* is able to buffer pH in the intestinal tract to approximately 5.5 to 6.0 in the anterior hindgut and approximately 6.0 to 6.5 in the posterior hindgut (Zimmer and Topp, 1997). Presuming that the *P. dilatatus* has a similar digestive physiology, at these pHs the metal-rich granules, if present, do not seem likely to become available, since dissolution occurs at lower pH. Bioavailability of Cd stored in granules depends on the form and granule elemental composition, but is likely to be higher at lower pH levels (Nott and Nicolaidou, 1994).

In both *Thlaspi* species, HDP displayed the highest contribution to the estimated AEs, accounting for approximately half of the estimated Cd AE by isopods (21.9% out of the total 36.4% for *T. arvense* and 13.0% out of the total 26.4% for *T. caerulescens*), and this is in closer agreement with the proposed TAM fraction (Wallace and Luoma, 2003). Similar results were obtained by other authors through direct evidence of bioavailability of Cd bound to HDP (Cheung and Wang, 2005; Zhang and Wang, 2006), indicating that Cd-HDP as part of TAM was partially bioavailable to fish (Zhang and Wang, 2006) and marine snails (Cheung and Wang, 2005).

Conclusions

The concept of TAM as defined by Wallace and Luoma (2003) is not supported by the data presented here. In contrast with results in marine food chains obtained by other

authors, Cd bound to HSP is relatively less available and seems to contribute in lesser extent to the trophic transfer of Cd than other fractions obtained by a centrifugal fractionation procedure. However, the AE of compartment-specific Cd was consistent across the different plant species. These results point to the ecological relevance of the subcellular Cd distribution in plants, which directly influences the trophic transfer of Cd to the animal consumer, and highlight that a shift in Cd subcellular distribution in plants due to different detoxifying mechanisms may have a direct important impact in trophic transfer to the animal consumer. Although predicted Cd AEs from the different plants were overestimated in two of the plants studied, they helped to elucidate the observed Cd AE in isopods, providing the specific contribution of each subcellular fraction on the trophic transfer of Cd.

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General discussion and concluding remarks

Chapter 5

General discussion and concluding remarks

Ecotoxicology has emerged as a distinct subject of interdisciplinary character only recently, and was early defined as the branch of toxicology concerned with the study of toxic effects caused by natural or synthetic pollutants, to the constituents of ecosystems, animal (including human), vegetable and microbial, in an integral context (Truhaut, 1977). The ultimate goal of this approach is to be able to predict the effects of pollution and thereby, provide information to facilitate the regulation of chemical use and to set maximal environmental limits and, should a pollution incident occur, the most efficient and effective action to remediate the detrimental effects can be identified (Connell et al., 1999; Walker et al., 2001). In this context, the main aim of this work was to improve the scientific knowledge concerning the toxic effects of cadmium (Cd) accumulation for the plant itself and to study the consequent trophic transfer of this metal to the animal consumers.

Firstly, the main physiological and genotoxic effects of Cd to plants were addressed (Chapters 2 and 3). Phytotoxicity of Cd has been largely studied in fundamental plant physiology and several reviews have been published on the specific effects of this metal on plants (Prasad, 1995; Sanitá di Toppi and Gabbrielli, 1999). However, the mechanisms of Cd toxicity are not fully understood in plants and need to be clarified before this extensive existing knowledge can be transposed to more applied areas of scientific research, such as ecological risk assessment of metal contaminated soils. Therefore, Chapter 2 describes the cascade of events and enzymatic protection strategies of Cd-induced senescence in lettuce plants over 14 days of exposure to 100 μM, with a view to discriminate the most pertinent responses to Cd for further application/use as biomarkers of Cd stress. It is demonstrated that Cd stress induced senescence in lettuce plants which was generally manifested as photosynthetic efficiency reduction, nutrient imbalances, malonaldehyde (MDA) production, and by a decrease in the overall antioxidant capacities of lettuce plants over the 14 days of exposure. These alterations were accompanied by an inhibition in the classical endpoint, shoot growth, at the end of exposure.

Despite the absence of a reduction in chlorophyll content in Cd-exposed plant leaves, the analysis of the dark-adapted values of F_v/F_m (F_v, variable fluorescence, and F_m, maximal fluorescence) demonstrated a significant decrease in photosynthetic efficiency of photosystem II. This parameter is widely used by plant physiologists and is considered as a sensitive indicator of plant photosynthetic performance (Maxwell and Johnson, 2000), and thus, has the potential to be used as a reliable marker of Cd-induced senescence.

Cadmium exposure induced oxidative stress. At the concentration used, Cd sequentially altered catalase (CAT), guaicol peroxidase (POX), and superoxide dismutase (SOD) over the 14-day exposure. Peroxidase, which was significantly induced at the early days of exposure, and SOD seemed to act in combination to reduce the impact of Cd toxicity, especially in young leaves. However, the antioxidant capacities were found to be reduced at the end of the exposure period, mainly through the inhibition of CAT. This might have led to the accumulation of ROS which are known to cause lipid peroxidation. Malonaldehyde is a measurable product of lipid peroxidation and was found to be induced in lettuce leaves exposed to Cd at the 7th day of exposure. Therefore, together with photosynthetic efficiency, antioxidant enzymes and MDA content are at this point suggested as potential biomarkers of Cd stress in higher plants. Indeed, MDA content has already been suggested by other authors as a biomarker of metal stress in plants for pollution purposes (Zhang et al., 2007). The responses obtained with these endpoints are not Cd-specific; therefore, in further laboratorial tests and/or studies in real scenario conditions, they should not be used separately, but used together in an integrated approach with several endpoints, also including classical endpoints (e.g. growth).

The endpoints analysed were evaluated over time in lettuce plants exposed to a singular concentration of 100 μM Cd, which is only expected in worst case scenarios. This concentration was toxic to lettuce plants causing senescence. As the antioxidant capacities were found to be exceeded, it would be interesting to follow the antioxidant enzymes cascade and the lipid peroxidation at lower exposure to evaluate plant responses at lower environmental concentrations of Cd.

In this study, hydroponic culture provided consistent and reproducible levels of contamination in plants, avoiding complications associated with the adsorption of the contaminant to organic and inorganic soil components. However, the factors that are likely to alter the bioavailability of Cd in soils are an important aspect of exposure. Therefore, examining the responses of the endpoints suggested here with increasing concentrations of Cd, from lower environmental relevant concentrations up to the one used in this work, both in hydroponic and soil conditions will be of great relevance in order to test the suitableness of these markers for field monitoring purposes.

Cadmium is known to induce genotoxicity in plants, mainly indirectly through the production of ROS and/or by inhibiting DNA repair (Panda and Panda, 2002). In order to address part of the first objective of this dissertation, in Chapter 3 an evaluation of the Cd genotoxicity in plants was performed. In Chapter 3.1 the mutagenic effects of Cd were evaluated in 5-week-old lettuce plants exposed for a further 14 days to a medium

containing 100 μM of Cd. Considering the microsatellite or simple sequence repeat (SSR) loci analysed the uniform SSR patterns observed seem to suggest that Cd generated no genetic instability under the experimental conditions used.

It is known that the effects of metals are strongly dependent on the age of the plant at the time of exposure; the older the plant the greater the amount of metal that can be tolerated because metals accumulate at metabolically inactive sites such as cell walls and vacuoles (Fodor, 2002). This might explain the absence of observable genotoxic effects in the study described in Chapter 3.1. In a different study (Chapter 2), but under the same exposure conditions the plants were already severely affected by the toxic effects of Cd by the end of the 14-day exposure, becoming necrotic after that period. Therefore, it was decided to assess the genotoxic effects in younger plants, over a longer period of time using the lower concentration of 10 μM Cd^{2+}, which is considered an environmentally relevant concentration (Sanitá di Toppi and Gabbrielli, 1999) and this formed the basis of Chapter 3.2.

In Chapter 3.2 microsatellite instability was examined in leaves and roots of lettuce plants exposed for 28 days to 10 μM Cd. The uniform patterns found for the nine SSRs analysed seem to suggest that the Cd treatment generated no MSI instability on lettuce leaves. However, a deletion of 2 bp in one lettuce root SSR suggested MSI in this organ. Since Cd content was greater in roots than in leaves, roots are likely exposed to higher internal concentrations of Cd^{2+} than leaves, which might explain the different responses obtained by the two organs.

A similar approach to those performed in this dissertation was conducted by Paiva (2008) who evaluated Cd genotoxicity through the analysis of selected SSRs in hyperaccumulator *T. caerulescens* and in non-accumulator *T. arvense* plants exposed to 10 μM of Cd. However, no MSI was detected in neither of these plants (Paiva, 2008).

Although, the aforementioned trials have demonstrated the utility of the method, further longer-term trials, on plants grown under more realistic exposure conditions need to be performed in order to demonstrate the genotoxicity that might occur under natural conditions. Moreover, since the suggested molecular mechanisms of Cd genotoxicity include not only direct damage of DNA (Hossain and Huq, 2002) but, also direct inhibition of DNA mismatch repair (Jin et al., 2003; Slebos et al., 2006), the effect of Cd exposure might potentiate the genotoxic effects of other environmental mutagens as already shown for other metals (Deng et al., 2005). Therefore, assessment of Cd genotoxic effects in plants using mixtures of environmental mutagens with different modes of action seems like an important line of research to be followed in future.

The studies presented in Chapter 3 of this thesis could have benefited from the use of a positive control. The application of a mutagenic compound (such as EMS, ethyl methanesulphonate) in increasing concentrations might provide an indicator of the sensitivity of the technique for genotoxicity assessment purposes. Also, Cd genotoxicity assessment in other plant species, specifically in Cd-sensitive plants (natives or Cd-sensitive mutants) would help clarify the utility of SSRs as an assay for plant genotoxicity.

In Chapter 3.3 a flow cytometric (FCM) assessment of Cd genotoxicity (e.g. clastogenesis) was performed in three plants with different metal accumulation and detoxification capacities (*L. sativa*, *T. caerulescens* and *T. arvense*). No ploidy changes were found in the plant species analysed. However, statistically significant changes in nDNA content and the G_0/G_1 full peak coefficient of variation (FPCV) values were detected in *L. sativa* but not in *Thlaspi* spp. These results suggested that Cd stress may have lead to clastogenic damage as a consequence of loss of chromosome portions, because nDNA content was found to be diminished. These data, complemented other genetic assays previously performed (SSRs), which together with physiological and biochemical data obtained in Chapter 2 have provided further insights into the phenomenon of Cd-induced stress and resulting effects in plants.

Moreover, the results obtained with this FCM approach, highlighted the usefulness of this technique to screen and monitor *in vivo* genotoxic effects of environmental pollutants. However, Loureiro et al (2006) have highlighted the confounding effects of cytosolic compounds that can interfere with the FCM methodology. Therefore, improvement of this technique for use in some recalcitrant species (e.g., *Thlaspi* spp.) is still required.

Among the various aspects of ecotoxicology, the trophic transfer of contaminants along food-chains has recently generated a great deal of interest and research, particularly in regard to the implications for consumers of metals sequestered within prey species (Rainbow, 2002; Vijver et al., 2004; Wang and Rainbow, 2006; Rainbow, 2007). Traditionally, dietary toxicity studies have added contaminants directly to the food source. However, contaminants that are biologically incorporated into live prey are likely to be sequestered into various sub-cellular compartments and are likely to be bound within various chemical complexes. These compartmentalized chemical species may have very different bioavailabilities from Cd salts artificially added to the food to predator species. In addition, the degree to which metals are transferred within a food chain is not easily predictable, because both the metal-binding properties of the plant/prey species and subsequent bioavailability to the consumer/predator are likely to be highly variable.

The subcellular partitioning model (SPM) arose as an improved method to predict metal toxicity following its application in aquatic food chains (e.g. Wallace and Lopez, 1997; Wallace et al., 1998; Wallace and Luoma, 2003). As in aquatic food chains, the understanding, prediction and mitigation of toxicity associated with soil metal pollution will certainly be improved by knowledge of the mechanisms of metal detoxification and partitioning in plants, and how those different mechanisms will affect metal assimilation and transfer to consumers, including extreme cases such as metal trophic transfer from hyperaccumulator plants, but also in accumulator crops that might have direct impacts on human food chains.

One of the main questions of this work "How does Cd subcellular distribution in plants affect assimilation of Cd by an animal consumer?" was addressed in Chapter 4. In this study it was demonstrated that Cd assimilation efficiencies (AE) were significantly lower in isopods (*Porcellio dilatatus*) fed *T. caerulescens* leaves (10.0 ± 0.92%) than in those fed *T. arvense* (15.0 ± 1.03%). Moreover, under similar experimental conditions Calhôa et al. (2006) demonstrated much higher AE among isopods fed *Lactuca sativa* leaves (52 ± 5%). Each of these plant species possesses different strategies to store and detoxify Cd; therefore, the subcellular distribution of Cd in the leaves of these three plant species was examined to help explain the differences in Cd AE. The distribution of Cd between the four different subcellular fractions obtained by differential centrifugation revealed significant differences between the plant species. This, together with the direct assessment of isopod Cd AE from individual subcellular fractions of *L. sativa, T. caerulescens* and *T. arvense* plant-leaves, resulted in vital information to help explain the observed differences in Cd assimilation by isopods. On the basis of our results, Cd bound to heat-stable proteins (HSP) was the least bioavailable to isopods (14.4-19.6%), while Cd bound to heat-denatured proteins (HDP) was the most trophically available to the isopod (34.4-52.8%).

Trophically available metal (TAM), as defined by Wallace and Luoma (2003), includes metal bound to the HSP fraction; however, their definition is not supported by the data presented in Chapter 4. In contrast with results in marine food chains obtained by other authors, Cd bound to HSP is relatively less available in the terrestrial food chain examined in Chapter 4 and seems to contribute in lesser extent to the trophic transfer of Cd than other fractions obtained by the centrifugal fractionation procedure used.

In order to confirm the lower bioavailability to isopods of Cd bound to the HSP fraction of plant leaves, the direct assessment of the bioavailability of a Cd-cystein compound (e.g. Cd-phytochelatin (PC) and Cd-methallothionein (MT)) should be

performed in further studies. Moreover, as the digestive system of the animal is an important factor that affects metal assimilation, a different plant-herbivore food chain might shed more light on the low bioavailability of HSP bound Cd, and whether it is specific to some consumers and not others. Indeed, studies on mammals indicate that MT-Cd is assimilated via the mice (Sugawara and Sugawara, 1991) and rat (Groten et al., 1991) gut, to a lesser degree than Cd from $CdCl_2$ amended diets. The differences observed were attributed, by the authors, to the slower transport of the intact Cd-MT across the intestinal mucosa when compared to the absorption of Cd^{2+}. In agreement with these results, lower Cd AEs were found in isopods fed biologically contaminated lettuce which is known to produce PCs against Cd-stress, than in isopods fed $CdCl_2$ amended leaves (Calhôa et al., 2006). However, the contrary was demonstrated in rainbow trout fed biologically contaminated amphipods; fish fed Cd contaminated amphipods exhibited higher absorption efficiency than those fed $CdCl_2$ amended trout diet (Harrison and Curtis, 1992). These authors further speculated that Cd associated with strong biological ligands, such as the sulfur moieties that form the functional groups in cysteine and MT, are more easily absorbed by the fish gut than Cd^{2+}, that must compete with essential ions such as Ca^{2+} for binding sites on ion-transport system (Harrison and Curtis, 1992).

The AEs of compartment-specific Cd were consistent across the different plant species studied. These results point out the ecological relevance of the subcellular Cd distribution in plants, which directly influence the trophic transfer of Cd to the animal consumer, and highlight that a shift in Cd subcellular distribution in plants due to different detoxifying mechanisms may have a direct and important impact in trophic transfer to the animal consumer.

A mass balance equation was used to predict Cd AEs from the different plants, using data of subcellular distribution of Cd and of isopod Cd AE from individual subcellular fractions. Although the AEs predicted from the feeding study in which isopods were fed individual fractions (i.e. debris, organelles, HDP, HSP) were overestimated in two of the plants studied, they helped to elucidate the observed Cd AE in isopods, providing the specific contribution of each subcellular fraction on the trophic transfer of Cd. It should be pointed out that for each fraction only a proportion of Cd was bioavailable, suggesting Cd speciation in each subcellular fraction, with different Cd-species having differences in their bioavailability. This is obviously true for cell debris fraction, where Cd bound to cell walls carbohydrates should be differently assimilated from Cd bound to metal-rich granules. But also the other fractions might not present a homogeneous pool of bioavailable Cd. For instance, the organelles fraction is a well defined fraction in terms of plant subcellular

components (e.g. chloroplasts, mitochondria). However, the different organelles might have relevant differences in composition (e.g. different membrane proteins), with a variable capacity of the different components to bind Cd^{2+}. A proportion of the Cd bound to the organelle components of the plant species analysed might not be bioavailable to isopods, while another is. The same rationale is applied to the cytosolic proteins present in the HDP fraction, differences in the amino-acid composition and tertiary configuration might lead to proteins with stronger Cd binding ligands than others.

As far as is known, this was the first time that a subcellular fractionation method has been used in plants with the purpose of explaining metal trophic transfer from plants to consumers. It should be kept in mind that the fractionation protocol adopted in this work aims at a pragmatic separation of fractions. The protocol used originated from the discipline of molecular biology and has in this way been validated; however, the operationally defined fractions should be examined further to confirm their composition, particularly in regard to metal speciation. For instance, the likely occurrence of Cd bound to organic acids such as malate in electrodense granules inside vacuoles is an important issue, since it is an important metal detoxification pathway in plants (Ueno et al., 2005). Moreover, after confirming the presence of metal-rich granules that might accumulate in the acidic environment of vacuoles through complementary techniques (e.g. energy dispersive X-ray microanalysis or electron energy loss spectroscopy), their separation from cell debris fraction would be useful for further studies. The solubilization of metal-rich granules during homogenization might occur, depending on its elemental composition and on the pH of the homogenization buffer. Knowledge of the nature of metal-rich granules will certainly help to improve the fractionation protocol by providing an extra, better defined subcellular metal fraction. In addition, it would be relevant to perform the analysis of the composition of the HSP fraction through chromatographic techniques, in order to confirm the presence of MT and PC and/or to reveal the presence of other type of compounds that might bind Cd.

Summarizing, steps were made towards the understanding of the physiological and genotoxic effects induced by Cd in plants. However, testing these main effects as reliable biomarkers of Cd stress in plants remains a problem yet to be solved. Concerning the issue of Cd accumulation in plants and its implications to the trophic transfer to consumers, a new and promising approach was performed which provided a better understanding of metal trophic transfer in terrestrial food chains.

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