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Review on Some Emerging Endpoints of Chromium (VI) and Lead Phytotoxicity

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1. Introduction

Metals occur naturally in the environment as constituents of the Earth's crust and they tend to accumulate and persist due to their stability and mainly because they cannot be degraded or destroyed. However, and despite that in some cases (e.g. mercury) high levels occur naturally, for most situations, anthropic activities are among the primary causes for metal pollution. Examples of important sources of metal contamination come from industrial applications, mining, smelters, combustion by-products and fuel. From these sources, contaminants can enter the ecosystem as airborne particles, wastewaters and sludge, polluting not only sites near the source but locations thousands of kilometers apart. Studies like the ones of Murozumi et al. (1969), Hong et al. (1994) or McConnell and Edwards (2008) demonstrated the extension and persistence of metals in the environment. These studies also showed that contamination of the environment with these pollutants started way before the industrial revolution with evidence of pollution originating from Roman mining and smelters in 500 B.C. (Nriagu, 1996).

Due to the above reasons and to their toxicity to human health and environment, metal toxicity has become an increasing target of studies in humans, animals and plants. Of what is generally conceived, toxicity originates through a very complex pattern of metal interactions with cellular macromolecules, metabolic and signal transduction pathways and genetic processes (Beyersmann and Hartwig, 2008). Among the different models available to study metal toxicity, plants present some unique features that make them interesting subjects. Firstly, much of human diet depend directly from plants products like fruits and vegetables or indirectly as fodder given to livestock. Secondly, by lacking the ability to escape from contaminated sites, plants evolved mechanisms to handle exposure to toxicants, from the amount that is taken from the surroundings, to strategies of sequestration and inactivation in sub cellular compartments or even to the ability of tolerating putative deleterious effects of metals.

Regarding the amount of pollutant accumulated, three categories of plants were proposed by Baker (1981): *(1) excluders*: those that grow in metal-contaminated soil and maintain the shoot concentration at low level up to a critical soil value above which relatively unrestricted root-to-shoot transport results; *(2) accumulators*: those that concentrate metals in

the aerial part; *(3) indicators*: where uptake and transport of metals to the shoot are regulated so that internal concentration reflects external levels, at least until toxicity occurs.

The toxicity of metals, and of their compounds, largely depends on their bioavailability, i.e. the mechanisms of uptake through the cell's membrane, intracellular distribution and binding to cellular macromolecules (Beyersmann and Hartwig, 2008). Although the relative toxicity of different metals to plants can vary with plant genotype and experimental conditions, most act through one of the following: changes in the permeability of the cell's membrane; reactions of sulphydryl (–SH) groups with cations; affinity for reacting with phosphate groups and active groups of ADP or ATP; replacement of essential ions and oxidative stress (Patra et al., 2004). Through these, some of the most common, and often unspecific symptoms, of metals phytotoxicity are: growth inhibition, nutrient imbalance, disturbances in the ion and water regime (e.g. Gyuricza et al., 2010), photosynthetic impairment (e.g. Hattab et al., 2009b) and genotoxicity (e.g. Monteiro et al., 2010).

Most of the metals of greater environmental concern have been currently included in the classical and ill-defined group of "heavy metals". This is an unclear term for a group of elements that present metallic proprieties and normally include transition metals, some metalloids, lanthanides, and actinides. Some years ago, this term has been considered meaningless and misleading by the IUPAC due to the contradictory definitions and its lack of a coherent scientific basis (Duffus, 2002). It has been since then progressively abandoned by the scientific community, but still remains widespread in many reports, mostly reporting to any metallic element with relatively high density and which is toxic in low concentrations. Among the elements referred to as "heavy metals", 13 have been considered by the European Union to be of the highest concern: arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), mercury (Hg), manganese (Mn), nickel (Ni), lead (Pb), tin (Sn) and thallium (Ti). From these, some have been target of many investigations (e.g. Cd) while for others, the level of knowledge about the mechanism of toxicity are highly unsatisfactory (e.g. Cr). Interestingly, despite it being one of the first metals with known reports of human poisoning, not enough research have been undertaken to clarify Pb's mechanism of toxicity and even some conflicting data have been reported (García-Lestón et al., 2010).

In this review we´ll discuss some of the most relevant and updated data on Cr and Pb toxicity in plant cells, and explore some of the emerging techniques to diagnose cyto and genotoxicity.

2. Chromium: The element

Chromium was discovered in 1797 as part of the mineral crocoite, used as pigment due to its intense coloration. As a matter of fact, the name chromium is derived from the Greek word "χρώμα" (chroma- color) due to that propriety of the element. Chromium is the 21st most abundant element in Earth's crust with an average concentration of 100 ppm, ranging in soil between 1 mg/kg and 3000 mg/kg; in sea water from 5 µg/L to 800 µg/L and in rivers and lakes between 26 µg/L and 5.2 mg/L. Normally, Cr is mined from chromate but native deposits are not unheard off. One of the most interesting characteristics of this metal is its hardness and high resistance to corrosion and discoloration. The importance of these proprieties resulted among others in the usage of this metal in the development of stainless

steel, which together with chrome plating and leather tanning, are the most important applications of this element and the main sources of Cr pollution of the environment. Chromium is highly soluble under oxidizing conditions and forms, exhibiting a wide range of possible oxidation states (from -2 to +6), being that +3 [Cr(III)] and +6 [Cr(VI)] are the most stable forms. Under reducing conditions, Cr(VI) converts to Cr(III) that is insoluble, but this form is strongly absorbed onto the surface of soil particles.

3. Chromium: Uptake and assimilation by plants

Chromium is a common contaminant of surface waters and ground waters because of its occurrence in nature, as well as anthropic sources (Babula et al., 2008). Cr(III) and Cr(VI), being the most stable are also the important in terms of environmental contamination. The most important sources of Cr(III) are fugitive emissions from road dust and industrial cooling towers; also, Cr(VI) compounds are still used in the manufacture of pigments, in metal-finishing and chromium-plating, in stainless steel production, in hide tanning, as corrosion inhibitors, and in wood preservation (Shtiza et al. 2008).

Very few studies have attempted to elucidate the transport mechanisms of Cr in plants, but factors like oxidative Cr state or its concentration in substrate play important roles (Babula et al., 2008). Of what is known, due to its higher solubility and thus, bioavailability, Cr(VI) is more toxic at lower concentrations than Cr(III), which tend to form stable complexes in soils (Lopez-Luna et al., 2009). Also, the pathway of Cr(VI) transport is thought to be an active mechanism involving carriers of essential anions such as sulfate (Cervantes et al., 2001). Fe, S and P are known also to compete with Cr for carrier binding (Wallace et al., 1976). Also Cr absorption and translocation have been show to be modified by soil pH, organic matter content and chelating agents, among others (Han et al., 2004).

Studies performed to elucidate the uptake mechanism of Cr have demonstrated that only Cr(VI) is detected in plant tissues. However some plants (such as soybean and garlic) have the capacity to reduce Cr(VI) to unstable intermediate like Cr(V) and Cr(IV), or eventually to the more stable form, Cr(III); this represents the detoxification pathway of Cr(VI) (Babula et al., 2008). As this mechanism of detoxification is performed readily in the roots and as Cr is immobilized in the vacuoles of the root cells, the amount of Cr translocated to the aerial portion of the plants is very little (Shanker et al., 2005).

4. Chromium: Phytotoxicity

4.1 General effects

The effects of Cr in some of the classical endpoints of heavy metal genotoxicity have received some attention by fellow researchers. Seed germination and plants growth are two of the parameters that have been studied thoroughly. Results indicate that Cr provokes growth inhibition of roots in species like *Salix viminalis* (Prasad et al., 2001), *Caesalpinia pulcherrima* (Iqbal et al., 2001), wheat (Chen et al., 2001) and mung bean (Samantaray et al., 1998).

Shanker et al. (2005) hypothesized that root growth inhibition due to Cr toxicity could be due to inhibition of root cell division/root elongation or to the extension of cell cycle. Aerial part growth (measured by effects on shoot length and on reduction of leaf number and area) has also been proven to be negatively affected by Cr in species like rice (Singh et al., 2006)

wheat, oat and sorghum (Lopez-Luna et al., 2009). Justifications to these facts were proposed by Shanker et al. (2005) by stating that root growth inhibition, as well as its consequent and/or causal nutrient imbalance, could be behind low shoot development. In fact, chromium, due to its structural similarity with some essential elements, can affect mineral nutrition of plants in a complex way (Shanker et al., 2005) and there has been innumerous considerations regarding this issue, especially in crop species.

It has been demonstrated that very low concentrations of Cr (0.05–1 mg /L) promoted growth, and increased nitrogen fixation and yield in leguminosae (e.g. Hewitt, 1953). At higher concentrations and just giving a couple of examples, authors have successfully proven that this metal reduces the uptake of the essential elements Fe, K, Mg, Mn, P and Ca in *Salsola kali* (Gardea-Torresdey et al., 2005) and K, Mg, P, Fe and Mn in roots of soybean (Turner and Rust, 1971). The justification of nutrient imbalance has been pointed to competitive binding to common carriers by Cr(VI), to inhibition of the activity of plasma membrane's H^+-ATPase and to reduced root growth and impaired penetration of the roots into the soil due to Cr toxicity (Shanker et al., 2005).

4.2 Photosynthesis

Like other metals, Cr can affect photosynthesis severally and in many different steps, which can ultimately translate in loss of productivity and death. Shanker et al. (2005), in a review about Cr phytotoxicity, discussed that while Cr toxicity at the photosynthetic level was well documented in trees and higher plants, the exact target and mechanisms affected by this metal were poorly understood.

Cr(VI) can easily cross biological membranes and has high oxidizing capacity, generating reactive oxygen species (ROS) which might induce oxidative stress (Pandey et al., 2009). ROS are generated in normal metabolic processes like respiration and photosynthesis, being chloroplasts one of the main sites of reactive oxygen production and detoxification (Mittler, 2002). However, because the chloroplast has high amounts and complex systems of membranes rich in polyunsaturated fatty acids, this organelle might also be a target for peroxidation (Hattab et al., 2009b) and one of the ways by which photosynthesis is affected. A common parameter affected by Cr is the amount of photosynthetic pigments, which tends to decrease when plants or algae are exposed to high doses of this metal (Rodriguez et al., 2011, Subrahmanyam, 2008, Vernay et al., 2007). The results obtained by Juarez et al. (2008) using algae, demonstrated that, ROS caused structural damage to the pigment-protein complexes located in the thylakoid membrane (e.g. the destabilization and degradation of the proteins of the peripheral part of antenna complex), followed by the pheophytinization of the chlorophylls (substitution of Mg^{2+} by H^+ ions), and destruction of the thylakoid's membranes. It has also been demonstrated that Cr affects, and might even inhibit, pigment biosynthesis, among others, by degrading δ-aminolaevulinic acid dehydratase (Vajpayee et al., 1999), an essential enzyme in chlorophyll biosynthesis. Vernay et al. (2007) also presented evidence that this metal probably competed with Fe and Mg for assimilation and transport to the leaves and therefore affected different steps of pigment biosynthesis.

Another endpoint of Cr phytotoxicity is *Chl a* fluorescence; however, it was demonstrated that, within some of the common biomarkers of *Chl a* fluorescence, most parameters evaluated are somewhat resistant to Cr toxicity (namely the F_v/F_m). On the other hand, the

ones related to the fluorescence emission status of light adapted-plants have been shown to be highly affected by this metal (Subrahmanyam, 2008, Vernay et al., 2007). Several hypotheses explaining these results have been proposed, e.g., structural alterations in the pigment–protein complexes of PSII or impairment in energy transfer from antennae to reaction centers (like a diversion of electrons from the electron-donating side of PS I to Cr(VI) are the most endorsed (Shanker et al., 2005). Recently, Henriques (2010) implied that Cr(VI) might not be directly responsible for the damage to the chloroplast, as the valence state of Cr depends of the local pH and redox values. For instance, in irradiated chloroplasts, the previously mentioned conditions would favor the less toxic Cr(III) form over the highly toxic Cr(VI). Appenroth et al. (2000) demonstrated that Cr damaged the water oxidizing centers (WOC) associated to PSII and Henriques (2010) proposed that this could be explained by the reduction of the Ca and Mn availability, caused by Cr, which are fundamental in the structure and functioning of the WOC.

Besides the photochemical process, Cr is also known to cause distress in the biochemical aspects of photosynthesis. Vernay et al. (2007, 2008) discussed that despite that loss of biomass and wilting were common symptoms of Cr exposure, little was known about Cr effect on water status and gas exchange. Subrahmanyam (2008) also commented that it was unclear if Cr-induced inhibition of the photosynthetic process was also due (among others previously mentioned factors) to Cr-induced interference with the Calvin cycle's enzymes. In those reports, the authors proved that Cr consistently affected parameters like E (transpiration rate), g_s (stomatal conductance), A (photosynthetic rate) and C_i (substomatal CO_2 concentration). One of the main conclusions of those articles was that even though the decrease in g_s seemed to be responsible for the variation in water regulation status, the increase in Ci induced by Cr accumulation clears g_s as the responsible for the decrease in A. This also indicates as hinted by Subrahmanyam (2008) and by Vernay et al. (2007) that the reduction in A might lay in the functional status of the Calvin cycle enzymes. Unfortunately, the availability of data regarding Cr putative effects on the enzymes of the Calvin cycle is far less than what exists for other parameters.

The recent works of Dhir et al. (2009) and Bah et al. (2010) provided one of the first insights to Cr-induced effects at the Calvin cycle enzymes. Dhir et al. (2009) found a significant decrease in ribulose-bisphosphate carboxylase oxygenase (RuBisCO) activity induced by exposure to wastewaters (rich in Cr) from an electroplating unit and suggested that this results could be explained by: a substitution of Mg^{2+} in the active site of RuBisCO subunits by metal ions; decline in RuBisCO content as a result of oxidative damage; a shift in the enzyme's activity from carboxilation to oxygenation. On the other hand, Bah et al. (2010) performed a proteomic analysis of *Typha angustifolia*'s leaves exposed to metals and found that exposure to Cr induced the expression of ATP synthase, RuBisCO small subunit and coproporphyrinogen III oxidase. The authors then explained that their data were an evidence of a protective mechanism against metal toxicity at the photosynthetic level, which might be responsible for the metal tolerance displayed by *T. angustifolia*. Furthermore, the authors also suggested that the increased expression of ATP synthase was indicative of the high energetic requirements needed to cope with metal toxicity.

Recently we compared the Cr(VI) phytotoxicity using some photosynthetic endpoints in pea leaves exposed to this metal (up to 2000 mg / L) (Rodriguez, 2011). Our group demonstrated that Cr(VI) was more aggressive to the gas exchange, biochemical and

chloroplastidial morphology markers than to those related to the photochemical apparatus. However, exposure to higher Cr(VI) dosages induced significant negative effects on the photochemical apparatus, proving that despite having some degree of resistance to metal toxicity it can still be damaged by Cr(VI) (Rodriguez, 2011). In these analyses metal toxicity in photosynthesis, flow cytometry (FCM) was used in complement to the classical tools. Few reports have up to moment tried to apply FCM's potential to study chloroplast and there are even less reports focused on evaluating the effects of hazardous substances in these organelles. In that assay our group compared the information provided by FCM vs PAM fluorometry and pigment content was also carried, in order to assess if chloroplast auto-fluorescence emission, as measured by FCM, related to those classical techniques.

FCM is gaining importance in toxicological assays of the photosynthetic machinery. We demonstrated FCM reliability and its endowment of complementary data to conventional techniques as PAM in a recent exhaustive study with paraquat (Rodriguez et al., 2011c; Figure 1). In that study, FCM and PAM fluorometry presented a strong positive correlation value, even though FCM measurements were performed on isolated chloroplasts while for

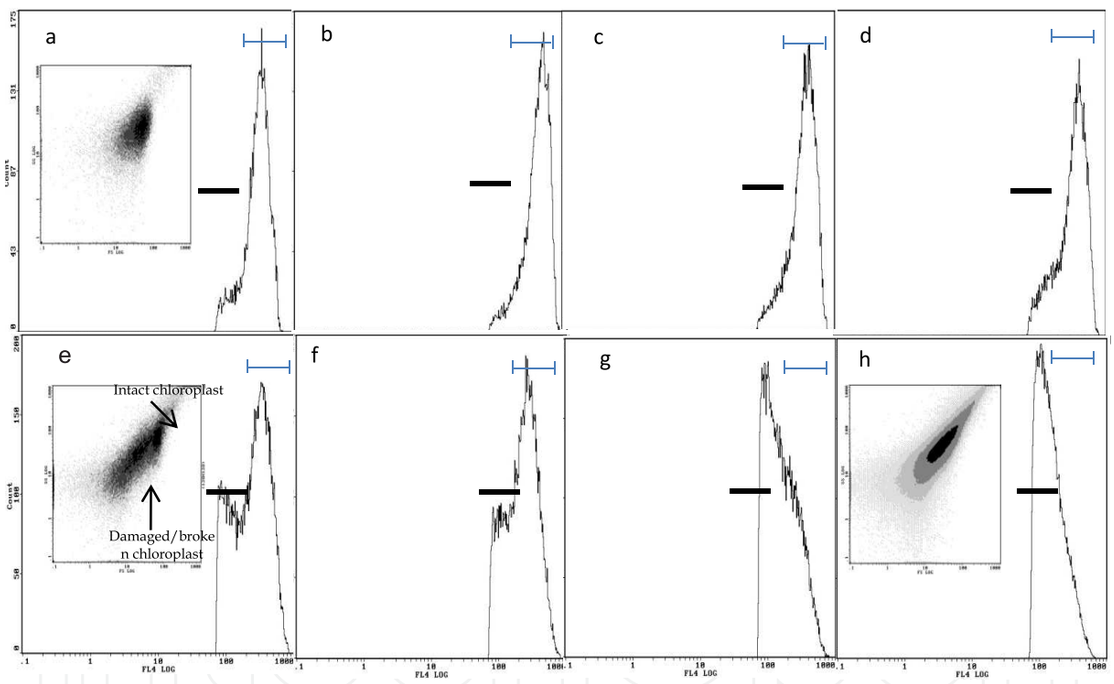

Fig. 1. Validation of FCM as measuring chloroplast fluorescence after stress: Histograms of relative fluorescence intensity (FL) of chloroplast isolated form plants exposed to a contaminant (paraquat). a) control, b) 3h of exposure, c) 6h of exposure, d) 9h of exposure, e) 12h of exposure, f) 15h exposure, g) 18h exposure, h) 24h exposure (Due to its similarity, the histogram for 21h of exposure was omitted). In each histogram 2 regions are marked: the ⊢⊣ region that defines population B (chloroplast with higher integrity, with higher fluorescence intensity and that shows a tendency to desapear with the increase of stress throughout the time); the ▬ region that has lower fluorescence intensity and appears to dominate with the stress. Inserted in histograms a), e) and h), are the respective cytograms of FS (volume) vs SS (granularity) of the isolated chloroplasts in a logarithmic scale. (Adapted from Rodriguez et al 2011c; Rodriguez 2011).

PAM fluorometry, intact leaves (i.e. still part of the plants) were used (Rodriguez, 2011; Rodriguez et al., 2011c). Also FCM was used in *Chlorell vulgaris* cultures: volume, granularity and algal autofluorescence intensity (FL) were determined by FCM, and it was demonstrated that algal density was the most affected parameter measured, while cell volume and, less, granularity were affected in a similar manner (Rodriguez et al., 2011 a).

Another unexplored endpoint of Cr-induced stress at the metabolic level is the variation in the amount of soluble sugars and starch accumulated in leaves. Besides being the fuel for carbon and energy metabolism, sugars also play a pivotal role as signaling molecules (Rolland et al., 2006). Therefore, the quantification of the sugar levels in the leaves could provide information of paramount importance in the characterization and understanding of Cr-induced phytotoxicity.

Despite the appalling lack of data, reports like the ones presented by Tiwari et al. (2009) and Prado et al. (2010) offer some insight into the effects of Cr at this level. Tiwari and co-workers (2009) found that exposure to increasing concentration of Cr caused a decrease in the amount of non-reducing sugars while the inverse was observed for reducing sugars. Prado et al. (2010) on the other hand observed that Cr exposure caused the levels of sucrose (transport sugar) to increase while the concentration of glucose decreased.

4.3 Genotoxicity

In animals and yeast, Cr (VI) has been extensively studied and shown to be highly toxic, inducing cell cycle arrest and causing carcinogenic effects (e.g. O'Brien et al., 2002, Salnikow and Zhitkovich, 2007, Zhang et al., 2001). Despite of the critical importance of Cr toxicity, we are still far away of having in plants, the same level of understanding that exists in other eukaryotes about the mechanisms of Cr genotoxicity. What serves as bases for understanding Cr genotoxicity in plants is what is known in other organisms; Cr is a special case as unlike other metals, when inside the cell, Cr interacts primarily and directly with DNA, forming DNA-protein and DNA-DNA cross links, making this element a highly mutagenic and carcinogenic toxicant. By this, while other metals are considered weakly mutagenic, mostly acting through the inhibition of DNA repair machinery, Cr acts directly on DNA causing genotoxicity directly.

Cr can also form complexes which can react with hydrogen peroxide and generate significant amounts of hydroxyl radicals that may directly trigger DNA alterations and other effects (Shi and Dalal 1990a,b). The mechanism of Cr(VI) detoxification by reductases creates unstable forms of Cr that are known to create ROS which are one of the most common causes of DNA degradation. It has been shown that Cr(V) reacted with isolated DNA to produce 8-hydroxydeoxyguanosine, whereas Cr(VI) performed this reaction only in the presence of the reductant glutathione (Faux et al. 1992).

In cultured mammalian cells, Cr(VI) induced superoxide and nitric oxide production (Hassoun and Stohs 1995), whereas treatment of cells with Cr(VI) in the presence of glutathione reductase generated hydroxyl radicals. This ROS, besides degrading DNA, can also affect Mitogenic-Activated Protein Kinsases (MAPK), which cause the deregulation of cell proliferation (tumor inducing effect), thus causing mutagenicity through an indirect path, besides the aforementioned direct interaction with DNA (Beyersmann and Hartwig, 2008).

Cr genotoxicity studies in plants are summarized in Table 1. Most of the researches performed in plants have demonstrated that Cr generates chromosomal aberration and micronuclei formation, which is understandable, as both of these are commonly used as genotoxicity endpoints in ecotoxicological assay. As it can be seen in table 1, there is also evidence of Cr related DNA degradation (Comet assay) and point-mutation (AFLP), and thus it is very likely that more research will confirm that at least part of what is known in animals can also be observed in plants.

Species	Reference	Dose	Technique	Effects
V. faba	(Chandra et al., 2004)	Tannery solid waste	Cytogenetic	Chromosomal and mitotic aberration
B. napus	(Labra et al., 2004)	$K_2Cr_2O_7$ (10 to 200 mg/L)	AFLP, SAMPL, DNA methylation analysis	Methylation changes, Mutation
A. thaliana	(Labra et al., 2003)	$K_2Cr_2O_7$ (2, 4 and 6 mg/L)	AFLP	DNA mutation
C. sativa	(Citterio et al., 2003)	$K_2Cr_2O_7$ (25µg/g and 50 µg/g soil)	FCM	ND
T. repens	(Citterio et al., 2002)	Contaminated soils from a steelworks- Up to 4810 mg/kg soil	AFLP, FCM	Mutation, DNA decrease
V. faba	(Wang, 1999)	Cr (contaminated soils)	MN	Dose-related increase of MN
A. cepa	(Matsumoto et al., 2006)	Tannery effluent	Cytogenetic	Chromossomal aberration
V. faba	(Koppen and Verschaeve, 1996)	$K_2Cr_2O_7$ (Up to 10^{-3}M)	COMET assay	Increase in %Tail DNA, Tail moment and Tail length
Tradescancia sp. V. faba	(Knasmuller et al., 1998)	$CrCl_3$, CrO_3 (from 0.75 to 10 mM)	MN	Dose-related increase of MN for *Tradescancia,* ND in *V. faba*
P. sativum	Rodriguez (2011b)	$CrCl_3$ (up to 2000 mg/L)	Comet Assay FCM	DNA damage Clastogenicity G_2/M arrest

Table 1. Literature survey of Cr genotoxic effect in plants.

Rodriguez et al. (2011) showed that flow cytometry (FCM) and Comet assays provided accurate and sensitive biomarkers of the DNA damage endpoint, detecting significant changes in both roots and leaves of plants exposed to Cr(VI) (Figure 2). The level of DNA damage observed in roots was significantly higher than that of the leaves. Roots had direct contact with the metals and it is known that in most cases, this organ acts like a barrier against metal translocation, which might justify why the higher level of DNA damage observed was in roots.

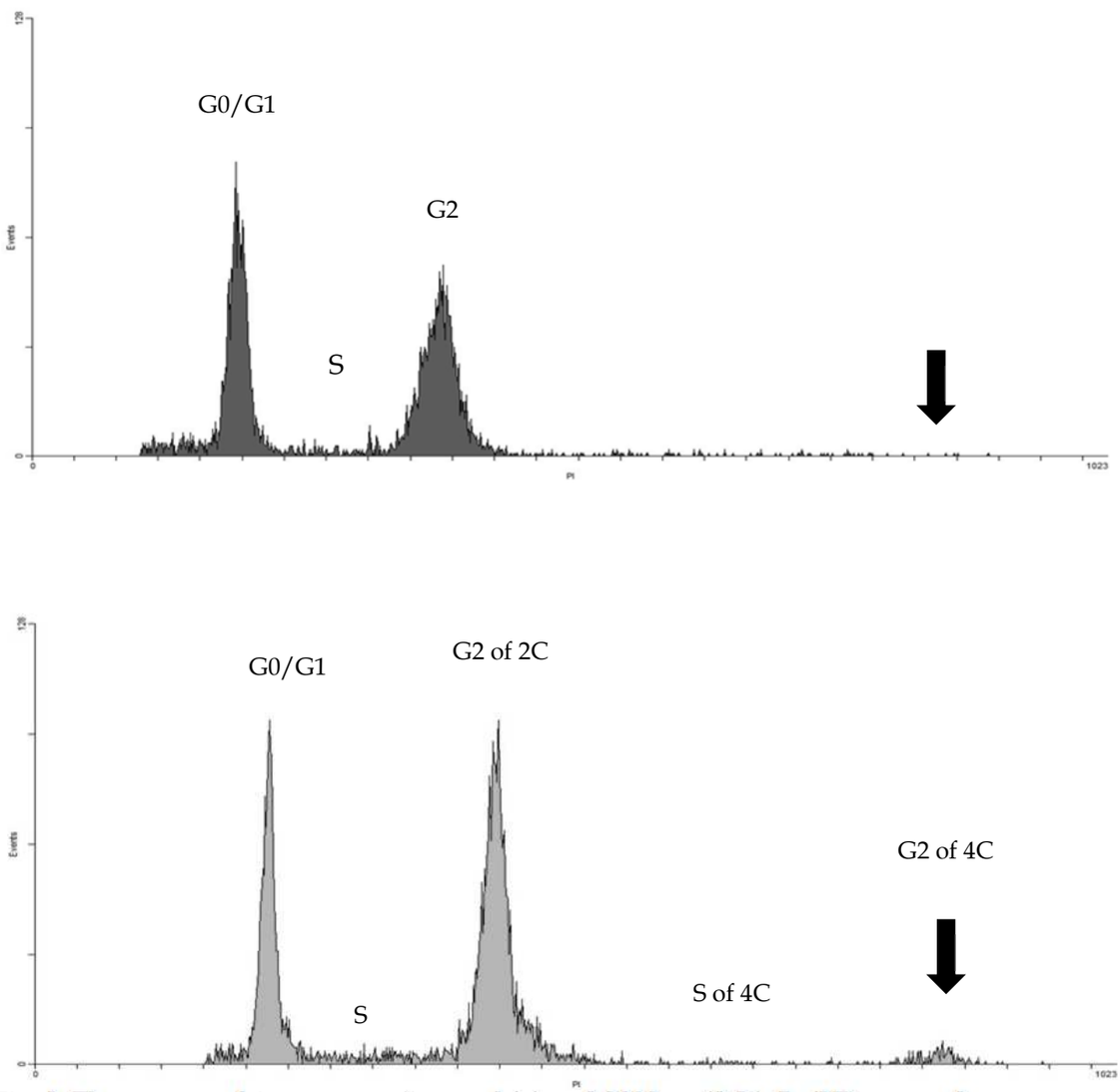

Fig. 2. Fluorescence histograms of control (a) and 2000mg/l (b) Cr (VI) exposed pea roots. Values are given in channels (X axis) and nº of events (Y axis). The arrow indicates the position of extra peak in the bottom histogram (not present in control, above) (adapted from Rodriguez et al 2011b).

Also, under the same Cr(VI) conditions, 40% of the individuals analyzed suffered polyploidization having both 2C and 4C levels. Rodriguez et al (2011) also demonstrated that the clastogenic data provided FCM supported those of Comet assays, and that both tools complemented each other in genotoxicity evaluations.

For the putative cytostatic effects induced by Cr(VI), we have recently showed that Cr(VI) (up to 2000 mg/L) induced few changes in *Pisum sativum* leaves and roots cell cycle progression and that these changes were dependent on the organ and on Cr(VI) concentration: pea leaves showed no significant variations in either cell cycle dynamics. Contrarily, in roots, exposure to 2000 mg/L resulted in cell cycle arrest at the G_2/M

checkpoint (Rodriguez et al., 2011). This may support that an arrest of the cell cycle at this checkpoint occurs when DNA synthesis has been compromised, to give cells extra time to either repair the damage (O'Connell and Cimprich, 2005) or activate an apoptosis-like program (Figure 3). In some cases though, cells might continue with proliferation without completing the damage repair (Carballo et al., 2006). Moreover, the evaluation of MSI helped to explain why, despite that significant DNA damage was detected in lower dosages, only at the maximum dosage an arrest at the cell cycle was observed, since signs of MSI could only be observed at that dosage (Rodriguez 2011).

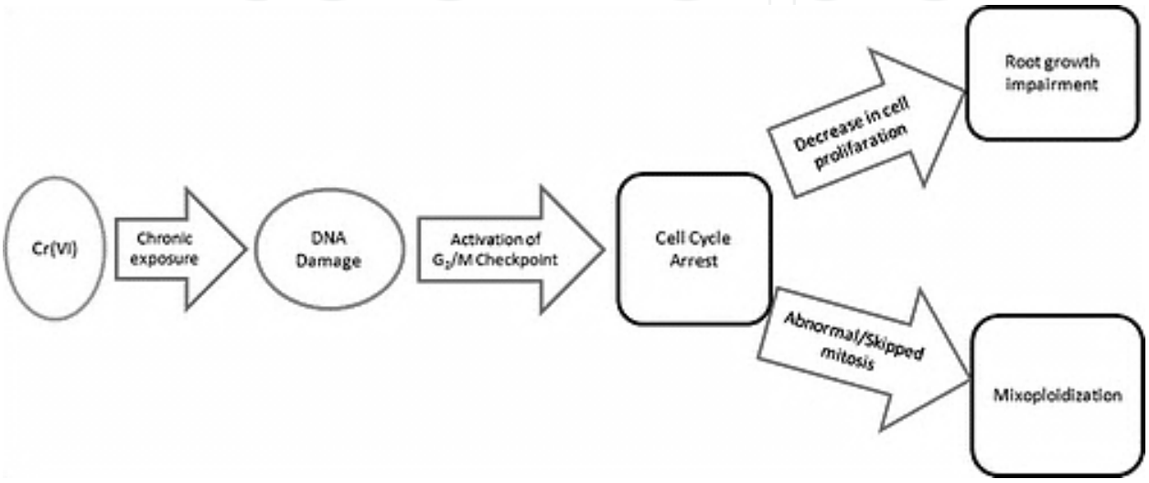

Fig. 3. Cr(VI), after inducing a critical level of DNA damage, leads to malfunction of the DNA repair system, which might in turn induce problems with the cell cycle/division machinery, causing arrest and in extreme cases, polyploidization (from Rodriguez et al., 2011b).

As demonstrated by the authors, flow cytometry (FCM) is a technique that can easily excel in genotoxic studies, allying high analytical speed with multiparametric analysis (with a single analysis can provide information on variations in DNA content and polyploidization, variations in cell cycle dynamics and also, DNA damage). In plants, FCM has been demonstrated to detected differences in DNA content as small as 1% (Pfosser et al., 1995), chromosome aberration in wheat-rye lines exposed to aluminium (Rayburn and Wetzel, 2002), DNA damage in lettuce plants exposed to Cd (Monteiro et al., 2010) and cell cycle arrest in *A .cepa* exposed to X-ray radiation (Carballo et al., 2006).

Another good technique for assessing genotoxicity is the Comet assay, which is a versatile and sensitive method for measuring single- and double-strand breaks in DNA (Collins et al., 2008). The simplicity inherent to sample preparation and the relatively small number of cells/nuclei analysis required to obtained robust results (Hattab et al., 2009a), the later which can be automated further reducing the time need to obtain results, can be accounted as the reasons for the dramatic increase of Comet assays application in genotoxicity studies. In plants, Comet assay has been proved to be very useful to study genotoxicity of heavy metals (e.g Hattab et al., 2009a; Gichner et al., 2006; 2008a).

5. Lead: The element

Lead (Pb) is a silvery-white highly malleable metal, with a low melting point and high density. Pb has had many applications since its discovery: The Egyptians used grounded lead ore as eyeliner with therapeutic proprieties; Pb based pigments were used as part of yellow red and white paint; in ancient Rome, Pb was used to build pipes for water transportation and not so long ago, tetraethyl lead was used in petrol fuels. Nowadays, this metal remains a major constituent of most batteries used in automobiles, is used in projectiles for firearms, and molten Pb is used as a coolant.

Releases of lead in the environment can occur naturally from the mobilization of Pb from the Earth's crust and mantle, such as volcanic activity and the weathering of rocks. However, these releases are very rare and the most significant sources of Pb discharge are those originated by anthropogenic activities.

Some of the most influential sources of Pb pollution are lead impurities in raw materials such as fossil fuels and other extracted and treated metals, mining, releases from incineration and installations for municipal waste, open burning and the mobilization of historical Pb releases previously deposited in soils, sediments and wastes. From these sources this pollutant can be transported thousands of kilometres through the air (burned fuel and air-borne particles like fly ash) and by rivers and oceans (discharges from industries and leakage from residues).

In the atmosphere, lead will deposit on surfaces or exist as a component of atmospheric particles. In the atmosphere, lead exists primarily as lead compounds. The residence time ranges from hours to weeks. In the aquatic environment, lead can occur in ionic form (highly mobile and bio-available), organic complexes with dissolved humus materials (binding is rather strong and limits availability), attached to colloidal particles such as iron oxide (strongly bound and less mobile when available in this form than as free ions) or to solid particles of clay or dead remains of organisms (very limited mobility and availability).

6. Lead: Uptake and assimilation by plants

The speciation of lead differs whether it is in fresh water, seawater or soil. In fresh water lead primarily exists as the divalent cation (Pb^{2+}) under acidic conditions, and forms $PbCO_3$ and $Pb(OH)_2$ under alkaline conditions. Lead speciation in seawater is a function of chloride concentration and the primary species are $PbCl_3^-$ > $PbCO_3$ > $PbCl_2$ > $PbCl^+$ > and $Pb(OH)^+$.

In soil, lead is generally not very mobile. The downward movement of elemental lead and inorganic lead compounds from soil to groundwater by leaching is very slow under most natural conditions. Clays, silts, iron and manganese oxides, and soil organic matter can bind lead and other metals electrostatically (cation exchange) as well as chemically (specific adsorption). Biotic factors like soil pH, content of humic acids and amount of organic matter influence the content and mobility of lead in soils. Despite the fact that lead is not very mobile in soil, lead may enter surface waters as a result of erosion of lead-containing soil particles. All these factors will influence the bioavailability of Pb and thus the toxicity level of this heavy metal.

To become metabolized by plants, elements need to be transported, at some point, through the plasma membrane of the roots. (Kučera et al., 2008) reported that once in contact with

plants, Pb was transported by CPx-type ATPases, a subgroup of P-type ATPases, that pump essential and non-essential metals such as Cu^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+} across the plasma membrane.

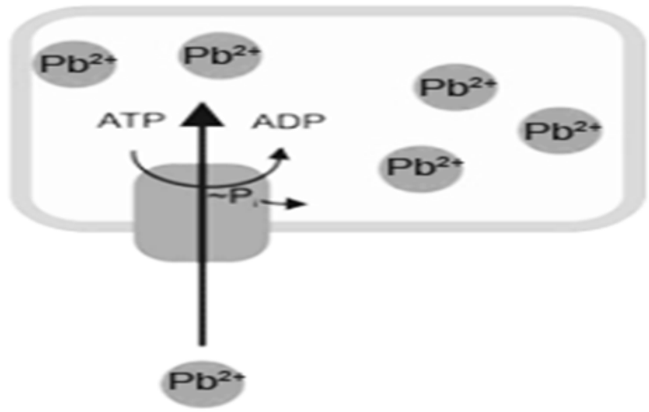

Fig. 4. Pb transport by CPx-type ATPases (adapted from the model of Dr. Mathias Lübben)

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To become metabolized by plants, elements need to be transported, at some point, through the plasma membrane of the roots. (Kučera et al., 2008) reported that once in contact with plants, Pb was transported by CPx-type ATPases, a subgroup of P-type ATPases, that pump essential and non-essential metals such as Cu^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+} across the plasma membrane.

Plants absorb Pb usually accumulating it in the roots (Carruyo et al., 2008; Hanc et al., 2009), acting like a natural barrier. Although, a small portion can also be translocated upwards to stems, leaves (Hanc et al., 2009) and seeds being the increase level, directly proportional to the amount of exogenous lead.

Authors have studied the effect of pH variation in Pb uptake, in different plant species: in low pH soils (3.9), increased mobility of lead was observed, resulting in higher uptake (Ernst et al., 2000); Gorlach et al. (1990), working Italian ryegrass found that with increasing soil pH (3.9–6.7), Pb uptake was reduced. Also, and in addition to soil factors, the species and genotype also dictate Pb's uptake and accumulation.

Once inside the root cortex, Pb moves in the apoplastic space, using the transpiration conductive system (Wierzbicka, 1999; Hanc et al., 2009)). It can also bypass the endodermis and gain symplastic access in the young root zone and in sites of lateral root initiation (Eun

et al., 2000). Pb has been shown to enter and move within the cytoplasm and proteins mediating cross-membrane movement of Pb have been identified (Kerper and Hinkle, 1997; Arazi et al., 1999). Most of the Pb absorbed by roots exists as extracellular precipitate (as phosphate and carbonate)or is bound to ion exchangeable sites in the cell walls (Sahi et al., 2002). The unbound Pb is moved through Ca channels accumulating near the endodermis (Huang and Cunningham, 1996; Antosiewicz, 2005). Depending on the plant species exposed, different cellular types can be used to store Pb: in wheat, Pb is fixed to the cell wall of roots but it can be removed as a complex using citric acid (Varga et al., 1997). Peralta-Videa et al. (2009) on the other hand discuss the accumulation of Pb in the phloem tissues of *Prosopis* sp. associated with *Glomus deserticola,* suggesting that it was transported to the leaves and returned through the phloem to the plant organs.

7. Lead: Phytotoxicity

7.1 General effect

The first reported uses of lead date back to 4000 BC, and toxicological effects have been linked to lead since antiquity. Lead is known to bioaccumulate in most organisms, whereas it is generally not biomagnified up the food web.

Pb its known to negatively affect some of the most classical endpoints of plant toxicity like germination rate, growth and dry mass of roots and shoots (Ekmekçi et al., 2009; Munzuroglu and Geckil, 2002;). In general, effects are more pronounced at higher concentrations and durations. In some cases, lower concentrations stimulate metabolic processes and enzymes involved. The major processes affected are seed germination, seedling growth (shoot and root growth), photosynthesis, plant water status, mineral nutrition, and enzymatic activities. Visible symptoms include chlorotic spots, necrotic lesions in leaf surface, senescence of leaf and stunted growth. Germination of seeds is drastically affected at higher concentrations. Development and growth of root and shoot in seedling stage are also affected, roots being more sensitive.

Lead reduced the uptake and transport of nutrients in plants, such as Ca, Fe, Mg, Mn, P and Zn, by blocking the entry or binding of the ions to ion-carriers making them unavailable for uptake and transport from roots to leaves (Xiong, 1997). This in turn affects several physiological and biochemical processes, among which photosynthesis is one of the most affected.

7.2 Photosynthesis

Photosynthesis is one of the processes most sensitive to lead: the substitution of the central atom of chlorophyll, magnesium, by lead in vivo prevents photosynthetic light-harvesting in the affected chlorophyll molecules, resulting in a breakdown of photosynthesis (Küpper et al., 1996). Higher concentrations of lead significantly affected plant water status causing water deficit.

The deleterious effects of this metal in several physiological parameters have been addressed in several species: John et al. (2009) found in *Brassica juncea* exposed to this metal, growth impairment and decrease in pigments content; Kosobrukhov et al. (2004) working with *Platango major* showed that Pb can affect g_s, pigment content, and light and dark

reactions; Bibi and Hussain (2005) demonstrated that the *A*, *E* and g_s of *Vigna mungo* plants were significantly affected when exposed to Pb. The total chlorophyll content and relative content proportion of Chl *a* and *b* were reduced, through inhibition of chlorophyll biosynthesis (Ernst et al., 2000; Van Assche and Clijsters, 1990; Sengar and Pandey, 1996). Cenkci et al. (2010) found that the content in carotenoids was less affected than chlorophylls by Pb and suggested that this was so because carotenoids protect chlorophyll from photo-oxidative destruction and therefore, a reduction in carotenoids could have a serious consequence on chlorophyll pigments.Limitation of photosynthesis by reduced activity of Calvin cycle enzymes, e.g. RuBisCO activity was reported for several plant species exposed to Pb (Vojtěchová and Leblová, 1991; Moustakas et al., 1994). Lee and Roh (2003) found that exposure to Cd induced significant decrease in RuBisCO activity which was associated to the amount of RuBisCO protein; this might be a hint to the decrease in RuBisCO activity observed with Pb exposure, it is possible that Pb as Cd cause a decrease in RuBisCO protein. More recently, Bah et al. (2010) proved that Pb caused the up-regulation of carbohydrate metabolic pathway enzymes; APX and GRSF; RuBisCO activase, Mg-protoporphyrin IX chelatase, fructokinase, a chloroplast precursor and plastocyanin suggests. With those results, the authors concluded that what was observed was part of a strategy to cope with Pb toxicity, by increasing carbohydrate metabolism (fruktokinase), photosynthesis (RuBisCO activase, Mg-protoporphyrin chelatase and plastocyanin) and defense response (APX and GRSF). They also concluded that despite that the strategy was responsible for the high tolerance of *T. angustifolia* to Pb toxicity, this had a high energetic cost.

Transpiration intensity, osmotic pressure of cell sap, water potential of xylem, and relative water content were significantly reduced after 24 and 48h of exposure to Pb (Parys et al., 1998). Lead also reduces the size of stomata but increases their number and diffusion resistance.

The mechanism(s) of this metal toxicity on photosynthesis is still a matter of speculations, this may be partly due to the differences in experimental design, but it almost certainly involves electron transport in light reactions and enzyme activity in the dark reactions (Romanowska et al., 2006). Despite of the fact that the mechanism by which Pb affects the photosynthetic apparatus is unclear, evidence indicates that this metal causes severe effect to the photosynthetic status of plants and thus, it of vital importance to carry studies to better understand Pb's toxicity.

Similarly to the studies with Cr(VI), Rodriguez (2011) evaluated the Pb -induced toxicity on the photosynthetic status of *Pisum sativum* plants. The endpoints measured involved gas exchange, Calvin cycle enzymes activity, amount of soluble sugars and starch, pigment content and fluorescence emission. Moreover, chloroplast structure and functional status variation as function of Pb toxicity were also demonstrated in pea leaves by FCM.

7.3 Genotoxicity

The chemical form of Pb only affects lead transport from the medium into the plants and all forms had similar effects on mitosis. The iodides had a greater mutagenic effect than the nitrates, perhaps because the latter dissolved completely in the solution and were supplied as ions, rather than molecules as in the cases of the iodides (Radecki et al., 1989).

Pb toxicity has been linked to carcinogenicity and the genotoxic effects of this metal have been studied thoroughly in animals and humans. Nevertheless, data related to the

mutagenic, clastogenic and carcinogenic properties of inorganic lead compounds are still conflicting (García-Lestón et al., 2010). Hartwig et al. (1990) working with V79 Chinese hamster cells exposed to Pb and UV radiation concluded that Pb alone did not induced DNA damage but magnified that caused by UV rays, this they said, was due to Pb interference with the repair machinery. This might be due to Pb ability to substitute calcium and/or zinc in enzymes involved in DNA processing and repair leading to an inhibition of DNA repair and an enhancement in the genotoxicity when combined with other DNA damaging agents (García-Lestón et al., 2010). The major mechanisms putatively involved in Pb genotoxicity are summarized in Figure 5.

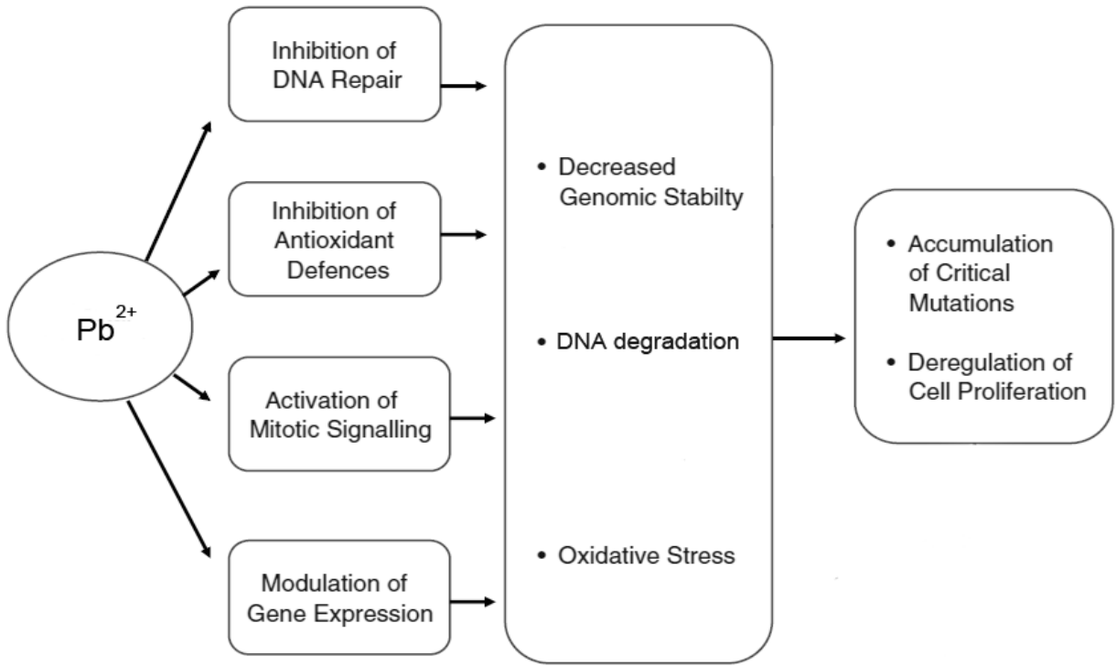

Fig. 5. Major mechanism of Pb genotoxicity. Adapted from Beyersmann and Hartwig (2008).

Valverde et al. (2001) demonstrated that despite that Pb did not cause DNA damage in in vitro DNA, production of lipid peroxidation and an increase in free radical levels were observed, suggesting that Pb exposure cause genotoxicity and carcinogenicity by indirect interactions, such as oxidative stress. These investigations support the current thesis stating that the way of action of Pb might be through ROS formation and interference with the DNA repair mechanism (Beyersmann and Hartwig, 2008), rather than a direct interaction with DNA as it is seen with Cr.

Animal cell proliferation has also been demonstrated to be sometimes affected by Pb exposure, by increasing proliferative lesions in the kidney, below cytotoxic concentrations. This stimulation indicates that genotoxicity and accelerated growth stimuli may act in concert in lead-induced carcinogenicity in mammals (Beyersmann and Hartwig, 2008).

In plants and despite of the importance of Pb pollution and risk associated to the environment, the mechanism and effects of Pb toxicity are far less known than in animals. Of what is known, most of the Pb absorbed remains in the root with only a small fraction

being translocated to the shoots (Patra et al., 2004). There, Pb has been demonstrated to cause chromosome aberration (Carruyo et al., 2008) in *A. cepa*; DNA degradation in lupin and tobacco (Gichner et al., 2008b, Rucinska et al., 2004) and genomic instability in turnips (Cenkci et al., 2010). Lead nitrate proved to be a weak mutagen but owing to its high toxicity had a synergistic effect in combination with ionizing radiation in some populations (Patra et al., 2004). Lead in particular, has been demonstrated to increase Comets formation, thus having genotoxic effects, at short term exposure, in tobacco (Gichner et al., 2008b) and lupin (Rucinska et al., 2004). Pb genotoxicity studies in plants are summarized in Table 2.

Recently our group demonstrated that leaves from Pb-exposed plants showed a slight increase in DNA degradation at the highest tested concentration, while in roots, significant changes in cell cycle dynamics were observed at G_0/G_1 and G_2. In these roots, significant damages of DNA were shown by increases of tail moment (TM) and of full peak coefficient of variation (FPCV). The authors suggested that Pb induced a blockage of cell cycle at the G_2/M checkpoint due to severe degradation of the DNA (Rodriguez 2011).

Species	Reference	Dose	Technique	Effects
P. sativum	(Gabara et al., 1995)	10^{-4} M	DNA synthesis	Diminished DNA synthesis
V. faba	(Chang-qun and Huan-xiao, 1995)	Pb^{2+} (NR)	cytogenetic	Mitotic stage shortened and interphase prolonged
A. cepa	(Rank and Nielsen, 1998)	Wastewater Sludges	cytogenetic	Anaphase-Telophase chromosome aberration
H. vulgare *C. sativum* *A. cepa*	(Bhowmik, 2000)	$Pb(NO_3)_2$ (0.001 to 1 mg/Kg)	cytogenetic	Redution of mitotic index, increase of chromosomal aberration, Polyploidy
A. thaliana (transgenic)	(Kovalchuk and Yao, 2011)	Pb^{2+} (0.002 to 0.83 mg/L)	Trasngenic plant reporter gene	Increase in the mutation frequency
L. luteus	(Rucinska et al., 2004)	$Pb(NO_3)_2$ (150 and 350 mg/l)	Comet assay	DNA damage
N. tabacum	(Gichner et al., 2008b)	Pb^{2+} (200 µM to 0.4 mM)	Comet Assay	DNA damage
B. rapa	(Cenkci et al., 2010)		RAPD	Genomic template instability
L. sativa	(Ritambhara and Girjesh, 2010)	$Pb(NO_3)_2$ (25 to 300 ppm)	cytogenetic	Abnormal chromosome migration
V.faba	(Shahid et al., 2011))	$Pb(NO_3)_2$ (5 µM)	Cytogenetic	MN and mitotic index
P sativum	Rodriguez 2011	$Pb(NO_3)_2$ (up to 2000 mg/L)	Comet Assay FCM	DNA damage Clastogenicity G_2/M arrest

Table 2. Literature survey of Pb genotoxic effect in plants.

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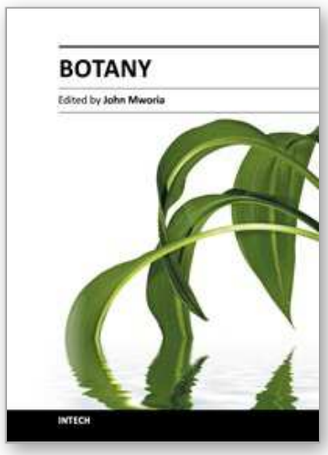

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This book is devoted to botany and covers topical issues in this diverse area of study. The contributions are designed for researchers, graduate students and professionals. The book also presents reviews of current issues in plant-environment interactions making it useful to environmental scientists as well. The book is organized in three sections. The first section includes contributions on responses to flood stress, tolerance to drought and desiccation, phytotoxicity to Chromium and Lead; the second has aspects of economic botany including a review of Smut disease in sugarcane and properties of plant extract used Tassaboount date juice; the last covers topical issues on morphogenesis and genetics on cotton fiber special cell, secretory glands Asphodelus aestivus flower ,pollen tube growth in Leucojum aestivum , morphological studies of Ardisia crenata complex, and hybrid lethality in the Genus Nicotiana.

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