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**Titre :**
Induction de la phytotoxicité du Plomb chez Vicia faba L. : rôles de l'absorption et de la spéciation
Lead-induced toxicity to Vicia faba L. in relation with metal celluptake and speciation

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# Avant-propos

Au cours de ma thèse j'ai travaillé au laboratoire d'écologie fonctionnelle ECOLAB, sur le campus de l'ENSAT-INPT. Je remercie E. Chauvet et A. Lebrihi, respectivement directeurs du laboratoire Ecolab et de l'ENSAT pour les conditions de travail favorables dont j'ai pu bénéficier. Cette thèse a été réalisée dans le cadre d'une bourse Franco-Pakistanais, gérée par l' « Higher Education Commission of Pakistan » (HEC) et la Société Française d'Exportation de Ressources Educatives (SFERE) pour la période de septembre 2007 à décembre 2010. Le travail a été financé par le « Ministère français de l'Enseignement Supérieur et de la Recherche'' (MESR, enseignementsup-recherche.gouv.fr) et par un projet national de l'INSU EC2CO-CYTRIX. Je remercie ces organismes pour leur soutient qui a participé à la réalisation de ce travail de thèse. Camille Dumat et Eric Pinelli enfin, mes deux directeurs de thèse, m'ont promulgué tout au long de la thèse leurs précieux conseils scientifiques et pédagogiques. Ils ont toujours été disponibles et à l'écoute afin que mon travail de thèse avance efficacement et je les en remercie sincérement.

Cette thèse est rédigée principalement en anglais, avec l'accord de l'école doctorale Sciences De l'Univers, de l'Environnement et de l'Espace (SDU2E), Université de Toulouse, France. Plusieurs chapitres (synthése bibliographique et résultats) correspondent à des publications acceptées, soumises ou en cours de soumission.

Par ailleurs, j'ai pu bénéficier d'un poste de moniteur de l'enseignement supérieur durant ces 3 années de thèse. J'ai réalisé mes trois années d'enseignement à l'INP-ENSAT (Institut National Polytechnique-Ecole Nationale Supérieure Agronomique de Toulouse) sous la direction de Mme Camille DUMAT, principalement dans les deux premières années du cycle ingénieur (travaux dirigés et travaux pratiques du module de Physico-chimie et fertilisation des sols et évaluation de sols par des tests ecotoxicologique). J'ai également enseigné en mastère « ingénierie des risques » et module de biogéochimie de l'environnement la modélisation de la spéciation des métaux en solution. Ce poste de moniteur m'a permis de bénéficier des formations du Centre d'Initiation à l'Enseignement Supérieur de Midi-Pyrénées, très profitables car je souhaiterais par la suite devenir enseignant-chercheur.

# Forewords

During my PhD, I worked in the Laboratory of Functional Ecology (ECOLAB) at the campus of INP-ENSAT. I thank E. Chauvet and A. Lebrihi, respectively, the directors of Ecolab and ENSAT for favorable working conditions that I have enjoyed. This thesis was funded by the Higher Education Commission of Pakistan and the French Embassy for a period from November 2007 to December 2011. The scholarship was supervised by the French Society for Export of Educational Resources (SFERE). The experimental work was funded by the "French Ministry of Higher Education and Research'' (MESR, enseignementsup-recherche.gouv.fr) and EC2CO-CYTRIX project. I thank these organizations for their support, which has enabled the realization of this work.

This thesis is written primarily in English, with the consent of the Graduate School "Science of Universe, Environment and Space (SDU2E)", University of Toulouse, France. The different sections of chapters (literature review and results) correspond to the publications accepted, submitted or being submitted.

I was also recruited as lecture during these three years of thesis at INP-ENSAT (National Polytechnic Institute-National Higher School of Agriculture Toulouse). I conducted my teaching services under the guidance of Dr. Camille Dumat. During this period, I participated in teaching classes of M2R (M.Sc. level) on «Physico-chemistry and fertilization of soil: agronomic effectiveness and environmental safety» and "evaluation of soil through eco-toxicological tests». I also taught M.Sc. classes of risk engineering and a module "environmental biogeochemistry" concerning the speciation modeling of metals in solution. This job also allowed me to participate in 15 days training classes and 15 days practical session of teaching organized by Initiation Center for Higher Education (CEIS) in Midi-Pyrenees. These training classes provide me an opportunity to discuses teaching skill, methods and problems with other lectures and specialized teacher of different domains.

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A bundle of thanks to my friends *Arshad, Nafees, Waheed, Saad, Ali, Hussnain, Saqlain, Umar, Tameez, Saima* and of course special thanks to doctor company (*Rashid, Toufeer, Rizwan, Majid, Ilyas, Kaleem and Muazzam*) for their help and cooperation.

I am also indebted to my colleagues at EcoLab and office fellows *Bertrand, Uzu,* Damien, Timothée, *Lobat, Thierrry, Désirée, Floriane, Eva and Roman* for their nice company and help during my stay at Ecolab.

Cordial and sincere obligations are rendered to my loving mother and affectionate father, whose hands always raised with unlimited affection by virtue of which I could reach at this position. A special thanks to my wife "*Asma*" who always shares my problems with an ultimate solution, and cooperated at each and every crucial step of my life. My humble thanks to my sisters, brothers *Ihsan* and *Zafar*; nice *Arij*, *Isha*, *Zahra* and bleu eyed *Zeenat*, and nephews *Taha* and *Umar*. I dedicate this thesis in honor of my family.

Finally thanks to HEC (Higher Education Commission, Pakistan) and SFERE France for giving me the opportunity to study in this beautiful country with the most professional environment.

*Shahid*

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**List of abbreviations**

| | |
|---|---|
| ABA | Abscisic acid |
| ABC | ATP Binding Cassette |
| AFNOR | Association Française de NORmalisation |
| ALAD | Delta-aminolevulinic acid dehydratase |
| ANOVA | Analysis of Variance |
| APX | Ascorbate Peroxidase |
| ATP | Adenosine Tri Phosphate |
| BHT | Butylated Hydroxy Toluene |
| CA | Citric Acid |
| CAT | Catalase |
| CEC | Cation Exchange Capacity |
| Chl | Chlorophyll |
| Chl-a | Chlorophyll a |
| Chl-b | Chlorophyll b |
| COOH | Carboxyl Group |
| DHA | Dehydroascorbate |
| DHAR | Dehydroascorbate Reductase |
| DM | Dry Matter |
| DNA | Deoxyribonucleic acid |
| DOC | Dissolved Organic Carbon |
| DTPA | Diethylene triamine pentaacetic acid |
| DW | Dry Weight |
| EDHA | Ethylenediamine-N, N'-bis(2-hydroxyphenylacetic acid) |
| EDDS | Ethylenediamine-N,N'-disuccinic acid |
| EDTA | Ethylenediaminetetraacetic Acid |
| EHPG | N,N'-Ethylenebis-[2-(o-hydroxyphenyl)]-glycine |
| ESFA | Elliott Soil fulvic acid |
| FA | Fulvic Acid |
| GPOX | Guaiacol Peroxidase |
| GR | Glutathione Reductase |

| | |
|---|---|
| GSH | Reduced Glutathione |
| GSSG | Glutathione Oxidized |
| $H_2O_2$ | Hydrogen Peroxide |
| HA | Humic Acid |
| HSs | Humic Substances |
| HSD | Honestly Significant Difference |
| IAA | Indole Acetic Acid |
| ICP-OES | Induced Coupled Plasma-Optical Emission Spectrometry |
| LMWOAs | Low Molecular Weight Organic Acids |
| LOX | Lipoxygenase |
| LPO | Lipid Peroxidation |
| MDA | Malondialdehyde |
| MDHAR | Monodehydroascorbate Reductase |
| MH | Maleic Hydrazide |
| MI | Mitotic Index |
| MN | Micronuclei |
| MSW | Municipal Solid Waste |
| MTs | Metallothioneins |
| NADH (P) | Reduced Nicotinamide Adenine Dinucleotide Phosphate |
| NBT | Nitro Blue Tetrazolium |
| NC | Negative Control |
| NTA | Nitrilotriacetic acid |
| $O_2^-$ | Oxide ion |
| $O_2$ | Oxygen |
| $O_2^{\cdot -}$ | Superoxide anion radicl |
| $O_2^{2-}$ | Peroxide ion |
| OD | Optical density |
| $OH^-$ | Hydroxyl anion |
| $OH^{\cdot}$ | Hydroxyl radical |
| OH | Alcoholic Group |
| OM | Organic Matter |
| PCA | Principal Component Analysis |

| | |
|---|---|
| PCS | Phytochelatin Synthetase |
| PCs | Phytochelatins |
| POX | Peroxidase |
| PSI | Photosystem I |
| PSII | Photosystem II |
| REACH | Registration, Evaluation, Authorisation and Restriction of Chemical substances |
| ROS | Reactive Oxygen Species |
| -SH | Thiol Group (or sulfhydryl) |
| STEM | Scanning Transmission Electron Microscope |
| SOD | Superoxide Dismutase |
| SOM | Soil Organic Matter |
| SRFA | Suwannee River fulvic acid |
| TBARS | Thiobarbituric Reactive Species |
| TCA | Trichloroacetic Acid |
| *V. faba* | *Vicia faba* |
| WHAM | Windermere Humic Aqueous Model |

**Résumé**

Peu d'études concernent actuellement l'influence de la spéciation du plomb (polluant métallique toxique, persistant et très présent dans les écosystèmes) sur sa phyto-toxicité. Pourtant, l'absorption des métaux, leur translocation et les mécanismes impliqués dans leur phyto-toxicité peuvent être fortement modifiés par ce paramètre. Dans ce contexte, l'objectif de la thèse était d'étudier l'influence de la spéciation chimique du plomb (formation de divers complexes organométalliques) sur sa phytodisponibilité et sa phytotoxicité.

Des plants de *V. faba* ont été exposés pendant 1 à 24 h en hydroponie à 5 µM de nitrate de plomb seul ou chélatés à des degrés variables par différents ligands organiques : acide éthylènediamine, acide citrique et substances humiques. Les pourcentages de plomb libre et chélaté ont été calculés puis utilisés pour la conception du dispositif expérimental. La phytotoxicité induite par le plomb a été évalué pour les différentes conditions en mesurant : les activités d'enzymes antioxydantes, la génotoxicité, la peroxydation des lipides, les concentrations d'espèces réactives de l'oxygène, des pigments photosynthétiques et la génotoxicité.

La phytotoxicité est fonction de la spéciation des métaux et de la durée d'exposition. Selon la nature du ligand organique, les mécanismes impliqués différent. L'EDTA aurait un rôle protecteur : l'absorption du Pb par les racines est accrue, alors que sa translocation et sa phyto-toxicité sont réduites de façon dose-dépendante. En revanche, l'acide citrique ne modifie pas le transfert du plomb, mais retarde cependant l'induction de sa phytotoxicité. Finalement, les acides fulviques appliqués à 25 $mg.L^{-1}$, réduisent la toxicité du Pb en limitant son absorption. En outre, l'efficacité et la sensibilité des tests écotoxicologiques, en relation avec l'absorption et la spéciation, ont été comparées (analyse en composantes principales) et discutés. Ce travail trouve donc des applications pour le développement de tests d'écotoxicité pertinents pour évaluer la qualité des milieux.

**Abstract**

Lead (Pb) is a known toxic and persistent pollutant, which does not have any essential role in the metabolism of living organism. Only few studies concern Pb-induced phyto-toxicity in relation to its speciation, which can nevertheless influence metal uptake, translocation and mechanisms involved in phyto-toxicity. In this context, the objective of this thesis was to study the influence of chemical speciation of Pb (formation of various organometallic complexes) on its phytoavailability and phytotoxicity.

*Vicia faba* seedlings were exposed for 1-24 hourd (h) in controlled hydroponic conditions to 5µM of Pb nitrate alone and chelated to varying degrees by different organic ligands i.e. ethylenediaminetetraacetic acid (EDTA), citric acid and two types of humic substances (Suwannee River fulvic acid and Elliott Soil fulvic acid). Visual Minteq and WHAM VI metal speciation softwares were used to estimate the chelated and free Pb cations concentration in nutrient solution. These calculations were used to design the experimental layout. The effect of these organic ligands on Pb-induced toxicity to *V. faba* was assessed by measuring five antioxidant enzyme activities (superoxide dismutase, ascorbate peroxidise, guaiacol peroxidase, catalase, glutathione reductase), lipid peroxidation, reactive oxygen species, photosynthetic pigment and genotoxicity.

Pb-induced phytotoxicity is the function of metal speciation and duration of exposure. EDTA has a protective role: the absorption of Pb by the roots is increased, whereas its translocation and phyto-toxicity are reduced in a dose-dependent. In contrast, citric acid does not alter the transfer of Pb, but delays the induction of its phytotoxicity. Finally, fulvic acids applied at 25 $mg.l^{-1}$, reduce the toxicity of Pb by limiting its absorption. Moreover, the efficiency and sensitivity of ecotoxicological tests was compared in relation with Pb uptake and speciation using principal component analysis. This work is, therefore, applicable for the development of ecotoxicity tests relevant to assessing environmental quality.

## Introduction générale

Le plomb (de symbole Pb et de numéro atomique 82) est un métal gris-bleu, ductile, dense, résistant à la corrosion avec un faible pouvoir conducteur. C'est pour ces nombreuses propriétés physico-chimiques, que ce métal a été largement utilisé par l'homme depuis les égyptiens. De nos jours, le plomb continue à être utilisé dans de nombreux processus industriels (Sharma et Dubey, 2005). Ceci a conduit à une augmentation significative de la concentration de ce métal dans tous les compartiments environnementaux biotiques et abiotiques (Sammut et al. 2010; Grover et al. 2010).

Les principales sources d'émission de plomb dans l'environnement ont été ou sont encore, l'utilisation des carburants d'origine fossile complétés en plomb, l'exploitation des mines, les anciennes peintures à base de plomb, la plomberie, les munitions, les boucliers de rayons X, le brasage, les conteneurs pour les liquides corrosifs. Les émissions de plomb dans le sol et l'eau peuvent également se produire suite à l'épandage des boues d'épuration ou lors du rejet d'effluents agricoles traités (Singh and Agrawal, 2010). Selon l'USGS, la production en 2009 de plomb extrait des mines en Chine, Australie et États-Unis, était respectivement de 1690, 516 et 400 millions de tonnes.

Le plomb est le 36$^{ème}$ élément le plus abondant dans l'écorce terrestre (Arias et al. 2010; Grover et al. 2010) avec une concentration moyenne de 13 mg $kg^{-1}$ (Nriagu, 1978). Dans la croûte terrestre, le plomb s'observe le plus souvent sous la forme de sulfures de plomb dans le minerai (la galène), produit final de la désintégration de trois éléments naturellement radioactifs: l'uranium, le thorium et l'actinium. Les teneurs naturelles de Pb dans les sols sont généralement inférieures à 50 $mg.kg^{-1}$ (Pais et Jones, 2000). En raison d'une relativement faible mobilité dans les sols, le plomb est généralement peu soluble et disponible pour les plantes (Garcia et al. 2003; Arshad et al. 2008). Il est donc nécessaire de déterminer le comportement biogéochimique du plomb dans l'environnement.

Le plomb peut toutefois se retrouver dans les sols sous des formes plus mobiles et disponibles comme l'ion libre $Pb^{2+}$, complexée par des ligands organiques ou absorbée à la surface de particules colloïdales (Sammut et al. 2010; Vega et al. 2010). Cette fraction mobile du plomb peut être absorbée par les organismes vivant et contaminer la chaîne alimentaire. L'absorption du plomb par les plantes est influencée par de nombreux facteurs liés au sol tels que la composition, la granulométrie, mais également des facteurs liés à la plante comme la production d'exsudats racinaires (Cecchi et al. 2008; Punamiya et al. 2010).

L'une des principales voies de pénétration du plomb dans les plantes est le franchissement des membranes cellulaires via des canaux ionique à forte affinité pour le calcium (Pourrut et al, 2008). Une fois dans les racines, le plomb est généralement peu transféré vers les parties aériennes des plantes, mais a tendance à être piégé dans les parois ou les cellules racinaires. Excepté dans le cas des hyper accumulateurs, environ 95 % du plomb absorbé serait présent dans les racines (Gupta et al. 2010; Jiang and Liu 2010).

Bien que les plantes aient de nombreux systèmes de détoxification pour limiter l'interaction des métaux potentiellement toxiques avec des molécules biologiques, ces métaux entraînent souvent des effets néfastes pour les organismes. L'accumulation excessive de plomb dans les tissus est toxique pour la plupart des plantes, conduisant à des diminutions de la germination, de l'allongement racinaire, de la biomasse, et une inhibition de la biosynthèse chlorophyllienne (Sharma et Dubey, 2005; Brunet et al. 2009; Piotrowska et al. 2009; Sing et al. 2010). La présence de plomb dans des plantes induit également la production d'espèces réactives de l'oxygène qui perturbent le statut redox des cellules, causant un stress oxydant (Pourrut et al. 2008; Liu et al. 2008; Grover et al. 2010; Yadav, 2010). Cette fonctionnalité est connue pour être une cause majeure de la toxicité des métaux lourds. Cependant, les mécanismes d'origine de cette toxicité de plomb ne sont pas encore connus.

En termes de santé humaine, les empoisonnements au plomb ont jalonné l'histoire des civilisations. Dans le contexte des habitats vétustes et de la proximité des friches industrielles non contrôlées, les enfants peuvent êtres plus particulièrement touchés avec des cas de déficience mentale détectés chez les jeunes enfants (Davies, 1995 ; Lamphear, 1998). Le plomb a en conséquence été classé comme produit chimique préoccupant dans le cadre de la réglementation Européenne REACH (ce 1907 / 2006, d'enregistrement, d'évaluation, d'autorisation et de restriction des produits chimiques). Il a également été signalé en 2003 comme la 2$^{ème}$ substance la plus dangereuse (critères de fréquence d'apparition, toxicité et potentiel d'exposition de l'homme) par l'ATSDR (Agence pour les substances toxiques et du registre de la maladie).

Plusieurs travaux étudient le potentiel bio-toxique du plomb. La majorité de ces études concernent la teneur totale du métal. Pourtant, les effets potentiels des éléments toxiques dans l'environnement sont fortement influencés par leur spéciation au sens large : distribution physico-chimiques et spéciation chimique. Le rôle de l'ion libre ou chélaté avec des molécules organiques solubles a en particulier été mis en avant dans plusieurs études

(Dumat et al. 2006; Kopittke et al. 2008; Uzu et al. 2009; Sammut et al. 2010, Shahid et al, 2010). Si la concentration totale en métal, facile à mesurer reste un indicateur de la qualité des milieux, elle est un indicateur peu pertinent de la toxicité. Evaluer la toxicité métallique en relation avec le transfert et la spéciation constitue donc un sujet de recherche important pour les années à venir.

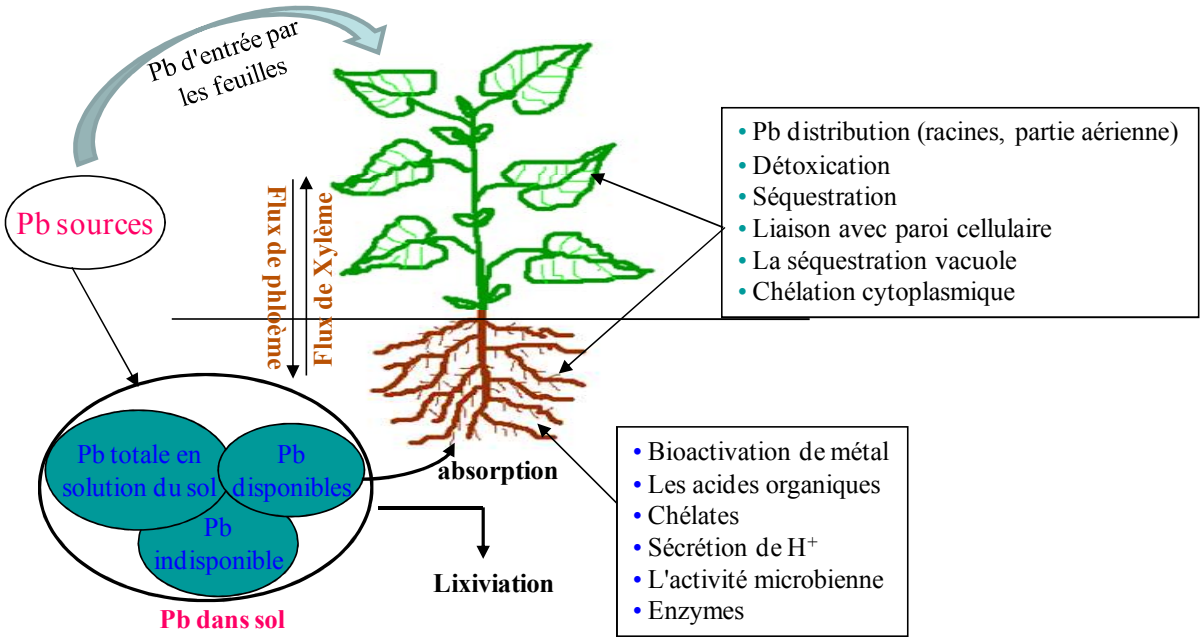


**Figure-1.** Comportement biogéochimique du plomb dans le sol, l'absorption par les racines et l'accumulation ainsi que les mécanismes de détoxication adoptée par les plantes contre la toxicité du plomb.

C'est pourquoi, dans cette thèse, nous avons choisi d'étudier le rôle de la spéciation du plomb sur son transfert et sa toxicité. En raison de sa grande sensibilité au Pb et de sa large utilisation lors d'études précédentes (Hajjouji et al. 2007; Pourrut et al. 2008; Probst et al. 2009; Marcato-Romain et al. 2009a; Marcato-Romain et al. 2009b), *Vicia faba* a été choisie comme un biomarqueur pour évaluer la toxicité métallique. La concentration de 5 µM Pb $(NO_3)_2$ a été choisie car selon Pourrut et al. (2008), elle induit une génotoxicité sur *V. faba* tout en restant représentative d'une pollution environnementale. Trois ligands organiques (EDTA, acide citrique et substances humiques) aux caractéristiques contrastées (taille, nature et pouvoir complexant) ont été sélectionnés pour complexer le Pb en solution. Les niveaux de concentrations appliquées ont été choisis pour atteindre différents taux de complexation, en

utilisant des modèles de spéciation : Visual Minteq logiciel version 2,60 et Modèle humique aqueuse de Windermere VI (Windermere Humic Aqueous Model VI). Ces modèles ont en effet permis de calculer les concentrations de Pb chélaté et libre en solution nutritive pour les différentes expériences. Les traitements d'exposition des plantes au plomb (avec divers ajouts de complexant) ont été réalisés sur des durées courtes allant de 1 à 24 h, afin de suivre la cinétique de réaction de *V. faba* lors des différents tests d'écotoxicologie.

Les études réalisées visent d'une part, à analyser les transferts de plomb des solutions nutritives vers les différents compartiments des plantes en fonction de sa spéciation et d'autre part de pouvoir corréler les effets observés non pas à la concentration totale en plomb dans les plantes mais à sa spéciation. Nous avons donc étudié les effets du plomb en présence des différents chélates sur différents marqueurs de stress. Nous avons ciblé notre étude sur le contenu chlorophyllien, la peroxydation lipidique, la production des espèces réactives de l'oxygène et des activités enzymatiques impliqués dans la lutte contre le stress oxydatif (catalase, superoxyde dismutase, gluthation réductase, ascorbate peroxydase, et guaiacol peroxydase). Enfin, nous avons étudié les effets de la spéciation du plomb sur la génotoxicité.

Le premier chapitre de cette thèse présente une revue bibliographique divisée en deux sections: (1A) la toxicité du plomb chez les plantes (1B) les effets de la spéciation de plomb sur son absorption et sa toxicité. Le matériel végétal et les techniques utilisées dans ce travail sont présentés dans la 2$^{ème}$ chapitre de matériel et méthodes. Le 3$^{ème}$ chapitre de cette thèse présente les résultats sous la forme de trois publications (Sections 3A, 3B et 3C). La première publication (Section 3A) traite de l'influence de spéciation sur la phyto-toxicité induite par le plomb. L'article suivant (Section 3B) présent la relation mise en évidence entre la génotoxicité induite par le plomb et sa spéciation. La dernière publication (Section 3C) porte sur les effets de deux substances humiques sur la toxicité du plomb. Ce travail se termine par une discussion générale et conclusion qui ouvre sur des perspectives.

## General introduction

Pb (symbol Pb and atomic number 82) is a bluish-gray, ductile, dense metal, resistant to corrosion and a poor electrical conductor. Due to these and many other physico-chemical properties, this metal has a long history of use, dating to the earliest civilizations of Greeks, Romans and Egyptians (Cheng and Hu, 2010). Pb continues to be used widely in many industrial processes, which led to a significant enhanced concentration of this metal in all environmental compartments, soil, water, air, living organisms (Sammut et al. 2010; Grover et al. 2010). Figure-2 represents the movement of this metal in ecosystem (source-soil-plant) along with factors affecting its behaviour in soil, and detoxification mechanisms in plants.

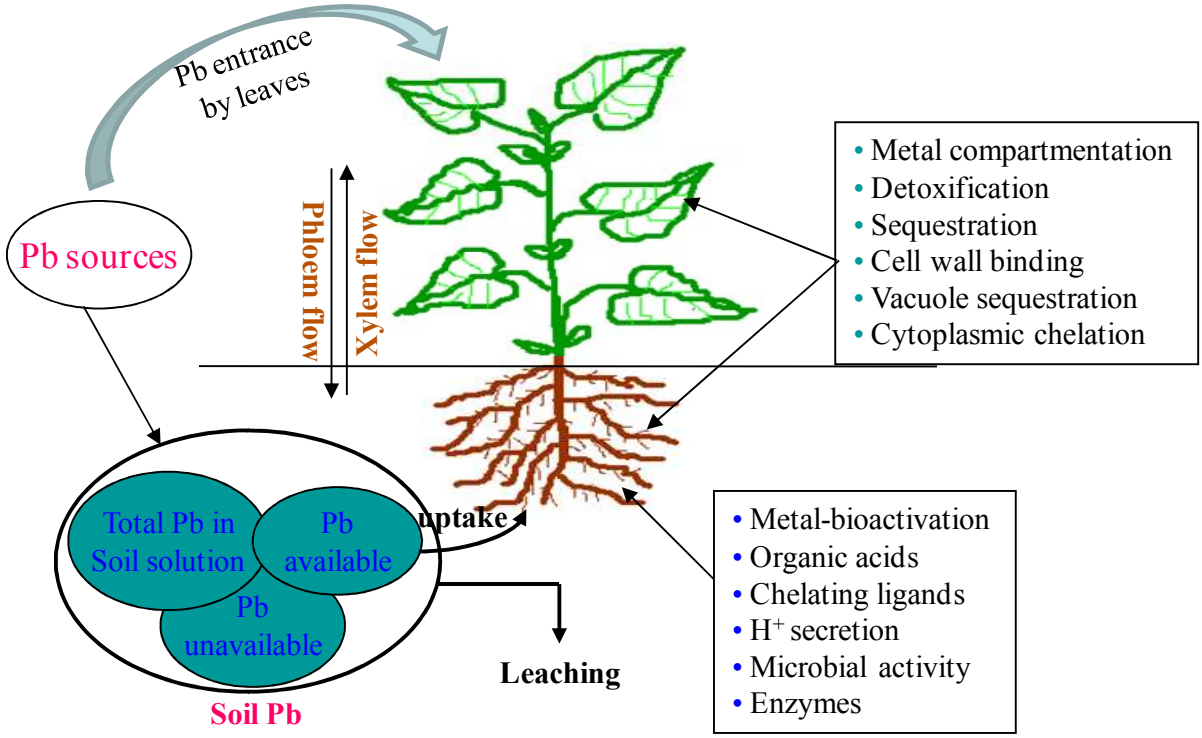


**Figure-2.** Pb biogeochemical behaviour in soil, uptake by plant roots and accumulation as well as different detoxification mechanisms adopted by plants against Pb toxicity.

The main sources of Pb pollution in environment include burning of fossil fuels, mining, Pb-based paints, plumbing, ammunition, x-ray shields, solder, containers for corrosive liquids, weapons manufacturing, and stained glass windows (Sharma and Dubey, 2005; Saifullah et al. 2009). Emissions of Pb to soil and water can also occur with the application of sewage sludge to land and the discharge of treated effluent (Singh and Agrawal, 2010). According to USGS (United State Geological Survey), the mine production

of recoverable Pb in 2009 was 1690, 516 and 400 thousand metric tons, respectively by China, Australia and USA (Figure-3).

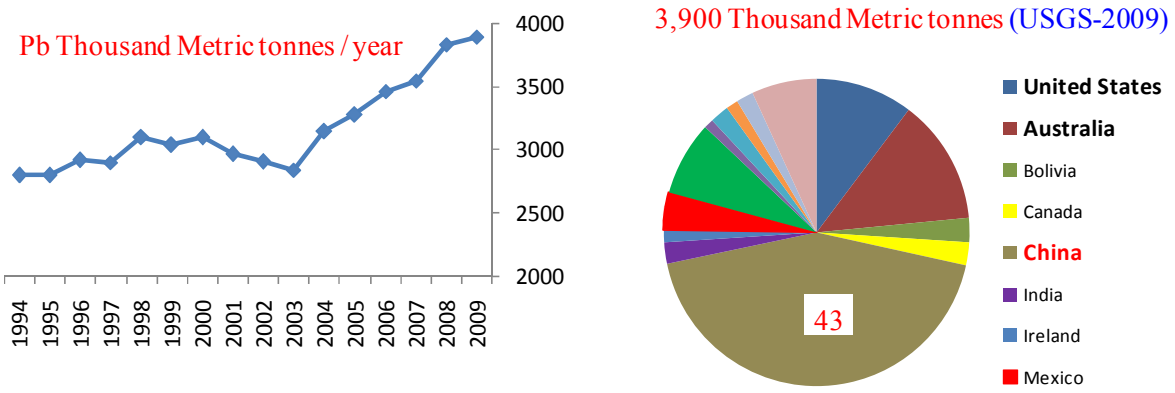


**Figure-3.** World-wide annual production of Pb. Pi chart shows the major Pb producing countries.

Pb is the 36$^{th}$ most abundant element and the most abundant heavy metal in the earth's crust (Arias et al. 2010; Grover et al. 2010) with an average concentration of 13 mg kg$^{-1}$ (Nriagu, 1978). In the earth's crust, Pb occurs in the form of Pb-sulfide in galena ore as the end-product of the radiometric decay of three naturally-occurring radioactive elements: uranium, thorium and actinium. The natural levels of soil Pb remained below 50 mg.kg$^{-1}$ (Pais and Jones, 2000). In soil, Pb is thought to be insoluble and unavailable for plant uptake because of its immobile nature (Cheng and Hu, 2010; Padmavathiamma and Li 2010). Therefore, it is necessary to determine the geochemical behaviour of Pb in the environment.

Once Pb is released into the environment, it becomes available for exposure to receptors. Although, Pb forms stable complexes and tend to store mainly in the soil but a mobile fraction can be absorbed by living organisms (Sammut et al. 2010; Vega et al. 2010) and go well in the food chain. Pb absorption by plants is affected by many soil factors such as composition/particle size, surface area of roots, substances released by roots including organic acids, mycorrhization, the rate of transpiration, and the quantity of Pb in a growth medium (Cecchi et al. 2008; Punamiya et al. 2010).

One of the main pathways for Pb to enter the plant is through the roots via calcium ion channels (Pourrut et al, 2008). Once in the roots, Pb is not translocated to the shoots of a plant, but tends to be sequestered in root cells. Approximately 95 % or more of absorbed Pb

in most plants is located in the plant roots unless the plant is a hyperaccumulator or chelate-assisted translocation processes are taking place (Gupta et al. 2010; Jiang and Liu 2010).

Although, plants have many detoxification systems to limit the interaction of ions with biological molecules (Figure-3), Pb induces a whole range of deleterious effects to plants. Excessive Pb accumulation in plant tissue is toxic to most plants, Pbing to a decrease in seed germination, root elongation, decreased biomass, and inhibition of chlorophyll biosynthesis (Sharma and Dubey, 2005; Brunet et al. 2009; Piotrowska et al. 2009; Sing et al. 2010). While inside a cell, Pb affects photosynthesis, respiration, mineral nutrition, and enzymatic reactions as well as a number of other physiological parameters (Sharma and Dubey, 2005). The presence of Pb in plants also induces the production of reactive oxygen species, which disrupt the redox status of cells, causing oxidative stress (Pourrut et al. 2008; Liu et al. 2008; Grover et al. 2010; Yadav 2010; Gill and Tuteja, 2010). This feature is known to be a major cause of heavy metals toxicity. However, the mechanisms behind this toxicity are not yet known.

Pb poisoning has been documented throughout the history of human civilizations. Children are particularly at risk to Pb poisoning. Pb toxicity has been shown to cause mental impairment in young children (Costa de Almeida et al. 2010; Liu et al. 2010; Wilhelm et al. 2010). Due to these and many other known toxical impact of Pb, this metal has now been characterized as chemical of great concern in the new European REACH regulation (EC 1907 / 2006, Registration, Evaluation, Authorization and Restriction of Chemicals) and was reported as the 2$^{nd}$ most hazardous substances based on the frequency of occurrence, toxicity, and the potential for human exposure by Agency for Toxic Substances and Disease Registry (ATSDR, 2003).

Although, several studies investigate the potential toxicity of Pb to living organisms, but majority relates to the total metal content. However, the potential effects of toxic elements in the environment are strongly influenced by their speciation in the broadest sense: physical and chemical distribution and chemical speciation. The role of the free ion or chelated with organic molecules soluble in particular has been highlighted in several studies (Dumat et al. 2006; Kopittke et al. 2008; Uzu et al. 2009; Sammut et al. 2010, Shahid et al. 2011). This makes total concentration, although relatively easier to measure, an unreliable indicator of toxicity. Predicting metal toxicity with respect to its applied form is, therefore, the pursuing of researchers.

In this project, we chose to identify the role of Pb speciation on its transfer and toxicity. *Vicia faba* plant was chosen as a model plant for evaluating metal toxicity due to its high sensitivity to Pb and common use in literature (El Hajjouji et al. 2007; Pourrut et al. 2008; Probst et al. 2009; Marcato-Romain et al. 2009a; Marcato-Romain et al. 2009b). The concentration of 5 µM $Pb(NO_3)_2$ was chosen because according to Pourrut et al. (2008), it induces genotoxicity to *V. faba* while remaining quite low and representative of environmental pollution. Three organic ligands (EDTA, HSs, and LMWOAs) were selected to chelat Pb in nutrient solution. These organic ligands vary greatly from one another with respect to molecular weight, molecular size, functional groups and metal complexing ability. The applied levels of all the three organic ligands were chosen using speciation models: Visual Minteq software version 2.60 (Gustafsson, 2008) and Windermere Humic Aqueous Model VI (Tipping et al. 1998). These models calculated the chelated and free metal ion concentration in nutrient solution, which were used to design experimental layout. All the treatments were applied for 1-24 h keeping in view the high sensitivity of *V. faba* to Pb and short-lived production of ROS before being scavenged rapidly by antioxidants.

In the present study, firstly, we analyse the transfer of Pb from nutrient solutions to different plant parts and, secondly, correlate the effects observed to Pb speciation rather than the total Pb concentration in plant tissues. The effects of Pb was examined in the presence of different chelates using different stress markers; photosynthetic pigments contents, lipid peroxidation, reactive oxygen species production and antioxidant enzyme activities, which are indirect markers of oxidative stress. Finally, relationship was developed between Pb-induced genotoxicity and Pb speciation.

The first chapter of this thesis presents a bibliographic review divided into two sections: (1A) mechanisms of Pb uptake, toxicity, and detoxification in plants and (1B) effects of Pb speciation on its uptake, toxicity in the presence of organic ligands. A presentation of the plant material and techniques used to meet our objectives is performed in a second chapter. The third chapter of this thesis presents our results in the form of three publications (Sections 3A, 3B and 3C). The first publication (Section 3A) addresses the influence of speciation on Pb induced phyto-toxicity comparing different ecotoxicological stress markers of differing sensitivities. The next article (Section 3B) traces the relationship between Pb-induced genotoxicity and speciation. The last article (Section 3C) focuses on the effects of humic substances on Pb-induced oxidative stress, which highlighted the importance of metal speciation in natural condition.

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# Chapter 1:

# Literature Review

Forwords

Before starting the experimental research work, a literature review was carried out to know the seminal works done in this field and to identify gaps in the literature. This review provided the intellectual context for present work and helped to put this work into perspective.

As described earlier, Pb is a toxic pollutant of environmental concern and its toxicity depends on its speciation. The literature of review is divided into two sections. First concerning the biogeochemical behaviour and toxicity of Pb, whereas second revealing the importance of Pb speciation towards its behaviour in the environment. These two sections were written in review article form. In the thesis, the figures are added to these review articles to explain in detail the literature review.

- **Section 1A:** Pb uptake, toxicity and detoxification in plants

- **Section 1B:** Review of Pb availability and toxicity to plants in relation with metal speciation; role of synthetic and natural organic ligands

# Section 1A:

## Pb uptake, toxicity and detoxification in plants

*-Publication-*

M. Shahid, C. Dumat, B. Pourrut, P. Winterton and E. Pinelli. 2011. Pb uptake, toxicity and detoxification in plants. Reviews of Environmental Contamination and Toxicology Vol 213.

# Pb Uptake, Toxicity and Detoxification in Plants

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## 1 Introduction

Plants are the target of a wide range of pollutants that vary in concentration, speciation and toxicity. Such pollutants mainly enter the plant system through the soil (Arshad et al. 2008), or via the atmosphere (Uzu et al. 2010). Among common pollutants that affect plants, Pb is among the most toxic and frequently encountered (Cecchi et al. 2008; Grover et al. 2010; Shahid et al. 2011). Pb continues to be used widely in many industrial processes, and occurs as a contaminant in all environmental compartments (soils, water, the atmosphere and living organisms). The prominence of environmental Pb contamination results both from its persistence (Islam et al. 2008; Andra et al. 2009; Punamiya et al. 2010), and from its present and past numerous sources. These sources have included smelting, combustion of Pbed gasoline or applications of Pb-contaminated media (sewage sludge and fertilizers, Figure-1) to land (Piotrowska et al. 2009; Gupta et al. 2009; Sammut et al. 2010; Grover et al. 2010). In 2009, production of recoverable Pb from mining operations was 1690, 516 and 400 thousand metric tons by China, Australia and the USA, respectively (USGS).

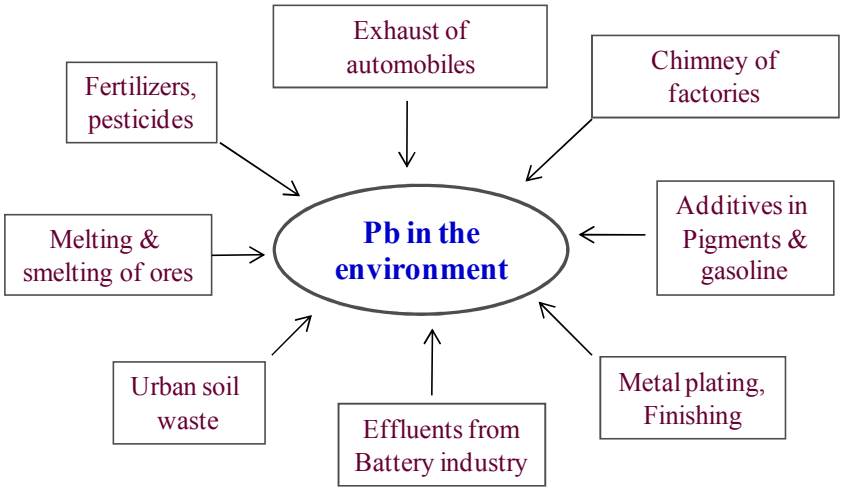


**Figure-1.** Anthropogenic source of Pb in the environment (Sharma and Dubey, 2005).

Despite a long history of its beneficial use by humankind, Pb has no known biological function in living organisms (Maestri et al. 2010), and is now recognized as a chemical of great concern in the new European REACH regulations (EC 1907/2006; Registration, Evaluation, Authorization and Restriction of Chemicals). Moreover, Pb was reported as being the second most hazardous substance, after arsenic, based on the frequency of occurrence, toxicity, and the potential for human exposure by the Agency for Toxic Substances and Disease Registry (ATSDR 2003). The transfer of Pb from polluted soils to plants was

therefore widely studied, especially in the context of food quality, use in phytoremediation, or in biotesting (Arshad et al. 2008; Uzu et al. 2009).

Pb is known to induce a broad range of toxic effects to living organism, including those that are morphological, physiological and biochemical in origin. This metal impairs plant growth, root elongation, seed germination, seedling development, transpiration, chlorophyll production, lamellar organization in the chloroplast, and cell division (Sharma and Dubey 2005; Krzesłowska et al. 2009; Gupta et al. 2009, 2010; Maestri et al. 2010). However, the extent of these effects varies, and depends on the Pb concentration tested, the duration of exposure, the intensity of plant stress, the stage of plant development, and the particular organs studied. Plants have developed various methods for responding to toxic metal exposures. They have internal detoxification mechanisms to deal with metal toxicity, that includes selective metal uptake, excretion, complexation by specific ligands and compartmentalization (Gupta et al. 2009; Krzesłowska et al. 2010; Maestri et al. 2010; Sing et al. 2010; Jiang and Liu 2010).

The various responses of plants to Pb exposure are often used as tools (bioindicators) in the context of environmental quality assessment. To develop tools that are relevant for ecotoxicological studies, it is essential to understand the mechanisms involved in plant uptake, transfer and toxicity. This is especially true in selected research areas, such as choice of plant species, when polluted soils are under study (i.e., reduced transfer when studying vegetables, or increased transfer when phytoextraction is desired). For example, legumes are considered more suitable to grow on contaminated soil than are Umbelliferae, Liliaceae, Compositae and Chenopodiaceae because they take up reduced amounts of Pb (Alexander et al. 2006). The reduced Pb uptake by vegetables minimizes the threat of Pbs introduction to the food chain. In contrast, phytoextraction requires plants that can sequester excessive amounts of Pb in their biomass without incurring damage to basic metabolic functions (Arshad et al. 2008; Zaier et al. 2010). Pelargonium (Arshad et al. 2008) and *Brassica napus* (Zaier et al. 2010) are characterized as Pb hyperaccumulators, and they can extract huge amounts of Pb from contaminated soil without showing morpho-phytotoxicity symptoms. Indeed, these plants have efficient natural detoxification mechanism to alleviate Pb toxicity. In the present review, we propose to trace the relationship that exists between Pb uptake, accumulation, translocation and toxicity, in plants.

## 2 Retention, Mobility and Bio-availability of Pb in Soil

Pb occurs naturally in the earth's crust (Arias et al. 2010) and its natural levels remain below 50 $mg.kg^{-1}$ (Pais and Jones 2000). But, anthropogenic activities often modify the amount and nature of Pb species present in soil. In soils, Pb may occur as a free metal ion, complexed with inorganic constituents (e.g., $HCO_3^-$, $CO_3^{2-}$, $SO_4^{2-}$, $Cl^-$), or may exist as organic ligands (e.g., amino acids, fulvic acids, humic acids); alternatively Pb may be adsorbed onto particle surfaces (e.g., Fe-oxides, biological material, organic matter, clay particles) (Uzu et al. 2009; Tabelin and Igarashi 2009; Sammut et al. 2010; Vega et al. 2010). Anthropogenic-sourced Pb generally accumulates primarily in the surface layer of soil, and its concentration decreases with depth (Cecchi et al. 2008). Because of its strong binding with organic and/or colloidal materials, it is believed that only small amounts of the Pb in soil is soluble, and thereby available for plant uptake (Kopittke et al. 2008; Punamiya et al. 2010).

However, Pb behavior in soil, in the context of species, solubility, mobility and bioavailability, is largely controlled by complex interactions governed by many biogeochemical factors (Punamiya et al. 2010). These factors include pH (Kopittke et al. 2008; Lawal et al. 2010; Vega et al. 2010), redox conditions (Tabelin and Igarashi 2009), cation exchange capacity (Vega et al. 2010), soil mineralogy (Dumat et al. 2006), biological and microbial conditions (Arias et al. 2010), amount of Pb present (Bi et al. 2010; Cenkci et al. 2010; Lawal et al. 2010), organic and inorganic ligand levels (Padmavathiamma and Li 2010; Sammut et al. 2010; Shahid et al. 2011), competing cation levels (Kopittke et al. 2008; Komjarova and Blust 2009), and plant species involved (Kovalchuk et al. 2005; Bi et al. 2010; Liu et al. 2010). Such factors may act individually or in combination with each other, and may alter the soil behavior of the Pb present, as well as the rate of uptake by plants.

Pb bioavailability is strongly influenced by its speciation and, in particular, by the concentration of free Pb ions present (Dumat et al. 2006; Uzu et al. 2009; Sammut et al. 2010; Shahid et al. 2011). This is because the most significant plant uptake route for many cationic metals (and especially for the free metal ion) is via the soil solution in dissolved form (Punamiya et al. 2010). Moreover, the free Pb ion concentration in soils depends on the adsorption/desorption processes in which it participates (Vega et al. 2010). Figure-2 shows different interactive processes that influence the retention of Pb in soil.

## 3 Pb Behavior in Plants

### *3.1 Pb Uptake by Plants*

With the exception of the special conditions that exists for plants cultivated near metal recycling industries (Uzu et al. 2010), the main pathway by which plants accumulate metals is through root uptake from soils (Sharma and Dubey 2005; Uzu et al. 2009). One part of Pb present in the soil solution is adsorbed onto the roots, and then becomes bound to carboxyl groups of mucilage uronic acid, or directly to the polysaccharides of the rhizoderm cell surface (Seregin and Ivanov 2001). Pb adsorption onto roots has been documented to occur in several plant species: *Vigna unguiculata* (Kopittke et al. 2007), *Festuca rubra* (Ginn et al. 2008), *Brassica juncea* (Meyers et al. 2008), *Lactuca sativa* (Uzu et al. 2009) and *Funaria hygrometrica* (Krzesłowska et al. 2009, 2010). Once adsorbed onto the rhizoderme roots surface, Pb may enter the roots passively and follow translocating water streams. However, Pb absorption is not uniform along plant roots as a Pb concentration gradient from root apex can be observed (Tung and Temple 1996; Seregin et al. 2004). Indeed, the highest Pb concentrations can be found in root apices, where root cells are young and have thin cell walls (with the exception of root cap cells) that facilitate root uptake (Tung and Temple 1996; Seregin et al. 2004). Moreover, the apical area is the area where rhizodermic pH is the lowest, which increases solubility of Pb in the soil solution.

At the molecular level, the mechanism by which Pb enters roots is still unknown. Pb may enter the roots through several pathways, and a particular pathway is through ionic channels. Although, Pb uptake is a non selective phenomenon, it nonetheless depends on the functioning of an $H^+$/ATPase pump to maintain a strong negative membrane potential in rhizoderm cells (Hirsch et al. 1998; Wang et al. 2007). Inhibition of Pb absorption by calcium is well-known (Garland and Wilkins, 1981; Kim et al. 2002), and is associated with competition between these two cations for calcium channels (Huang and Cunningam 1996). Several authors have demonstrated that $Ca^{2+}$-permeable channels are the main pathway by which Pb enters roots (Wang et al. 2007; Pourrut et al. 2008). The use of transgenic plants have shown that Pb can penetrate into roots through alternative non-selective pathways, such as cyclic nucleotide-gated ion channels (Arazi et al. 1999, Kohler et al. 1999), or via low affinity cation transporters (Wojas et al. 2007). Figure-2 shows the processes, which control the uptake of Pb from soil and its translocation to shoot.

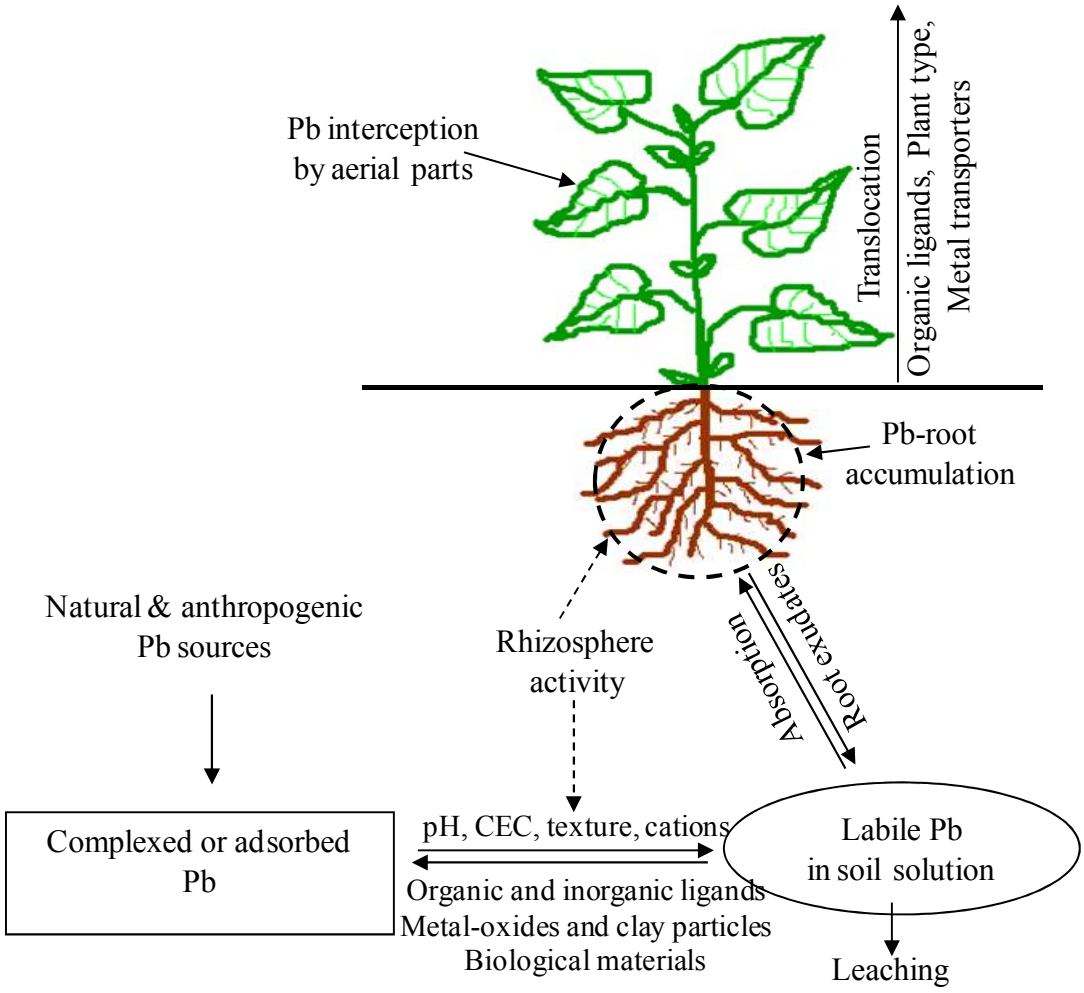


**Figure-2.** Factors affecting Pb solubilisation in soil, uptake by roots and translocation to aerial parts.

The reduced uptake and translocation of Pb to aerial plant parts of vegetables is considered to be beneficial in preventing Pb from entering the food chain. However, reduced uptake and translocation of Pb to aerial plant parts, when plants are used to remediate polluted soils, is a major problem. Indeed, soil remediation requires plants (hyper-accumulators) that can take high Pb levels up and translocate it to aerial plant parts with no, or minimal toxicity. The amount of Pb that moves from soil to penetrate into a plants can be measured by what is called "the transfer factor"; this factor is defined as the ratio that exists between the concentration of Pb in the plant vs. the concentration of Pb in the soil (Arshad et al. 2008; Bi et al. 2010; Liu et al. 2010). This transfer factor will be different for different plant species, and will change as soil physical and chemical properties are altered (Arshad et al. 2008; Bi et al. 2010; Liu et al. 2010). Generally, plants having a transfer factor greater

than one are categorized as hyper-accumulators, whereas those with transfer factor less than one are termed as non-accumulators of Pb (Arshad et al. 2008).

### *3.2 Pb Accumulation in Plants*

Once Pb has penetrated into the root system, it may accumulate there, or may be translocated to aerial plant parts. For most plant species, the majority of absorbed Pb (approximately 95% or more) is accumulated in the roots, and only a small fraction is translocated to aerial plant parts, as has been reported in *Vicia faba*, *Pisum sativum* and *Phaseolus vulgaris* (Piechalak et al. 2002; Małecka et al. 2008; Shahid et al. 2011), *Vigna unguiculata* (Kopittke et al. 2007), *Nicotiana tabacum,* (Gichner et al. 2008), *Lathyrus sativus* (Brunet et al. 2009), *Zea mays* (Gupta et al. 2009), *Avicennia marina* (Yan et al. 2010), non-accumulating *Sedum alfredii* (Gupta et al. 2010) and *Allium sativum* (Jiang and Liu 2010). Although many metals display the translocation restriction phenomenon mentioned above, this phenomenon is not common to all heavy metals. Notwithstanding, this phenomenon in plants is both specific and very intense for Pb.

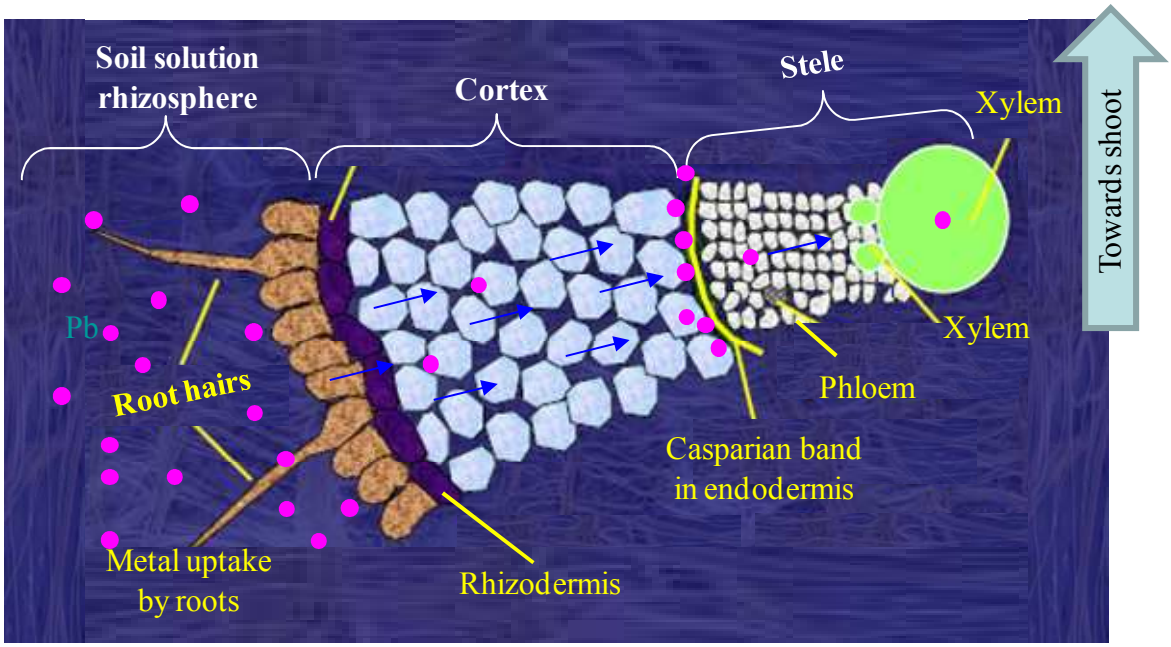


**Figure-3.** Schematic representation of root cross section. Factors affecting the radial diffusion and blockage of Pb in the roots.

When entering the root, Pb mainly moves by apoplast, and follows water streams until it reaches the endodermis (Tanton and Crowdy 1971; Lane and Martin 1977). There are several reasons why the transport of Pb from roots to aerial plant parts is limited. These

reasons include: immobilization by negatively charged pectins within the cell wall (Islam et al. 2007; Kopittke et al. 2007; Arias et al. 2010), precipitation of insoluble Pb-salts in intercellular spaces (Kopittke et al. 2007; Islam et al. 2007; Meyers et al. 2008; Małecka et al. 2008), accumulation in plasma membranes (Seregin et al. 2004; Islam et al. 2007; Jiang and Liu 2010), or sequestration in the vacuoles of rhizodermal and cortical cells (Seregin et al. 2004; Kopittke et al. 2007). Figure-3 shows the absorption of Pb by root hairs, radial diffusion in roots and its blockage by Casparian strip.

However, these reasons are not sufficient to explain the low rate of Pb translocation from root to shoot. The endoderm, which acts as a physical barrier, plays an important role in this phenomenon. Indeed, following apoplastic transport, Pb is blocked in the endodermis by Casparian strips and must follow symplastic transport. In endodermis cells, the major part of Pb is sequestered or excreted by plant detoxification systems (cf. 5.2).

Several hyperaccumulator plant species, such as *Brassica pekinensis* and *pelargonium,* are capable of translocating higher concentrations of Pb to aerial plant parts, without incurring damage to their basic metabolic functions (Xiong et al. 2006; Liu et al. 2008; Arshad et al. 2008). A specific hyperaccumulator species can accumulate more than 1000 ppm Pb (Maestri et al. 2010). Indeed, these plants exude substances from roots that dissolve metals in soil (Arshad et al. 2008) that increases uptake and translocation (by employing certain metal cation transporters/genes). Moreover, they can tolerate higher concentrations of Pb ions because they have various detoxification mechanisms, which may include selective metal uptake, excretion, complexation by specific ligands, and compartmentalization.

In addition, translocation of Pb to aerial plant parts increases in the presence of organic chelates like ethylenediaminetetraacetate (EDTA) (Liu et al. 2008; Zaier et al. 2010; Barrutia et al. 2010), or certain species of micro-organisms (Arias et al. 2010; Punamiya et al. 2010). Recently, Liu et al. (2010) reported that, in 30 *Brassica pekinensis* cultivars, increased soil Pb levels also increased the percent translocation to aerial plant parts. High concentrations of Pb are known to destroy the physical barrier of Casparian strip.

Transportation of metals from plant roots to shoots requires movement through the xylem (Verbruggen et al. 2009), and, when it occurs, is probably driven by transpiration (Liao et al. 2006). Arias et al. (2010) demonstrated high Pb deposition in xylem and phloem cells of mesquite plants by using X-ray mapping. After penetrating into the central cylinder

of the stem, Pb can again be transported via the apoplastic pathway. The Pb is then translocated to leaf areas via vascular flow (Sharma and Dubey 2005; Krzesłowska et al. 2010). While passing through the xylem, Pb can form complexes with amino or organic acids (Roelfsema and Hedrich 2005; Vadas and Ahner 2009; Maestri et al. 2010). However, Pb may also be transferred, in inorganic form, as is cadmium. To express degree of Pb translocation, some authors have used a translocation factor (Pb in aerial parts / Pb in roots) (Arshad et al. 2008; Uzu et al. 2009; Liu et al. 2010). When this factor is used, the numeric value is normally rather low, which indicates that Pb has been sequestered in the roots (Uzu et al. 2009; Liu et al. 2010). Figure-4 shows the apoplastic and symplastic movement of Pb inside the plant.

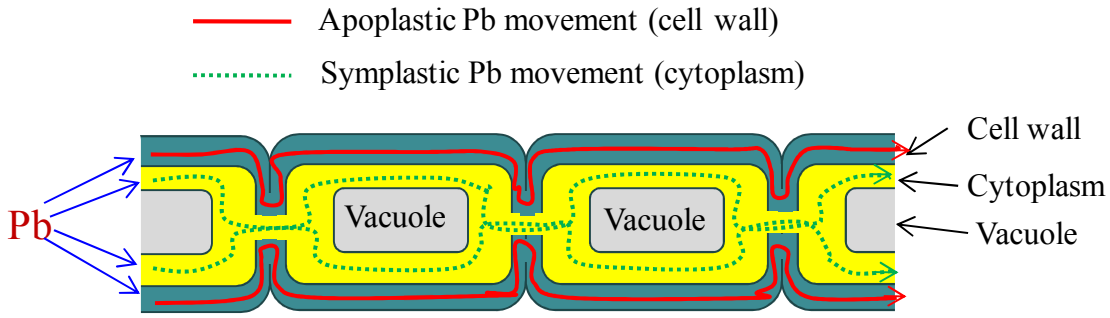


**Figure-4.** Apoplastic and symplastic movement of Pb in plant.

## 4 General Effects of Pb on Plants

Pb is a ubiquitous environmental pollutant that can directly or indirectly induce various morphological, physiological and biochemical dysfunctions. Figure-5 lists major negative effects (direct or indirect) of this metal on plant physiological processes.

### *4.1 Effects on Germination and Growth*

When plants are exposed to Pb, even at micromolar levels, adverse effects on germination and growth can occur (Kopittke et al. 2007). Germination is strongly inhibited by very low concentrations of $Pb^{2+}$ (Tomulescu et al. 2004; Islam et al. 2007). Pb-induced inhibition of seed germination has been reported in *Hordeum vulgare*, *Elsholtzia argyi*, *Spartiana alterniflora*, *Pinus halepensis*, *Oryza sativa*, and *Zea mays* (Tomulescu et al. 2004; Islam et al. 2007; Sengar et al. 2009). At higher concentrations, Pb may speed up germination, and simultaneously induce adverse affects on the length of radical and

hypocotyl in *Elsholtzia argyi* (Islam et al. 2007). Inhibition of germination may result from the interference of Pb with protease and amylase enzymes (Sengar et al. 2009).

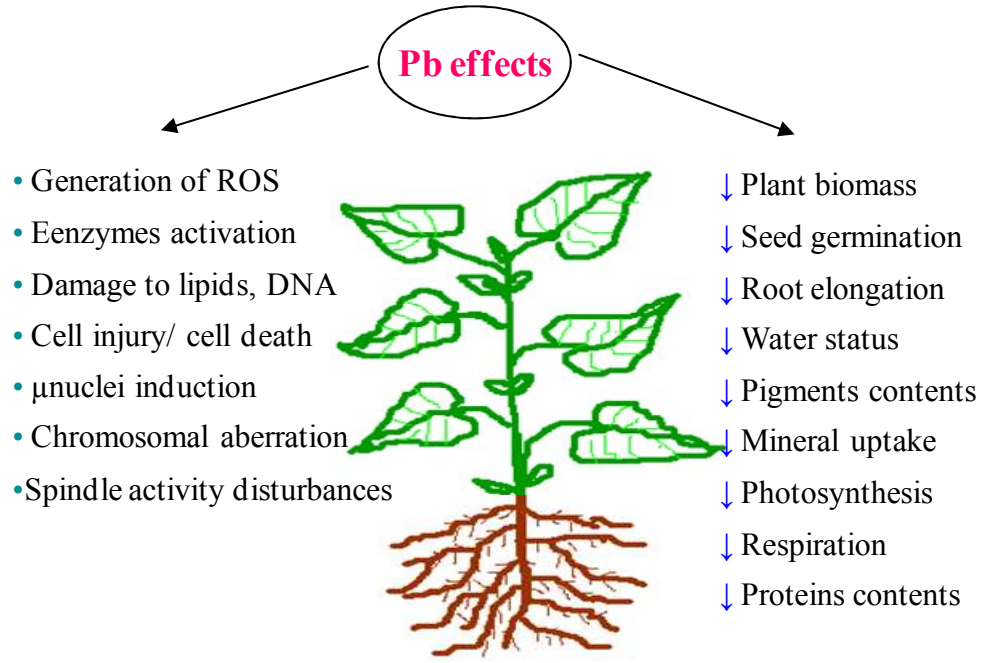


**Figure-5.** Pb-induced toxic effects to plants.

Pb exposure in plants also strongly limits the development and sprouting of seedlings (Dey et al. 2007; Gichner et al. 2008; Gopal and Rizvi 2008). At low concentrations, Pb inhibits the growth of roots and aerial plant parts (Islam et al. 2007; Kopittke et al. 2007). This inhibition is stronger for the root, which may be correlated to its higher Pb content (Liu et al. 2008). Pb toxicity may also cause swollen, bent, short and stubby roots that show an increased number of secondary roots per unit root length (Kopittke et al. 2007). Recently, Jiang and Liu (2010) reported mitochondrial swelling, loss of cristae, vacuolization of *endoplasmic reticulum* and dictyosomes, injured plasma membrane and deep colored nuclei, after 48-72h of Pb exposure to *Allium sativum* roots. Arias et al. (2010) reported significantly inhibited root elongation in Mesquite (*Prosopis* sp.).

Plant biomass can also be restricted by high doses of Pb exposure (Gopal and Rizvi 2008; Gichner et al. 2008; Islam et al. 2008; Piotrowska et al. 2009; Sing et al. 2010). Under severe Pb toxicity stress, plants displayed obvious symptoms of growth inhibition, with fewer, smaller, and more brittle leaves having dark purplish abaxial surfaces (Islam et al. 2007; Gupta et al. 2009). Plant growth retardation from Pb exposure may be attributed to nutrient metabolic disturbances (Kopittke et al. 2007; Gopal and Rizvi 2008), and disturbed

photosynthesis (Islam et al. 2008). In most cases, the toxic effect of Pb on plant growth is time- and dose-dependent (Dey et al. 2007; Gupta et al. 2009, 2010). However, the effect of low concentrations is not clearly established, and the observed growth inhibition is not necessarily correlated to a reduction in biomass (Kosobrukhov et al. 2004; Yan et al. 2010). Moreover, the effect of Pb toxicity varies with plant species, i.e., hyperaccumulators naturally tolerate more Pb toxicity than do sensitive plants (Arshad et al. 2008).

### *4.2 Effects on Proteins*

Similar to what occurs with other heavy metals, Pb interacts with cytoplasmic proteins. The effect of Pb on the total concentration of protein is unclear, although high concentrations may decrease the protein pool (Chatterjee et al. 2004; Mishra et al. 2006; Garcia et al. 2006; Piotrowska et al. 2009). This quantitative decrease in total protein content is the result of several Pb effects: acute oxidative stress of reactive oxygen species (ROS) (Piotrowska et al. 2009; Gupta et al. 2009), modification in gene expression (Kovalchuk et al. 2005), increased ribonuclease activity (Gopal and Rizvi 2008), protein utilization by plants for the purposes of Pb detoxification (Gupta et al. 2009), and diminution of free amino acid content (Gupta et al. 2009) that is correlated with a disturbance in nitrogen metabolism (Chatterjee et al. 2004). However, certain amino acids, like proline, increase under Pb stress (Qureshi et al. 2007). Such proteins play a major role in the tolerance of the plant to Pb. In contrast, low concentration of Pb increase total protein content (Mishra et al. 2006). This protein accumulation may defend the plant against Pb stress (Gupta et al. 2010), particularly for proteins involved in cell redox maintenance. If true, then such proteins act in a way similar to how ascorbate functions, or similar to how metals are sequestered by glutathione (GSH) or phytochelatins (PCs) (Brunet et al. 2009; Liu et al. 2009; Yadav 2010; Jiang and Liu 2010). In addition to a quantitative change, Pb can affect the qualitative composition of cell proteins. The protein profile of root cells in bean seedlings was modified after Pb exposure Pb (Beltagi 2005). Such modification can be correlated to the change that occurs in the transcriptome profile of several enzymes including: isocitrate lyase, cystein proteinase *SAG12*, serine hydroxymethyltransferase and arginine decarboxylase (Kovalchuk et al. 2005).

### *4.3 Water Status Effects*

The disruption of plant water status after Pb treatment has been addressed in many studies (Brunet et al. 2009). Results of such exposures show a decrease in transpiration, as

well as reduction of the moisture content (Barcelo and Poschenrieder 1990; Patra et al. 2004). Reduced transpiration may result from reduced leaf surface area for transpiration that is caused by decreased leaf growth (Elibieta and Miroslawa 2005). However, some plant species that have high stomatal density are capable of coping with such effects (Kosobrukhov et al. 2004; Elibieta and Miroslawa 2005). Pb reduces plant cell wall plasticity, and thereby influences the cell turgor pressure. The decrease in concentrations of molecules that control cell turgor, such as sugars and amino acids, further accentuate the phenomenon of Pb influence on turgor pressure (Barcelo and Poschenrieder 1990). The change in turgor pressure, particularly in the guard cells, interferes with stomatal opening and closing. To maintain cell turgor pressure, plants synthesize high concentrations of osmolytes, particularly proline under Pb stress conditions (Qureshi et al. 2007).

Stomatal opening/closing is controlled by abscisic acid (ABA), a phyto-hormone (Roelfsema and Hedrich 2005). The presence of $Pb^{2+}$ ions causes a large accumulation of ABA in roots and aerial plant parts (Parys et al. 1998; Atici et al. 2005; Cenkci et al. 2010), Pbing to stomatal closure (Mohan and Hosetti 1997). Stomatal closure strongly limits gas exchange with the atmosphere, and water losses by transpiration (Parys et al. 1998). According to Elibieta and Miroslawa (2005), the foliar respiration of plants is also reduced by Pb exposure, because the deposition of a cuticle layer, for example on *Glycine max* leaf surfaces, is affected. Moreover, a $CO_2/O_2$ imbalance in plants from Pb-induced oxidative phosphorylation and respiratory disorders may also disrupt plant water status.

### *4.4 Mineral Nutrition Effects*

Results from multiple studies demonstrate that nutrient uptake by plants is significantly affected by the presence of Pb (Chatterjee et al. 2004; Sharma and Dubey 2005; Gopal and Rizvi 2008). Although data are insufficient to allow a definitive conclusion to be drawn, it is known that Pb affects plant mineral uptake. However, it is known that Pb exposure decreases the concentration of divalent cations ($Zn^{2+}$, $Mn^{2+}$, $Mg^{2+}$, $Ca^{2+}$ and $Fe^{2+}$) in leaves of *Zea mays* (Seregin et al. 2004), *Oryza sativa* (Chatterjee et al. 2004), *Brassica oleracea* (Sinha et al. 2006), *Medicago sativa* (Lopez et al. 2007) *Vigna unguiculata* (Kopittke et al. 2007) and *Raphanus sativus* (Gopal and Rizvi 2008). But, it is not possible to conclude if this decrease results from blockage of root absorption, a decrease in translocation from roots to aerial plant parts, or a change in distribution of these elements in the plant. The effect of Pb on mineral accumulation in aerial plant parts, in most cases, follows a common

trend. In roots, the trend varies according to plant species, or the intensity of the imposed stress (Lopez et al. 2007; Kopittke et al. 2007; Gopal and Rizvi 2008).

The decreased absorption of nutrient in the presence of Pb may result from competition (e.g., those with atomic size similar to Pb), or changes in physiological plant activities. According to Sharma and Dubey (2005), the strong interaction of $K^+$ ions with Pb could result from their similar radii ($Pb^{2+}$: 1.29Å and $K^+$: 1.33Å): these two ions may compete for entry into the plant through the same potassium channels. Similarly, Pb effects on $K^+$-ATPase and -SH groups of cell membrane proteins cause an efflux of $K^+$ from roots. However, Pb does not cause nitrogen efflux. The general reduction in the concentration of inorganic nitrogen in all plant parts could be induced by the reduced activity of nitrate reductase, the rate-limiting enzyme in the nitrate assimilation process (Xiong et al. 2006; Sengar et al. 2009). Xiong et al. (2006) reported that Pb exposure (4 and 8 $mmol.kg^{-1}$) significantly decreased shoot nitrate content (70% and 80%), nitrate reductase activity (100% and 50%), and free amino acid content (81% and 82%) in *Brassica pekinensis*.

### *4.5 Photosynthesis Effects*

Photosynthesis inhibition is a well-known symptom of Pb toxicity (Xiong et al. 2006; Hu et al. 2007; Liu et al. 2008; Piotrowska et al. 2009; Sing et al. 2010; Cenkci et al. 2010). This inhibition is believed to result from the following indirect effects of Pb rather than from a direct effect:

- distorted chloroplast ultrastructure from the affinity Pb has for or protein N- and S- ligands (Elibieta and Miroslawa 2005; Islam et al. 2007),
- decreased ferredoxin $NADP^+$ reductase and delta-aminolevulinic acid dehydratase (ALAD) activity at the origin of chlorophyll synthesis inhibition (Gupta et al. 2009, Cenkci et al. 2010),
- inhibition of plastoquinone and carotenoids synthesis (Kosobrukhov et al. 2004; Chen et al. 2007; Liu et al. 2008; Cenkci et al. 2010),
- obstruction of the electron transport system (Qufei et al. 2009),
- inadequate concentration of carbon dioxide via stomatal closure (Romanowska et al. 2002, 2005, 2006),

- impaired uptake of essential elements such as Mn and Fe (Chatterjee et al. 2004; Gopal and Rizvi 2008) and substitution of bivalent cations by Pb (Gupta et al. 2009; Cenkci et al. 2010),
- inhibition of Calvin cycle enzymatic catalysis (Mishra et al. 2006; Liu et al. 2008), and,
- increased chlorophyllase activity (Liu et al. 2008).

However, these different effects vary by plant species. Generally, chlorophyll b is more sensitive than is chlorophyll a (Xiong et al. 2006). The mechanism of chlorophyll breakdown into phytol magnesium, and the primary cleavage product of the porphyrin ring occur in four consecutive steps. This reaction is catalyzed by chlorophyllase, Mg-dechelatase, pheophorbide a oxygenase, and red chlorophyll catabolite reductase. Loss of the typical chlorophyll green color occurs only after cleavage of the porphyrin ring (Harpaz-Saad et al. 2007). The decrease observed in photosynthetic activity is often a more sensitive measure than is pigment content.

### *4.6 Respiration Effects*

When exposed to Pb, photosynthetic plants usually experience harmful effects on respiration and adenosine triphosphate (ATP) content. Unlike the photosynthetic activity, the effect of Pb on respiratory activity has been little studied (Seregin and Ivanov 2001). All the studies carried out on respiratory activity deal with leaves, whereas, the effect of the $Pb^{2+}$ ions on the respiratory activity of roots remains unknown. Pb is reported to affect the activity of ribulose-bisphosphate carboxylase in $C_3$ plants that control $CO_2$ assimilation, without affecting oxygenase activity (Assche and Clijsters 1990). Therefore, it is quite possible that photosynthesis is significantly reduced without any effect on photorespiration being induced, thus increasing the relative rate of photorespiration to photosynthesis. Parys et al. (1998) reported that the $CO_2$ concentration of *Pisum sativum* in leaves increased significantly after exposure to Pb nitrate, most probably from the reduced photosynthetic, and increased respiration activity. Romanowska et al. (2002) stressed that $Pb^{2+}$-induced increases in respiration resulted only from dark (mitochondrial) respiration, while photorespiration was unaffected. The stimulation of dark respiration by Pb was observed in leaves or protoplasts of *Pisum sativum* and *Hordeum vulgare* (Romanowska et al. 2002, 2005, 2006). Moreover, the

stimulation of respiration was well correlated with increased production of ATP in mitochondria, resulting in the high energy demands of the plant to combat Pb effects, being met.

It has been also shown that divalent cations (like Pb, Zn, Cd, Co and Ni) can bind to mitochondrial membranes, disrupting the electron transport that could Pb to decoupling of phosphorylation (Romanowska et al. 2002, 2006). An increase in the respiratory rate of 20-50% was observed by Romanowska et al. (2002) in the detached leaves of $C_3$ plants (*Pisum sativum*, *Hordeum vulgare*) and $C_4$ plants (*Zea mays*), when they were exposed to 5 mM $Pb(NO_3)_2$ for 24hours. Glycine, succinate and malate substrates were more fully oxidized in mitochondria, isolated from Pb-treated *Pisum sativum* leaves, than in mitochondria from control leaves (Romanowska et al. 2002). Pb caused an increase in ATP content as well as an increase in the ATP/ADP ratio, in *Pisum sativum* and *Hordeum vulgare* leaves (Romanowska et al. 2005, 2006). Rapid fractionation of *Hordeum vulgare* protoplasts, incubated under conditions of low and high $CO_2$, indicated that the increased ATP/ADP ratio in Pb-treated leaves mainly resulted from the production of mitochondrial ATP. The activity of $NAD^+$-malate dehydrogenase in protoplasts of barley leaves treated with Pb was 3-fold higher than that in protoplasts from control leaves (Romanowska et al. 2005). Pb also significantly inhibited Hill reaction activity in spinach chloroplasts, in addition to photophosphorylation; moreover, Pb had a more conspicuous effect on cyclic photophosphorylation than on noncyclic photophosphorylation (Romanowska et al. 2008). Recently, Qufei and Fashui (2009) reported that the accumulation of $Pb^{2+}$ in photosystem II resulted in damage to its secondary structure, and induced decreased absorbance of visible light and inhibited energy transfer among amino acids. Moreover, Jiang and Liu (2010) reported mitochondrial swelling, loss of cristae and vacuolization of endoplasmic reticulum and dictyosomes during a 48-72 hour Pb exposure in *Allium sativum*.

### *4.7 Genotoxicity*

The antimitotic effect of Pb is one of its best known toxic effects on plants (Patra et al. 2004; Shahid et al. 2011). Indeed, Hammett (1928) demonstrated long ago that Pb induces a dose-dependent decrease in mitotic activity in root cells of *Allium cepa,* which was later described in detail by Wierzbicka (1999) and Patra et al. (2004). In *Vicia faba* roots, Pb shortened the mitotic stage and prolonged interphase, thus lengthening the cell cycle (Patra et al. 2004). The first step by which Pb induces plant toxicity is the binding of the $Pb^{2+}$ ion to

cell membranes and to the cell wall. This produces rigidity in these components and reduces cell division. The second step is the disruption of microtubules that are essential for mitosis. Pb exposure induces disturbances in the $G_2$ and M stages of cell division that Pbs to the production of abnormal cells at the c-mitosis (colchicine-mitosis) stage. This phenomenon is thought to be accentuated by direct or indirect interactions of Pb with the proteins involved in the cell cycle, such as cyclins. Cyclin activity is indirectly dependent on the concentration of GSH. The spindle activity disturbances caused by Pb may be transient in some cases, returning the mitotic index to initial levels. Figure-6 represents genotoxic potential of Pb in plant cells during cell division (mitosis)

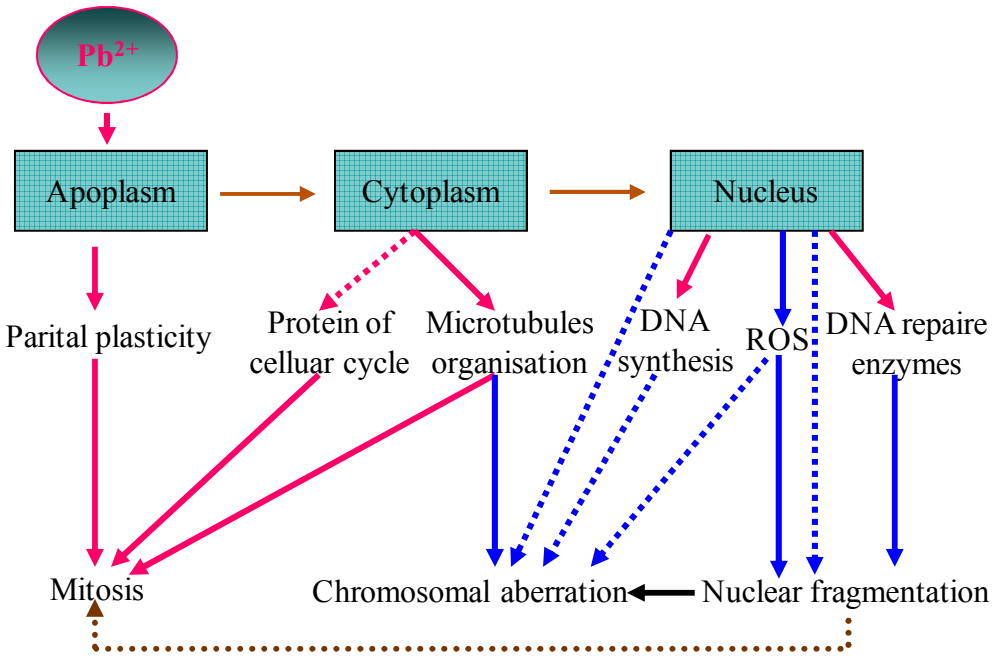


**Figure-6.** Representation of the documented links (solid arrows) and potential (dotted arrows) between Pb, mitosis and genotoxicity (Bertrand Thesis 2008).

Unlike antimitotic mechanisms, the mechanisms by which Pb causes genotoxicity are complex and not yet well understood. At low concentration, Pb did not induce a significant impact on mitosis, but did induce aberrations (chromosomal bridges during anaphase), loss of acentric fragments during meiosis, chromosomal fragmentation and micronucleus formation (National Toxicology Program 2003; Patra et al. 2004; Cecchi et al. 2008; Marcato-Romain et al. 2009; Grover et al. 2010; Barbosa et al. 2010; Shahid et al. 2011). The induction of chromosomal aberrations by Pb can be explained, in part, by its action of disrupting the microtubule network. Results of *in vitro* studies have demonstrated that Pb creates breaks in

single and double strands of DNA, and thereby affects horizontal DNA-DNA or DNA-protein links (Rucińska et al. 2004; Gichner et al. 2008; Shahid et al. 2011).

Pb may enter the nucleus (Małecka et al. 2008) and bind directly to the DNA or indirectly to protein. After binding to DNA, Pb disrupts DNA repair and replication mechanisms. Pb does not induce direct genotoxic effects until it becomes attached to naked DNA (Valverde et al. 2001). Pb can also affect replication by replacing the zinc in the Zn-finger pattern of the enzymes that intervene in DNA repair (Gastaldo et al. 2007). Recently, Cenksi et al. (2010) used a random amplified polymorphic DNA (RAPD) assay that amplifies random DNA fragments of genomic DNA, and they reported that genomic template stability was significantly affected by Pb exposure in *Brassica rapa.*

### *4.8 Oxidative Stress and Lipid Peroxidation*

ROS are produced during normal cell metabolism in the chloroplast, either as by-products of the reduction of molecular oxygen ($O_2$), or because of excitation in the presence of highly energized pigments. These ROS, such as superoxide radicals ($O_2^{\bullet-}$), hydroxyl radicals (·OH) and hydrogen peroxide ($H_2O_2$), are also generated following exposure to certain environmental agents. The Figure-7 presents different ROS along with chemical transformation of the molecular oxygen in a biological system.

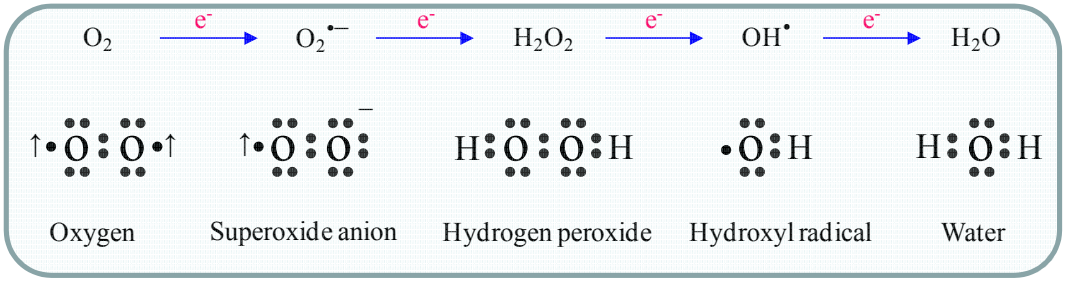


**Figure-7.** Schematic energy or chemical transformation (successive reduction) of the molecular oxygen in a biological system.

The production of ROS in the cells of aerobic organisms, defined as oxidative stress, is a well known feature of the toxicity of heavy metals, including Pb (Pourrut et al. 2008; Liu et al. 2008; Grover et al. 2010; Yadav 2010; Singh et al. 2010). However, the degree to which this feature is important is dependent on the metal type, specific form of the metal, plant species type, exposure time, etc. When ROS forms exhaust cellular antioxidant reserves, they

can rapidly attack and oxidize all types of biomolecules, such as nucleic acids, proteins and lipids (Reddy et al. 2005; Clemens 2006; Hu et al. 2007; Wang et al. 2007; Yadav 2010). Such attacks Pb to irreparable metabolic dysfunction and cell death.

b causes marked changes in the lipid composition of different cell membranes (Liu et al. 2008; Piotrowska et al. 2009; Grover et al. 2010; Yan et al. 2010; Singh et al. 2010). The polyunsaturated fatty acids and their esters that are present in lipids show high susceptibility to ROS (Dey et al. 2007; Gupta et al. 2009). Indeed, ROS removes hydrogen from unsaturated fatty acids and forms lipid radicals and reactive aldehydes, ultimately causing distortion of the lipid bilayer (Mishra et al. 2006). Pb-induced changes in lipid composition and potassium ion leakage were reported in *Zea mays* (Malkowski et al. 2002). Pb ions are known to induce lipid peroxidation, decrease the level of saturated fatty acids and increase the unsaturated fatty acid content of membranes in several plant species (Singh et al. 2010).

The oxidation of bis-allylic hydrogens on polyunsaturated fatty acids by ROS involves three distinct stages: initiation (formation of the lipid radical), progression (formation of lipid peroxyl-radical by reaction between lipid radical and oxygen) and termination (formation of non-radical products after bimolecular interaction of lipid peroxyl-radicals) (see details in the reviews of Gurer and Ercal 2000; Bhattacharjee 2005). These lipid membrane changes cause the formation of abnormal cellular structures, such as alterations in the cell membrane (Dey et al. 2007; Islam et al. 2008; Gupta et al. 2009), organelles (e.g., mitochondria), peroxisomes (Małecka et al. 2008; Liu et al. 2008) or chloroplasts (Choudhury and Panda 2004; Elibieta and Miroslawa 2005; Hu et al. 2007).

## 5 Mechanisms of Pb Tolerance

Plants respond to noxious effects of Pb in various ways, such as selective metal uptake, metal binding to the root surface, binding to the cell wall, induction of antioxidants, etc. There are several types of antioxidants to which plants may respond: non-protein thiol (NP-SH), cysteine, glutathione, ascorbic acid, proline and antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidise (APX), guaiacol peroxidase (GPX), catalase (CAT), and *glutathione reductase* (GR). However, the response varies with plant species, metal concentration and exposure conditions.

### *5.1 Passive Mechanisms*

Even when small amounts of Pb penetrate root cell membranes, it interacts with cellular components, and increases the thickness of cell walls (Krzesłowska et al. 2009, 2010). Pectin is a component of plant cell walls. Pb complexation with pectin carboxyl groups is regarded as the most important interaction by which plant cells can resist Pb toxicity (Meyers et al. 2008; Jiang and Liu 2010). Krzesłowska et al. (2009) observed that binding of Pb to JIM5-P (within the cell wall and its resultant thickening), acted as a physical barrier that restricted Pb access to the plasma membrane in *Funaria hygrometrica* protonemata. However, later, these authors stated that Pb bound to JIM5-P within the cell can be taken up or remobilized by endocytosis, together with this pectin epitope (Krzesłowska et al. 2010).

### *5.2 Inducible Mechanisms*

Recently, several authors have reported the presence of transporter proteins among plant cells that play an important role in metal detoxification, by allowing the excretion of metal ions into extracellular spaces (Meyers et al. 2008; Vadas and Ahner 2009; Maestri et al. 2010). The human divalent metal transporter 1 (DMT1), expressed in yeast, has been shown to transport Pb via a pH-dependent process (Bressler et al. 2004) in plants. Simultaneously, several ATP-binding cassette (ABC) carriers, such as AtATM3 or AtADPR12 at ATP-binding sites in *Arabidopsis,* were involved in resistance to Pb (Kim et al. 2006; Cao et al. 2009). Although, suspected to act against Pb, this detoxification mechanism has not yet been clearly confirmed. Transcriptome analysis has shown that the gene expression of these carriers is stimulated by Pb (Liu et al. 2009). Figure-8 shows the movement and binding of metals by transporter proteins.

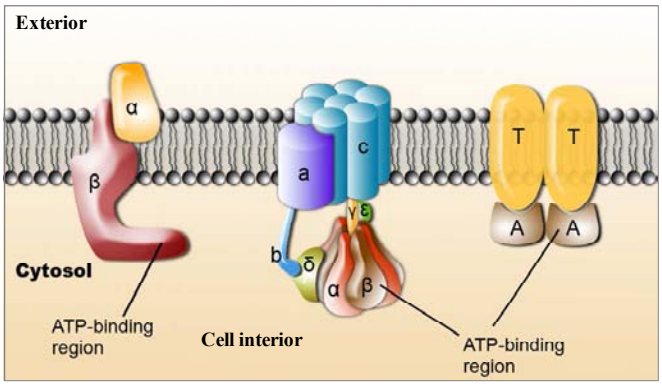


**Figure-8.** Metal binding and transportation by tansporter membranes.

Cellular sequestration is considered to be an important aspect of plant metal homeostasis, and in plant detoxification of heavy metals (Maestri et al. 2010). The Pb, that could be bound by certain organic molecules (Piechalak et al. 2002; Vadas and Ahner 2009), is sequestered in several plant cell compartments: vacuoles (Małecka et al. 2008; Meyers et al. 2008), dictyosome vesicles (Malone et al. 1974), endoplasmic reticulum vesicles (Wierzbicka et al. 2007) or plasmatubules (Wierzbicka 1998).

Cysteine and glutathione (GSH) are known to be non-enzymatic antioxidants in plants. An increase in cysteine content, in response to Pb toxicity, has been demonstrated in *Arabidopsis thaliana* (Liu et al. 2009). Glutathione protects plants from Pb stress by quenching Pb-induced ROS (Verbruggen et al. 2009; Liu et al. 2009). Moreover, as the substrate for phytochelatin (PC) biosynthesis, the glutathione-related proteins play an important role in heavy metal detoxification and homeostasis (Liu et al. 2009). Pb treatment can induce different GSH genes, including glutathione-synthetase,- peroxidase and -reductase, and -glutamyl cysteine synthetase. Glutathione can also enhance accumulation of proline in stressed plants, a role that is associated with reducing damage to membranes and proteins (Liu et al. 2009). Gupta et al. (2010) reported the role of GSH in Pb detoxification in *Sedum alfredii,* although this was accomplished without any induction of PC. This suggests that GSH may play an important role in detoxifying Pb, under stress conditions where PCs are absent. Figure-10 shows the chelation of heavy metals by cysteine, glutathione and phytochelatins.

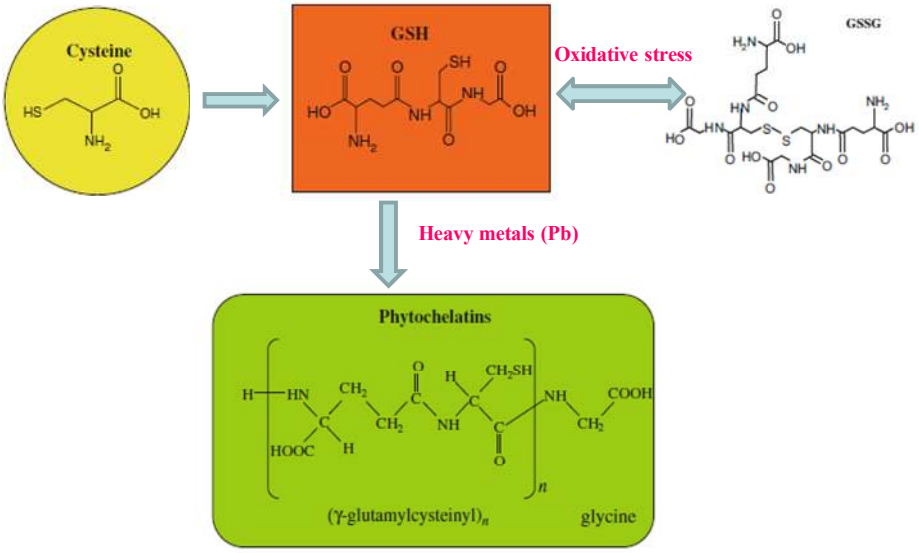


**Figure 1:** The chemical structure of cysteine, reduced glutathione, oxidized glutathione, and phytochelatins and their induction by Pb toxicity.

PCs and metallothioneins (MTs) are the best characterized metal-binding ligands in plant cells. These ligands belong to different classes of cysteine-rich heavy metal-binding protein molecules. PCs, the most frequently cited metal protective proteins in plants, are low molecular weight, metal-binding proteins that can form mercaptide bonds with various metals (Maestri et al. 2010), and play an important role in their detoxification in plants (Brunet et al. 2009; Liu et al. 2009; Gupta et al. 2010; Yadav 2010; Jiang and Liu 2010). These thiols are biologically active compounds, whose function is to prevent oxidative stress in plant cells (Verbruggen et al. 2009; Gupta et al. 2010). Their general structure is (γ-glutamyl-cys)*n* gly where *n* = 2-11, and they are synthesized by the action of γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase; PCS) from GSH (Yadav 2010).

Pb is known to stimulate the production of PC and activate PCS (Mishra et al. 2006; Clemens 2006; Andra et al. 2009; Vadas and Ahner 2009; Sing et al. 2010). It has been proposed that *in vivo*, phytochelatins are involved in the cellular detoxification and accumulation of several metals, including Pb, because of their ability to form stable metal-PC complexes (Clemens 2006; Yadav 2010). Phytochelatin sequesters soluble Pb in the cytoplasm before transporting it to vacuoles and chloroplasts (Piechalak et al. 2002; Małecka et al. 2008; Jiang and Liu (2010), thus reducing the deleterious effect of $Pb^{2+}$ in the cells. The mechanism regulating the passage of the Pb-PC complex through the tonoplast is, however, not yet known. Gisbert et al. (2003) reported significantly increased uptake and tolerance to Pb and Cd following the induction and over-expression of a wheat gene encoding for phytochelatin synthase (*TaPCS1*), in *Nicotiana glauca*.

### *5.3 Antioxidant Enzymes*

To cope with the increased production of ROS and to avoid oxidative damage, plants have a system of antioxidant enzymes that scavenge the ROS that are present in different cell compartments (Brunet et al. 2009; Singh et al. 2010; Gupta et al. 2010). Pb-induced toxicity may inhibit the activity of these enzymes, or may induce their synthesis (Table 1). However, Pb-induced inhibition or induction of anti-oxidant enzymes is dependent on metal type, specific form of the metal, plant species type, and the duration/intensity of the treatment (Islam et al. 2008; Gupta et al. 2009; Singh et al. 2010). Figure-10 shows the activation of antioxidant enzymes in plants when exposed to Pb.

Generally, Pb inhibits enzymatic activities, and, when this occurs, the values of the inactivation constant (Ki) ranges between $10^{-5}$ and $2.10^{-4}$ M (i.e., 50% of enzymatic activities are inhibited in this concentration range) (Seregin and Ivanov 2001). Enzyme inhibition results from the affinity Pb has for -SH groups on the enzyme (Sharma and Dubey 2005; Gupta et al. 2009). This is true for more than 100 enzymes, including ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) and nitrate reductase. Inactivation results from a link at either the catalytic site or elsewhere on the protein, and produces an altered tertiary structure. Pb can also produce the same effect by binding to protein-COOH groups (Gupta et al. 2009, 2010). Pb also interacts with metalloid-enzymes. Indeed, Pb can disrupt plant absorption of minerals that contain zinc, iron, manganese, etc., which are essential for these enzymes. Pb and other divalent cations also can substitute for these metals, and thereby inactivate enzymes, as occurs with ALAD (Gupta et al. 2009; Cenkci et al. 2010). The effect Pb has on ROS is constitutes another mechanism by which Pb exposure affects protein behavior (Gupta et al. 2009, 2010).

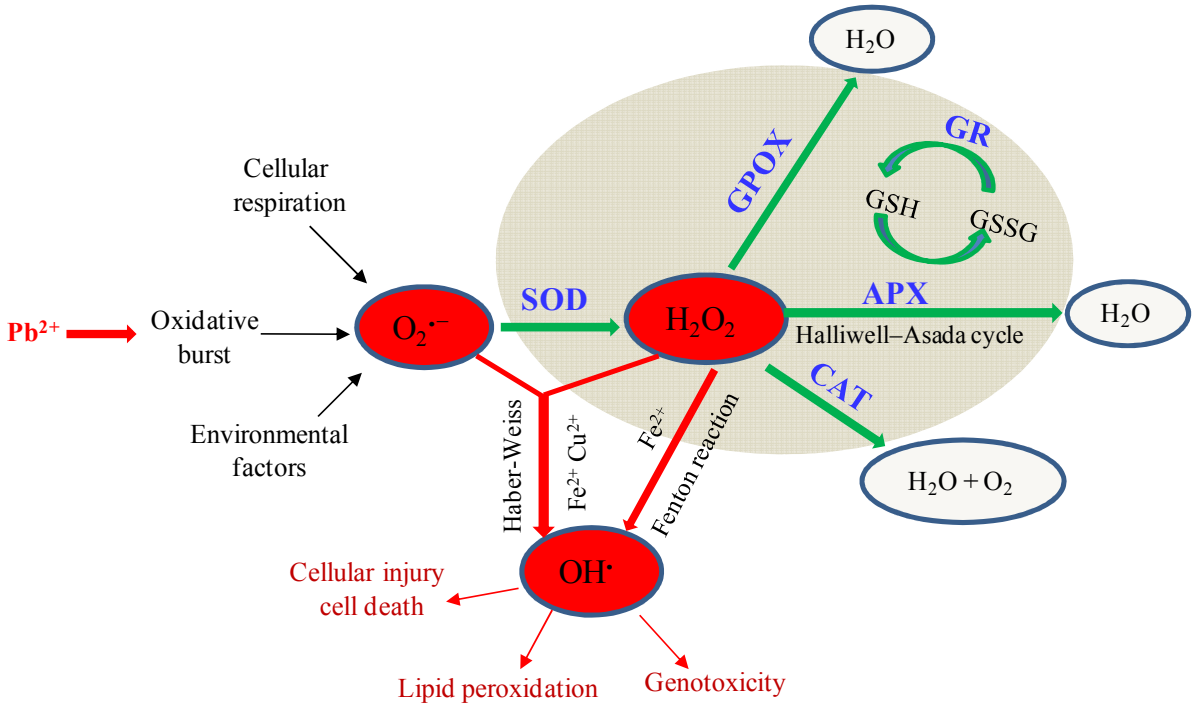


**Figure-9.** Pb-induced oxidative stress and activation of antioxidant enzymes. APX, Ascorbate Peroxidase; CAT, Catalase; GPOX, Guaiacol Peroxidase; GR, Glutathione Reductase; GSH, Reduced Glutathione; GSSG, Glutathione Oxidized; $H_2O_2$, Hydrogen Peroxide; $O_2^-$, Oxide ion ; $OH^·$, Hydroxyl radical ; SOD, Superoxide Dismutase.

Pb exposure is also known to stimulate the activities of certain enzymes (Table 1), but the mechanisms of action are, as yet, unclear. It has been proposed that Pb activates certain enzymes by modulating gene expression, or by restricting the activity of enzyme inhibitors (Seregin and Ivanov 2001). Indeed, antioxidant enzymes scavenge ROS, when they are produced in excess as a consequence of metal toxicity. Superoxide dismutase, a metallo-enzyme present in various cell compartments, is considered to be the first defense against oxidative stress (Mishra et al. 2006). It catalyses the dismutation of two superoxide radicals to $H_2O_2$ and oxygen, and thus maintains superoxide radicals at steady-state levels (Islam et al. 2008; Gupta et al. 2009). $H_2O_2$ is a very strong oxidant and requires quick removal to avoid oxidative toxicity; removal is achieved by the action of APX in the ascorbate-glutathione cycle, or by GPX and CAT in the cytoplasm, and in other cell compartments (Mishra et al. 2006). The role of GSH and glutathione reductase in the $H_2O_2$-scavenging mechanism in plant cells (Piechalak et al. 2002) is well established in the Halliwell-Asada enzyme pathway. Moreover, antioxidant enzymes may be activated from the increased concentration of their substrates, instead of direct interaction with Pb (Islam et al. 2008).

**Table-1.** Pb-induced activation (↑) and reduction (↓) of enzymatic activities in different plant species.

| Plant species | ↑ Enzyme activity | ↓ Enzyme activity | References |
|---|---|---|---|
| *Najas indica* | SOD, GPX, APX, CAT, GR | | Sing et al. 2010 |
| *Sedum alfredii* | SOD, APX | | Gupta et al. 2010 |
| *Zea mays* | SOD, CAT, AsA | | Gupta et al. 2009 |
| *Lathyrus sativus* | APX, GR, GST | | Brunet et al. 2009 |
| *Wolffia arrhiza* | CAT,APX | | Piotrowska et al. 2009 |
| *Raphanus sativus* | POD, Ribonuclease | CAT | Gopal and Rizvi 2008 |
| *Elsholtzia Argyi* | CAT | SOD, GPX | Islam et al. 2008 |
| *Kandelia candel* | SOD,POD,CAT | | Zhang et al. 2007 |
| *Bruguiera gymnorrhiza* | SOD,POD,CAT | | Zhang et al. 2007 |
| *Cassia angustifolia* | SOD, APX, GR, CAT | | Qureshi et al. 2007 |
| *Zea mays* | SOD,POD,CAT | | Wang et al. 2007 |
| *Triticum aestivum* | SOD, POX | CAT | Dey et al. 2007 |
| *Potamogeton crispus* | POD, SOD,CAT | SOD, CAT | Hu et al. 2007 |
| *Ceratophyllum demersum* | SOD, GPX, APX, CAT, GR | SOD, GPX, APX, CAT, GR | Mishra et al. 2006 |
| *Helianthus annuus* | GR | CAT | Garcia et al. 2006 |
| *Macrotyloma uniflorum* | SOD, CAT, POD, GR, GST | | Reddy et al. 2005 |
| *Cicer arietinum* | SOD, CAT, POD, GR, GST | | Reddy et al. 2005 |
| *Taxithelium nepalense* | APX, GPOX, CAT, | | Choudhury and Panda 2004 |
| *Oryza sativa* | SOD, GPX, APX, CAT, GR | GR, CAT | Verma and Dubey 2003 |

## 6 Conclusions and Perspectives

Pb is a major inorganic global pollutant and numerous studies have revealed its biogeochemical behavior and impact on the biosphere. Based on these studies, especially cited in this review, it is concluded that:

(1) Pb has been in use since antiquity, because of its many useful properties. The continued use of Pb in many industrial processes has increased its concentration to toxic levels in all environmental compartments.

(2) Pb forms stable complexes with different compounds in soil and tends to be stored in the soil. The fate and behavior of Pb in soil is affected by its form, solubility, mobility and

bioavailability, and is controlled by many biogeochemical parameters, such as soil pH, redox conditions, cation exchange capacity, soil mineralogy, biological and microbial conditions, amount and nature of organic and inorganic ligands present and competing cations.

(3) Pb enters plants mainly through the roots via the apoplast pathway or calcium ion channels. Pb can also enter plants in small amounts through leaves. Once in the roots, Pb tends to sequester in root cells. Only a limited amount of Pb is translocated from roots to shoot tissues, because there are natural plant barriers in the root endodermis (e.g., Casparian strips).

(4) Pb has no biological function and induces various noxious effects inside plants. Excessive Pb accumulation in plant tissue is toxic to most plants, Pbing to a decrease in seed germination, root elongation, decreased biomass, inhibition of chlorophyll biosynthesis, mineral nutrition and enzymatic reactions as well as a number of other physiological effects. The intensity of these effects vary depending on the duration of exposure, stage of plant development, studied organ and the concentration of Pb used for impact assessments.

(5) Pb-induced production of ROS is the major cause of its toxicity. These free radicals disrupt the redox status of cells, causing oxidative stress and DNA damage through oxidation, and Pb to irreparable metabolic dysfunction and cell death.

(6) Plants defend against Pb toxicity through several avoidance, or detoxification mechanisms. Plants resist Pb entry into their cells via exclusion, or they bind Pb to their cell walls or other ligands. Plants combat Pb-induced increased production of ROS by activating various antioxidant enzymes.

(7) The efficiency of detoxification mechanisms determines the final tolerance or sensitivity of plants to metal-induced stress. Plants that have efficient detoxification mechanisms are generally characterized as being hyper-accumulators. Such plants are useful in soil bioremediation for many metal types. Conversely, plants that do not efficiently cope with pollutants are sensitive to metal toxicity, and are often used in risk assessment studies.

In this review, we raise several questions that need attention in order to improve our understanding of the bio-geochemical behavior of Pb in different environmental compartments. Pb is known to interfere directly or indirectly with the genetic material, to induce ROS and modify (increase or decrease) the activities of certain enzymes in plants. These responses of plants to Pb toxicity are often used as tools, in the context of risk assessment. However, the mechanisms of action underlying the noxious effects of Pb in plants are still unknown.

Moreover, most field work performed on the effects of Pb on plants is based almost exclusively on the total metal content in polluted soil, even though this is of little significance from an environmental point of view. Indeed, the potential effects of Pb and other toxic elements in the environment depend on insights into their physico-chemical distribution, i.e., speciation. Therefore, if environmental scientists are to become better at predicting what the toxicity, or environmental impact, of Pb may be, then additional research on the form of Pb applied is essential.

## 7 Summary

Pb has gained considerable attention as a persistent toxic pollutant of concern, partly because it has been prominent in the debate concerning the growing anthropogenic pressure on the environment. The purpose of this review is to describe how plants take Pb up, and to link such uptake to the ecotoxicity of Pb in plants. Moreover, we address the detoxification mechanisms involved in the toxicity of Pb to plants and plant systems.

Pb has many interesting physico-chemical properties that makes it a very useful heavy metal. Indeed, Pb has been used by people since the dawn of civilization. Industrialization, urbanization, mining and many other anthropogenic activities have resulted in the redistribution of Pb from the earth's crust to the soil and to the environment.

Pb forms various complexes with soil components, and only a small fraction of the Pb present as these complexes in the soil solution are phytoavailable. Despite its lack of essential function in plants, Pb is absorbed by plants mainly through the roots from soil solution, and thereby may enter the food chain. The absorption of Pb by roots occurs via the apoplastic pathway, or via $Ca^{2+}$-permeable channels. The behavior of Pb in soil, and uptake by plants is controlled by its speciation, and by the soil pH, soil particle size, cation-exchange capacity, root surface area, root exudation, and degree of mycorrhizal transpiration. After uptake, Pb primarily accumulates in root cells, because of the blockage by Casparian strips within the endodermis. Pb is also trapped by the negative charges that exist on roots cell walls.

Excessive Pb accumulation in plant tissue impairs various morphological, physiological and biochemical functions in plants, either directly or indirectly, and induces a range of deleterious effects. It causes phytotoxicity by changing cell membrane permeability, reacting with active groups of different enzymes involved in plant metabolism and reacting with the phosphate groups of ADP or ATP, and replacing essential ions. Pb toxicity causes inhibition of ATP production, lipid peroxidation, and DNA damage by over production of

ROS. In addition, Pb strongly inhibits seed germination, root elongation, seedling development, plant growth, transpiration, chlorophyll production, and water and protein content. The negative effects that Pb has on plant vegetative growth mainly results from the following factors: distortion of chloroplast ultrastructure, obstructed electron transport, inhibition of Calvin cycle enzymes, impaired uptake of essential elements, such as Mg and Fe, as well as inducing a deficiency of $CO_2$ resulting from stomatal closure.

Under Pb stress, plants possess several defense strategies to cope with Pb toxicity. Such strategies include reduced uptake into the cell, sequestration of Pb into vacuoles by the formation of complexes, binding of Pb by phytochelatins, glutathione and amino acids, and synthesis of osmolytes. In addition, activation of various antioxidants to combat increased production of Pb-induced ROS constitutes a secondary defense system.

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# Section 1B:

## Review of Pb availability and toxicity to plants in relation with metal speciation; role of synthetic and natural organic ligands.

***-Publication-***

M. Shahid, E. Pinelli, P. Winterton and C. Dumat. Review of Pb availability and toxicity to plants in relation with metal speciation; role of synthetic and natural organic ligands (for Journal of Hazardous Materials)

# Review of Pb availability and toxicity to plants in relation with metal speciation; role of synthetic and natural organic ligands.

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**Abstract**

Biogeochemical behavior of lead (Pb), a persistent hazardous pollutant of environmental concern, strongly depends on its chemical speciation. Therefore, in this review, link between Pb speciation: presence of organic ligands and its environmental behavior has been developed. Both, biogeochemical and ecotoxicological data are discussed in environmental risk assessment context and phytoremediation studies. Three kinds of organic ligands selected for this review include: 1) ethylene diamine tetra-acetic acid (EDTA), 2) low molecular weight organic acids (LMWOAs) and 3) humic substances (HSs). For each organic ligand following points are developed: (i) effect on Pb speciation and behavior in soil; (ii) effect on Pb plant uptake and accumulation in different plant parts; and (iii) effect on Pb-induced phyto-toxicity.

Effects of organic ligands on Pb speciation are compared: how they can change Pb speciation modifying accordingly its fate and biogeochemistry in soil-plant system? **EDTA** forms soluble, stable and phytoavailable Pb-chelates due to high binding Pb affinity. **LMWOAs** can solubilize Pb in soil by decreasing soil pH or increasing soil organic contents, but have little effect on its translocation. Due to heterogeneous structure, **HSs** role is complex. Finally, influence of organic ligands towards Pb behavior in soil-plant system and phytotoxicity depends on their Pb binding capacity in addition to plant type and soil physico-chemical properties. In consequence Pb speciation knowledge is needed to discuss phyto-toxicity data and improve soil phytoremediation techniques.

**Key words:** Pb, speciation, organic ligand, EDTA, LMWOA, HS, biogeochemistry, ecotoxicology, soil phytoremediation studies.

## 1. Introduction

Despite considerable progress in recent years, Pb speciation in ecosystems, its transfer to the biosphere as well as associated ecological risks are still topical and remain an important research area (Uzu et al. 2010; Shahid et al. 2011a). Several previous studies and reviews have reported that Pb is hazardous material of environmental concern with high persistency. This metal has no biological function and can induce, directly or indirectly, various morphological, physiological and biochemical dysfunctions in plants such as

decrease in seed germination, plant growth, chlorophyll production. Pb also causes lipid peroxidation, oxidative stress and DNA damage in plants (Sharma and Dubey, 2005; Gupta et al. 2009; Saifullah et al. 2009; Sengar et al. 2009; Krzesłowska et al. 2010; Maestri et al. 2010; Sing et al. 2010; Yadav 2010; Gill and Tuteja, 2010; Shahid et al. 2011a). Figure-1 represents the different possible forms of a metal in the ecosystem.

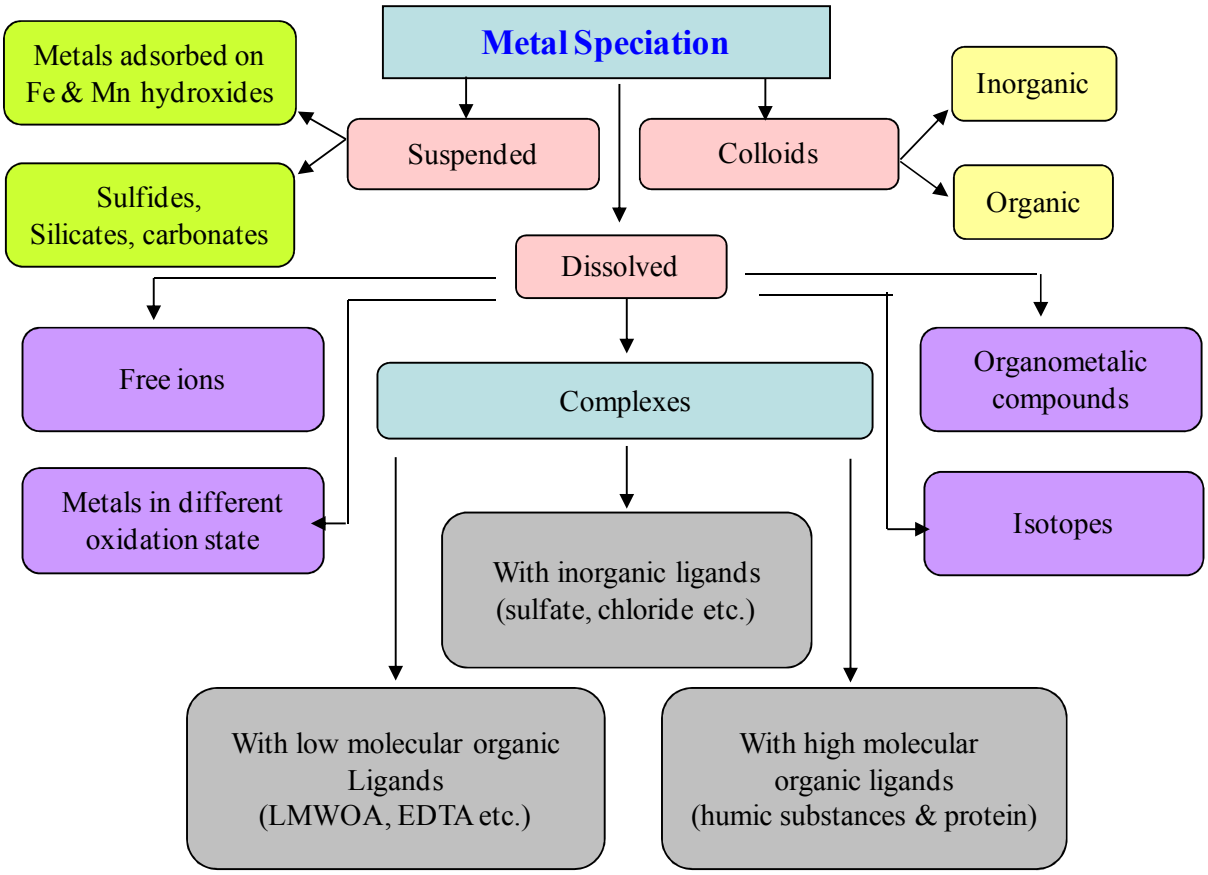


**Figure-1:** Speciation of a metal in the ecosystem.

However, biogeochemical Pb behaviour in ecosystems and its potential effects on plants are strongly influenced by its speciation (Dumat et al. 2006; Kopittke et al. 2008; Uzu et al. 2009; Shahid et al. 2011a,b). Speciation is the relative existence of a metal in different chemical forms under different environmental conditions. Pb in soils exists in several forms with different levels of solubility and bioavailability as follows: (i) dissolved (in soil solution), (ii) exchangeable (in organic and inorganic components), (iii) structural components of the lattices in soils and (iv) insolubly precipitated with other soil components (Cecchi et al. 2008; Uzu et al. 2009; Tabelin and Igarashi, 2009; Sammut et al. 2010; Vega et al. 2010). Usually, only the first two forms are considered as phytoavailable. In contrast, Pb which is an intrinsic part of the solid-phase minerals may not be phytoavailable (Lombi et al.

2003; Cecchi et al. 2008). This implies that Pb solubility and mobility in soil and plant uptake depends on its chemical speciation (Dumat et al. 2006). Several previous studies have described that free Pb cations concentration correlates better with its uptake and toxicity than total Pb concentration (Dumat et al. 2001 and 2006; Doig et al. 2007; Kim et al. 2010a; Shahid et al. 2011a). This makes total concentration, although easier to measure, an unreliable indicator of toxicity (Feng et al. 2005; Shahid et al. 2011a). Therefore, predicting the relevant species of Pb is very important step in improving our understanding of risk assessment or remediation studies.

Interactions between organic ligands and metals in natural media have been particularly studied due to their high affinity for metals (Ferrand et al. 2006; Quenea et al. 2009; Yip et al. 2010). Synthetic or natural organic ligands such as diethylene triaminepenta-acetic acid (DTPA), glycoletherdiamine tetra-acetic acid (EDGA), ethylene diamine tetra-acetic acid (EDTA), ethylene diamine disuccinate (EDDS), nitrilo tri-acetic acid (NTA), low molecular weight organic acids (LMWOAs) and humic substances (HSs) have been used extensively in Pb remediation techniques and to enhance micronutrient supply to plants (Ehsan et al. 2007; Evangelou et al. 2007; Saifullah et al. 2009 and 2010; Yip et al. 2010). These organic ligands desorb Pb from soil matrix into soil solution and facilitate its uptake by plant roots and translocation to shoot tissues at varying degree. Indeed, these ligands restrict Pb ions from playing their normal chemical roles by forming complexes (Shahid et al. 2011a). Fu and Wang (2011) reviewed the interaction between different chemical processes and organic ligands controlling Pb and other heavy metal kinetic in soil solution. The organo-metallic complexes vary greatly in term of stability, solubility and chronic toxicity (Hasegawa et al. 2010; Shahid et al. 2011a) compare to free metal ions. Many previous studies have presented the changes (increase or decrease) induced by organic ligands in Pb solubilisation, uptake, translocation and acute toxicity associated with Pb (Meers et al. 2005; Evangelou et al. 2007; Jamil et al. 2009; Kołodyńska et al. 2009; Yip et al. 2010; Hasegawa et al. 2010; Shahid et al. 2011a). Fig. 1 compares the effect of three types of organic ligands (EDTA, LMWOAs and HSs) on Pb solubilization in soil and uptake by plant.

Therefore, in order to better understand environmental biogeochemical Pb behavior it's necessary to make a link between its chemical speciation, mobility, solubility, phytoavailability and phyto-toxicity. How Pb chelation by organic ligands changes its speciation and in turn its biogeochemical environmental behavior is described in this review. Three types of organic ligands widely used in literature were selected on the basis of

contrasted influence and nature: EDTA as model of synthetic chelating agents, LMWOAs as model of root exudates and HSs as natural dissolved organic matter (DOM) present in soils. Figure-2 compares the effect of different organic ligands on metal solubilzation in soil and uptake by plants.

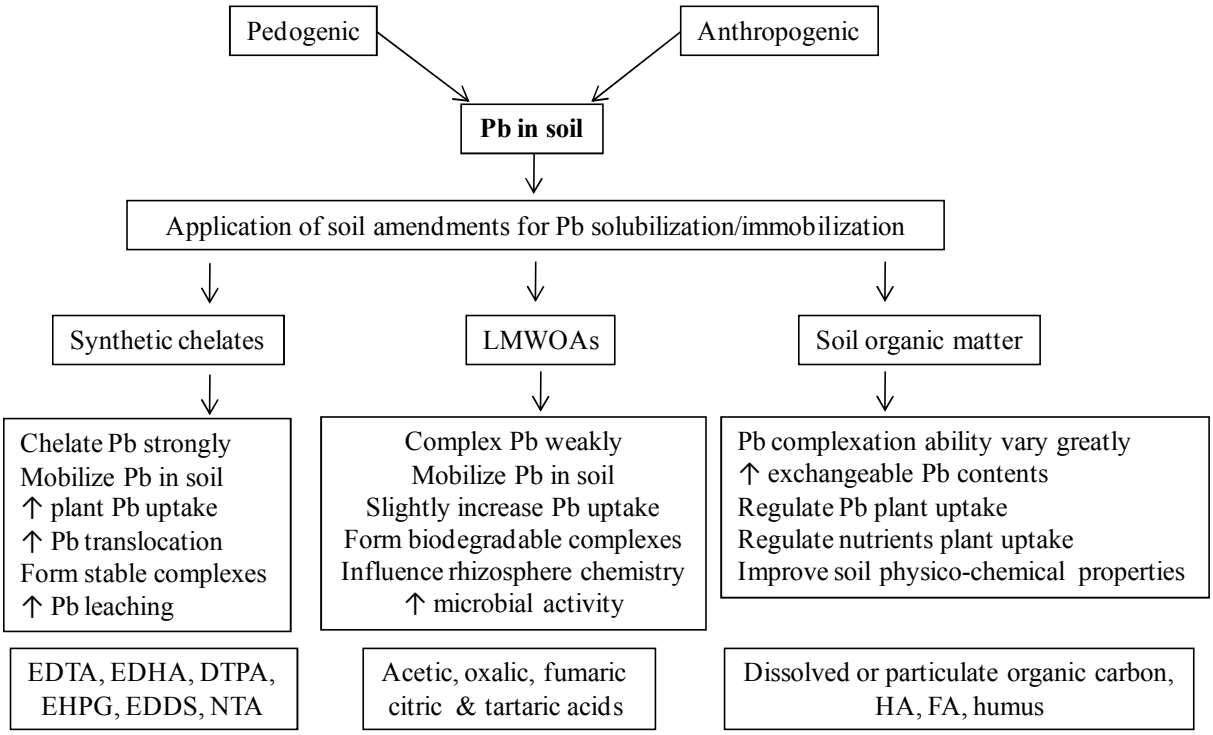


**Figure-2:** Comparison of different organic ligands towards their role in metal solubilzation in soil and uptake by plants.

## 2. EDTA

EDTA was patented in Germany in 1935 by F. Munz (Oviedo and Rodríguez, 2003) and was first reported by Wallace et al. (1974) for its ability to increase water-soluble metal concentrations in soils. EDTA is a hexahydric acid, which forms Pb-chelates. Strong Pb-EDTA interactions originate from six strong donor atoms in EDTA (two nitrogen atoms of amines and four oxygen atoms of carboxylates). These donor atoms surround Pb cation in the complex in order to achieve the maximal number (6) of possible donor–acceptor interactions (Kovács et al. 2010). In addition, EDTA anionic character also contributes to complex stabilization. Particularly, the four carboxylate oxygens can establish strong electrostatic attraction with the captured Pb cation. Pb-EDTA complex structure obtains octahedral shape in which two oxygens occupy axial positions, while the other two oxygens and the two nitrogens form the equatorial section while the Pb at the center (Kovács et al. 2010).

Many authors reported EDTA as the most efficient in increasing Pb solubility and phytoavailability in soils (Saifullah et al. 2009; Shahid et al. 2011a). It has been therefore widely used in remediation of Pb and other toxic metal polluted soils (Andra et al. 2009; Jamil et al. 2009; Sun et al. 2009; Xu et al. 2009; Barrutia et al. 2010). EDTA is also applied in chelation therapy for solubilisation of metals, as well as in numerous industrial applications such as washing and cleaning agents (Kovács et al. 2010; Kołodyńska 2011). Additional important applications of EDTA complexes appear as diagnostic and therapeutic agents for treating metabolic disorders and diseases (Kovács et al. 2010). Due to these numerous applications, EDTA is currently detected in the environment. Figure-3 shows the structural formula of EDTA and chelation of metals by EDTA.

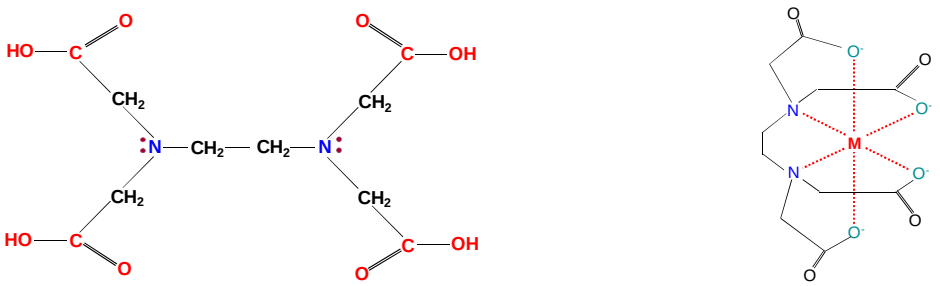


**Figure-3:** Structural formula of EDTA and metal-EDTA chelate.

### *2.1. Effect of EDTA on Pb speciation and behavior in soil*

Metal bioavailability is defined as the fraction of total metal soil content that can be absorbed by a biological target. Despite high total Pb soil concentration, only a low Pb fraction is readily available for plant uptake in the absence of organic ligands (Sharma and Dubey, 2005; Shahid et al. 2011b). This is because of most portions of the Pb in soil occur in residual form (Sun et al. 2009; Nogueira et al. 2010; Gharbi et al. 2010; Charriau et al. 2011) due to strong complexation with no biotic or biotic ligands (Uzu et al. 2009; Sammut et al. 2010; Vega et al. 2010; Shahid et al. 2011b). Therefore, Pb in soil is thought to be low soluble and plant available due to its immobile nature (Cheng and Hu, 2010; Padmavathiamma and, Li 2010; Shahid et al. 2011b).

In general, mobility and availability of Pb in soils are controlled by adsorption and desorption phenomena (Zeng et al. 2010; Ma et al. 2010; Hamidpour et al. 2010a; Hamidpour et al. 2010b; Waterlot et al. 2010; Xenidis et al. 2010; Rodríguez-Jordá et al. 2010; Shahid et al. 2011b), which in turn are associated with physico-chemical soil properties (Antoniadis et

al. 2008; Usman et al. 2008; Huang et al. 2011). Presence of EDTA increased Pb exchangeable fraction in soil. Pb solubilization results of a competition between adsorption by solid surfaces and formation of EDTA complex. According to many reports, EDTA can release Pb from several soil compartments, particularly Pb associated to Fe and Mn oxides and to organic matters (Lin et al. 2009; Udovic and Lestan, 2009; Park et al. 2011). Several authors observed a linear relationship between EDTA concentration and Pb mobilization in soils (Kołodyńska et al. 2009; Lin et al. 2009). When EDTA is applied to soil, formation of Pb–EDTA complexes shift precipitation and sorption equilibrium toward increased dissolution of Pb (Kołodyńska et al. 2009; Sun et al. 2009; Goel et al. 2010; Kołodyńska 2011) due to high binding affinity of EDTA for Pb (pK=17.8). Wu et al. (2003) and Nascimento et al. (2006) respectively noted a 600- and 217-fold increased Pb concentration in soil solution by EDTA.

However, EDTA effect on Pb solubilisation in soils greatly varied according to following factors: soil physico-chemical properties (texture/structure, pH, redox potential, cation exchange capacity, soil buffering capacity, fixation/aging) (Escudero et al; 2008; Kołodyńska et al. 2009; Lin et al. 2009; Saifullah et al. 2009; Kołodyńska 2011), soil biological and microbial conditions (Usman et al. 2009; Saifullah et al. 2009; Majumdar et al. 2010; Lin et al. 2010; Arias et al. 2010), concentration of other competing metals and EDTA (Kołodyńska et al. 2009; Udovic and Lestan, 2009; Kołodyńska 2011), Pb:EDTA stoichiometric ratio (Escudero et al; 2008; Kołodyńska et al. 2009; Lin et al. 2009; Saifullah et al. 2009), soil exposure time to contaminants, time and mode of EDTA application (Grčman et al. 2003; Wu et al. 2003; Shahid et al. 2011a) and combined application of EDTA with other amendments (Cui et al. 2004; Begonia et al. 2005; Luo et al. 2006; Labanowski et al. 2008; Saifullah et al. 2009; Yip et al. 2010; Hadi et al. 2010). These numerous influencing parameters could explain why it can appear contrasted and sometimes opposite results when comparing several studies dealing with EDTA-Pb-soil systems.

### *2.2. Effect of EDTA on Pb plant uptake and accumulation in different plant parts*

Although, Pb forms stable complexes and tend to store, mainly, in soil but a mobile fraction can be absorbed by living organisms (Sammut et al. 2010; Vega et al. 2010) and go well in food chain. EDTA is known to increase Pb uptake and translocation from roots to aerial parts, which is one of the most prominent features of its utilization in phytoremediation. Increased Pb uptake by EDTA has been documented in several recent

studies (Andra et al. 2009; Sun et al. 2009; Usman et al. 2009; Saifullah et al. 2009, 2010; Barrutia et al 2010; Shahid et al. 2011a). This increase in Pb uptake by plant roots is due to formation of Pb-EDTA complex, which is readily taken up by most of plant species (Liu et al. 2008a; Shahid et al. 2011a). It depends on substances released by roots including organic acids, types and varieties of plants, plant maturity (Shen et al. 2002; Usman et al. 2009) and planting density (Liphadzi et al. 2003). Different authors reported a wide variation in EDTA-induced increased Pb uptake by plants, i.e. Andra et al. (2009) reported 15 times increased Pb uptake by *Vetiveria zizanioides* roots, whereas Sun et al. (2009) demonstrated 161-fold increased Pb accumulation by *J. effuses* roots. Table 1 depicts effect of EDTA addition on Pb uptake and translocation by different plant species.

Several studies have shown that without chelators, major part of Pb (almost 95 %) tends to accumulate in root cells. This Pb translocation restriction phenomenon is due to precipitation as insoluble Pb-salts or immobilization by the negatively charged pectins within cell wall, precipitation in intercellular space, blockage by Casparian strip, accumulation in plasma membrane or sequesteration in the vacuoles of rhizodermal and cortical cells (Islam et al. 2007; Kopittke et al. 2007; Małecka et al. 2008; Arias et al. 2010; Jiang and Liu 2010). Pb accumulation in roots has been very well documented in many plants such as *Vicia faba*, *Pisum sativum* and *Phaseolus vulgaris* (Piechalak et al. 2002; Małecka et al. 2008; Shahid et al. 2011a), *Vigna unguiculata* (Kopittke et al. 2007), *Nicotiana tabacum,* (Gichner et al. 2008), *Pisum sativum*, *Lathyrus sativus* (Brunet et al. 2009), *Zea mays* (Gupta et al. 2009), *Avicennia marina* (Yan et al. 2010), *Sesuvium portulacastrum* and *Brassica juncea* (Zaier et al. 2010), non accumulating *Sedum alfredii* (Gupta et al. 2010) and *Allium sativum* (Jiang and Liu 2010). However, chelation of Pb by EDTA has been described to reduce Pb sequestration in roots by a number of workers in recent years (Andra et al. 2009; Jamil et al. 2009; Xu et al. 2009; Sun et al. 2009; Usman et al. 2009; Barrutia et al 2010; Chen et al. 2010). Lopez et al. (2005) found that EDTA addition at equimolar concentration to hydroponic medium increased Pb translocation to leaves by about 300 %. Liu et al. (2008a) observed that EDTA addition at 0.1mM concentration made it possible to raise Pb translocation to shoots from 17 to 31 %. Recently, Barrutia et al. (2010) reported that Pb chelation by EDTA resulted in 27 times higher Pb accumulation in shoots of *Rumex acetosa*.

Several previous studies have revealed the mechanism of EDTA-induced increased translocation to shoot tissues. These studies suggested that EDTA chelates Pb in nutrient/soil solution (Ruley et al. 2006; Usman et al. 2009; Xu et al. 2009; Shahid et al. 2011a) and this

chelate passes through free space of roots, which is continuous with surrounding soil solution (Shahid et al. 2011a). Presence of EDTA increases the root Pb flux through the apoplast and then increase Pb shoot/root ratio (Hernández-Allica et al. 2007). This is basically due to a reduced binding of Pb to extracellular cation exchange sites of the apoplast and cell walls (Tandy et al. 2006) due to neutral Pb-EDTA complex. Moreover, disruption of Casparian strip by EDTA might also be responsible for higher Pb accumulation in aerial parts (Wallace and Hale, 1962). Huang et al. (1997) also proposed that increased Pb uptake and translocation is probably due to damaged roots by EDTA.

However, some authors also reported no significant EDTA effect on Pb uptake (Vera et al. 2009; Athalye et al. 1995; Xu et al. 2007) and DelaRosa et al. (2007) concluded that EDTA application to Pb-contaminated agar media dramatically reduced total Pb acquisition in Tumbleweed plants. This inefficiency of EDTA on Pb uptake or translocation is most probably due to absence/ineffectiveness of transporter proteins responsible for Pb translocation to shoot tissues.

### *2.3. Effect of EDTA on Pb-induced toxicity to plants*

Excessive Pb accumulation in plant tissue is toxic to most plants. As seen above, presence of EDTA can increase Pb plant uptake and translocation. The question asked, is therefore, what is EDTA influence on Pb-induced toxicity to plants? Liu et al. (2008b) reported that Pb-induced decrease in pigment contents was reduced by EDTA. Several authors concluded that Pb is phytotoxic when present in free ionic form (Andra et al. 2009; Barrutia et al. 2010; Chen et al. 2010; Shahid et al. 2011a), whereas chelated Pb forms are less phytotoxic (Piechalak et al. 2003; Ruley et al. 2004; Shahid et al. 2011a). Hernández-Allica et al. (2007) reported that proper management of EDTA concentration can reduce its phytotoxicity. Table 1 presents EDTA effect on the Pb-induced phytotoxicity to various plants. Pb-induced oxidative stress due to over production of reactive oxygen species and increase in the activities of enzymes are also reported to be prohibited in the presence of EDTA. Huang et al. (2008) reported decreased Pb-induced lipid peroxidation and induction of superoxide dismutases and lysyl oxidase in presence of EDTA. Liu et al. (2009) presented that EDTA inhibited Pb induction of $H_2O_2$, malondialdehyde, ascorbate peroxidase, superoxide dismutases and dehydroascorbate reductase, in no accumulating *Sedum alfredii*. Ruley et al. (2004) reported that chelators mitigate Pb-induced oxidative stress by modulating anti oxidative enzyme activities in *Sesbania drummondii* seedlings. Despite higher Pb

concentrations in plant tissues, amount of total thiols and catalase activity in EDTA treated vetiver tissues was comparable to control (Andra et al. 2009), showing the protective role of EDTA against Pb toxicity. Protective role of EDTA against Pb-induced genotoxicity was also demonstrated by Shahid et al. (2011a) for *Vicia faba* root tips.

**Table-1.** Effect of EDTA on Pb uptake, translocation and toxicity to plants.

| EDTA | Pb | Duration | Crops | Uptake | Trans. | Effects | References |
|---|---|---|---|---|---|---|---|
| 15 µM | | 10 days | *V. zizanioides* | ↑ 15 | ↑ 24 | ↓ CAT activity and PC synthesis | Andra et al. 2009 |
| 500 µM | 67874 mg/kg | 52 hours | *R. acetosa* | NM | ↑ 27 | ↑ Stomatal conductance | Barrutia et al. 2010 |
| 8 mM | 57.93 mg/kg | 14 days | *J. effusus* | ↑ 161 | ↑ 78 | ↓ Plant growth & root dry biomass | Sun et al. 2009 |
| 3 mM | 115 mg/kg | 46 days | *Z. mays* | ↑ 1.3 | ↑ 5.4 | ↓ Root and shoot dry matter | Usman et al. 2009 |
| | | | *H. annuus* | ↑ 1.6 | ↑ 6 | ↓ Root and shoot dry matter | |
| 500 µM | 500 µM | 7 days | *P. radiata* | ↓ 17 | ↑ 7 | No effect on cell morphology | Jarvis and Leung 2002 |
| 3 mM | | | | NM | ↑ 4 | ↓ Dry matter yield | Neugschwandtner |
| 6 mM | | | | | ↑ 5 | ↓ Dry matter yield | et al. 2008 |
| 9 mM | | | | | ↑ 14 | ↓ Dry matter yield | |
| 1.2 mM | 2.4 mM | 7 days | *Z. elegans* | NM | ↑ 1.1 | ↓ Root length, ↑shoot length | Cui et al. 2007 |
| 2.4 mM | | | | | ↑ 1.5 | ↑ Root length, ↑shoot length | |
| 4.8 mM | | | | | ↑ 1.2 | ↓ Root length, ↑shoot length | |
| 2.4 mM | 4.8 mM | 7 days | | | ↑ 1.1 | ↓ Root length, ↓ shoot length | |
| 4.8 mM | | | | | ↑ 1.3 | No effect on Root length, ↑shoot length | |
| 9.6 mM | | | | | ↓ 1.1 | ↓ Root length, ↑shoot length | |
| 250 µM | 500 mg/kg | 2 days | *C. cardunculus* | ↑ 4 | ↑ 6 | ↑ Plant water transpiration | Hern´andez-Allica |
| 500 µM | | | | ↓ 1.1 | ↑ 10 | ↑ Plant water transpiration | et al. 2007 |
| 750 µM | | | | ↓ 2 | ↑ 8 | ↑ Plant water transpiration | |
| 100 mg/l | 500 mg/kg | | *S. drummondii* | ↓ 15 % | ↑ 4 | NM | Israr and Sahi 2008 |
| 5 mM | 44.2 mg/kg | 7 days | *Z. mays* | | ↑ 28 | ↓ Shoot and root dry weight | Luo et al. 2005 |
| | | 14 days | | | ↑ 26 | ↓ Shoot and root dry weight | |
| | | 7 days | *P. vulgaris* | NM | ↑ 50 | ↓ Shoot and root dry weight | |
| | | 14 days | | | ↑ 69 | ↓ Shoot and root dry weight | |
| 3 mM | 108 mg/kg | 28 days | *C. sativa* | NM | ↑ 7 | No effect on plant biomass | Meer et al. 2005 |
| | | | *H. annuus* | | ↑ 13 | | |
| | | | *B. rapa* | | ↑ 4 | | |
| | | | *Z. mays* | | ↑ 9 | | |
| 2 mM | 456 mg/kg | 10 days | *T. aestivum* | NM | ↑ 5 | ↑ Photosynthetic rate | Saifullah et al. 2010 |
| 4 mM | | | | | ↑ 5 | | |
| 8 mM | | | | | ↑ 9 | | |
| 1 mM | 3362 mg/kg | 30 days | *P. tomentosa* | ↑ 2 | ↑ 2 | ↑ total and root/shoot dry weight | Doumett et al. 2008 |
| 5 mM | | | | ↑ 2.5 | ↑ 3 | | |
| 10 mM | | | | ↑ 3 | ↑ 3 | | |
| 1.25 mM | 7500 mg/kg | 14 days | *S. drummondii* | ↑ 12 | ↑ 1.5 | ↑ Root, shoot length, chlorophyll a | Ruley et al. 2006 |
| 2.5 mM | | | | ↑ 12 | ↑ 1.9 | ↓ Root, shoot length, chlorophyll a | |
| 5 mM | | | | ↑ 27 | ↑ 2.2 | ↑ Root, shoot length, chlorophyll a | |
| 10 mM | | | | ↑ 42 | ↑ 2.8 | ↓ Root, shoot length, chlorophyll a | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2.5 mM | 800 mg/kg | 7 days | Mung bean | NM | ↑ 25 | ↓ Shoot dry matter, high necrotic leaves | Chen et al. 2004 |
| 5 mM | | | | | ↑ 24 | | |
| 2.5 mM | | | Buckwheat | | ↑ 65 | ↓ Shoot dry matter, necrotic leaves | |
| 5 mM | | | | | ↑ 71 | ↓ Shoot dry matter, plant deth | |
| 2.5 mM | | | Sunflower | | ↑ 7 | ↓ Shoot dry matter, necrotic leaves | |
| 5 mM | | | | | ↑ 7 | | |
| 2.5 mM | | | Cabbage | | ↑ 11 | ↓ Shoot dry matter, necrotic leaves | |
| 5 mM | | | | | ↑ 18 | | |
| 2.5 mM | | | Pea | | ↑ 104 | ↓ Shoot dry matter, necrotic leaves | |
| 5 mM | | | | | ↑ 139 | | |
| 2.5 mM | | | Mustard | | ↑ 16 | ↓ Shoot dry matter, necrotic leaves | |
| 5 mM | | | | | ↑ 27 | | |
| 2.5 mM | | | Maize | | ↑ 8 | ↓ Shoot dry matter, less necrotic leaves | |
| 5 mM | | | | | ↑ 15 | | |
| 2.5 mM | | | Sorghum | | ↑ 13 | ↓ Shoot dry matter, less necrotic leaves | |
| 5 mM | | | | | ↑ 24 | | |
| 2.5 mM | | | Wheat | | ↑ 22 | ↓ Shoot dry matter | |
| 5 mM | | | | | ↑ 31 | | |
| 2.5 mM | | | Barley | | ↑ 8 | ↓ Shoot dry matter | |
| 5 mM | | | | | ↑ 6 | | |

NM: Not mentioned, Trans: Translocation

However, few authors also reported increased Pb toxicity in presence of EDTA (Sun et al. 2009; Hadi et al. 2010). Geebelen et al. (2002) found reduced roots biomass, shortening of the stems and decrease in the fresh weight of *Phaseolus vulgaris* leaves when treated with Pb–EDTA complexes (10–200 mM) in Hoagland medium. Usman et al. (2009) reported that applying EDTA to Pb polluted soil depressed the plant growth, and significantly decreased dry matter of roots and shoots of *Helianthus annuus* plants. These authors suggested that EDTA increased Pb uptake and translocation to aerial parts and, thereby, the associated toxicity (Jamil et al. 2009; Xu et al. 2009; Chen et al. 2003; Ruley et al. 2006). According to Sarret et al. (2001), Pb-EDTA complex is not toxic but can dissociate after taken up by plants giving rise to increased free ionic $Pb^{2+}$ and hence the toxicity increased. A depressed photosynthetic activity was reported when *Sesbania drummondii* seedlings were grown in the solution culture containing 450 mg $l^{-1}$ EDTA alone (Ruley et al. 2004). Another hypothesis to explain increased Pb toxicity in presence of EDTA could be therefore due to EDTA influence. Moreover, EDTA application may increase the potential off-site Pb migration and leaching risk into aquifers (Grčman et al. 2003; Wu et al. 2004; Jiang et al. 2003; Núñez-López et al. 2008; Yip et al. 2010 and 2011; Park et al. 2011). Zhao et al. (2011) introduced a horizontal permeable barrier to reduce Pb and other heavy metals leaching to groundwater following EDTA application. Moreover, EDTA and Pb-EDTA may persist for several months in field (Zhao et al. 2010; Goel and Gautam, 2010). Recently, Wang et al. (2010)

proposed EDTA degradation by ozonolysis. Use of EDTA is therefore questionable and natural organic ligands could replace it for environmental uses.

### 3. LMWOAs

LMWOAs, which include acetic, oxalic, fumaric, citric, and tartaric acids originates from root exudation, decomposition of soil organic matter and by microbial metabolites (Strobel, 2001; Quartacci et al. 2009; Koo et al. 2010; Mucha et al. 2010; Kim et al. 2010d). These LMWOAs, can serve in soil for nutrient acquisition (Neumann and Römheld, 1999; Neumann et al. 1999), alleviation of anaerobic stress in roots, mineral weathering and microbial attraction (Magdziak et al. 2011). Recently, LMWOAs use as an alternate to synthetic chelating agents was found to be useful for Pb remediation (Quartacci et al. 2009; Najeeb et al. 2009). These compounds induce modification in the biogeochemical Pb behavior and fate in soil directly by affecting acidification, chelation, precipitation and redox reactions. These compounds can also act indirectly, through their effects on microbial activity, by changing physical and chemical properties of rhizosphere, root growth pattern and soil organic contents (Tao et al. 2004; Chopin et al. 2008; Najeeb et al. 2009; Quartacci et al. 2009; Koo et al. 2010; Martínez-Alcalá et al. 2008; Kim et al. 2010a, 2010b, 2010c and 2010d; Magdziak et al. 2011).

LMWOAs are usually present in soil solution in mM range (Sandnes et al. 2005; Magdziak et al. 2011), with rhizosphere concentration higher in comparison with bulk soil (Haoliang et al. 2007). However, their amounts and compositions are dependent on plant genotype, plant growth stage, plant transpiration rate, growing season and environmental conditions such as $CO_2$, light, pH, temperature, moisture, concentration and chemical form of nutrients and the presence of toxicants (Cawthray, 2003; XuandJi, 2003; Wu et al. 2003; Collins, 2004; Qin et al. 2004; Liao et al. 2006; Evangelou et al. 2007; Zeng et al. 2008; Quartacci et al. 2009; Mucha et al. 2010; Kim et al. 2010b and 2010c; Magdziak et al. 2011). LMWOAs release by plant roots and their composition vary with environmental conditions and physico-chemical rhizosphere properties. For example, LMWOAs are released at higher rate from roots during oxidative stress, under high level of Pb and iron, or low concentrations of calcium and phosphorus (Cawthray, 2003; XuandJi, 2003; Wu et al.2003; Collins,2004; Qin et al. 2004; Liao etal., 2006; Evangelou et al. 2007; Quartacci et al. 2009; Magdziak et al. 2011). Indeed, under Pb stress conditions, $H^+$-ATPases located at the plasma membrane level become activated resulting in the release of organic exudates from roots (Quartacci et al.

2009). Therefore, LMWOAs are suggested even more important than soil pH, because their release and role vary according to environmental conditions and plant needs (Huang et al. 1998; Qin et al. 2004). Figure-4 indicates the structural formula of some LMWOAs.

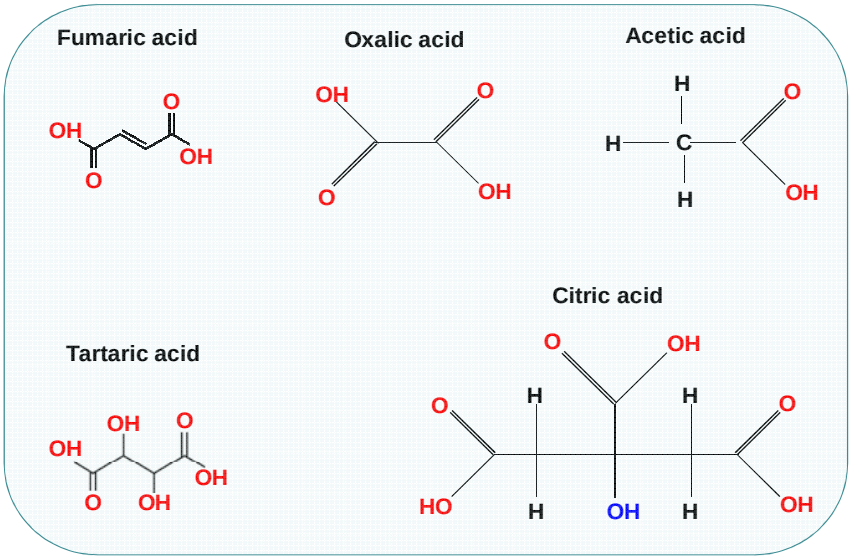


**Figure-4:** Structural formula of different LMWOAs.

### *3.1. Effect of LMWOAs on Pb speciation and behavior in soil*

Typically, rhizosphere consists of soil layer (1-5 mm) surrounding root cylinder (Tao et al. 2003; Chaignon et al. 2009). By modifying rhizosphere chemistry and processes operating (Chen et al. 2003; Evangelou et al. 2006; Kim et al. 2010a, 2010b, 2010c and 2010d; Mucha et al 2010) and supplying both protons and complexing anions, LMWOAs can facilitate Pb desorption from soil components (Strobel, 2001; Quartacci et al. 2009) and Pb-compounds dissolution (McBride, 1994). Debela et al. (2010) reported increased Pb release from pyromorphite, a presumably stable Pb-phosphate [$Pb_5(PO_4)_3Cl$] mineral, in contaminated soils by LMWOAs. Wu et al. (2003) stated that LMOWAs introduction into the soil resulted in 20–30 times increased Pb level in soil solution.

Because Pb adsorption on soils decreased at lower pH values (Chen et al. 2003; Evangelou et al. 2007; Kopittke et al. 2008; Lawal et al. 2010; Vega et al. 2010; Zhao et al. 2010; Soler-Rovira et al. 2010; Martínez-Alcalá et al. 2010), $H^+$ released by carboxylic acid groups of LMWOAs play a major role in Pb dissolution (Chen et al. 2003; Quartacci et al. 2009; Kim et al. 2010b). Fest et al. (2008) observed highest $CaCl_2$ Pb extractable at low pH. They suggested that Pb solubilisation was influenced by local competition between $H^+$ or

$Ca^{2+}$ and $Pb^{2+}$ for solid phase adsorption. Separately or simultaneously, the production of protons, exudates and metabolites released in rhizosphere by roots and microorganisms can modify soil pH by as much as one or two units depending, for instance, on the soil physico-chemical properties, plant type and composition of LMWOAs (Quartacci et al. 2009). However, Pb solubilization in rhizosphere is not always related to decrease in soil pH by LMWOAs. Recently Kim et al. (2010a, 2010b and 2010c) shows that Pb solubilization in alkaline soil by *Brassica juncea* was due to increase in soil dissolved organic carbon (DOC) contents and Pb-DOC complex formation.

From another side, few authors concluded that LMWOAs have little or no effect on Pb solubility (Shen et al. 2002; Wu et al. 2003; Vera et al. 2009) due to low metal binding capacity (Meers et al. 2005) or short half-life (1.5–5.7 days) in soils (Römkens et al. 2002). Actually, root exudates influence on Pb solubilization depends on soil physico-chemical conditions and microbial activity (Evangelou et al. 2006; Palma and Mecozzi, 2007; Liu et al. 2008a; Chen et al. 2008). In addition, LMWOAs effectiveness is function on plant species (Quartacci et al. 2009). For example Kim et al. (2010a, 2010b and 2010c) demonstrated that root exudates from *Brassica juncea* and *Helianthus annuus* increased soluble Pb contents in alkaline soil but decrease in acidic.

### *3.2. Effect of LMWOAs on Pb plant uptake and accumulation in different parts*

LMWOAs can influence Pb uptake by plants and translocation to aerial parts (Meers et al. 2005; Kim et al. 2010d; Sun et al. 2009). Liu et al. (2008a) reported that citric acid significantly increased Pb root uptake by accumulating and non accumulating *Sedum alfredii* plants, but increased Pb translocation to shoot was observed only for accumulating *Sedum alfredii.* Several other authors reported increased Pb translocation with LMWOAs (Chen et al. 2003; Luo et al. 2005). LMWOAs effect on Pb uptake and translocation in some plant species is shown in Table 2. Biodegradation of LMWOAs could reduce their influence on Pb solubility (Shen et al. 2002; Muhammad et al. 2009). However, interaction with Pb can reduce LMWOAs biodegradability (Dumat et al, 2006). Anyway, Liao et al. (2000) presented no significant LMWOAs effects on Pb uptake by *Lactuca sativa.* Chen et al. (2003) reported that citric acid had no significant effect on plant uptake or translocation. Some authors reported that LMWOAs effect on Pb uptake and translocation is less significant compare to synthetic chelates like EDTA (Sun et al. 2009; Chen et al. 2003; Evangelou et al. 2008; Shahid et al. 2011a). Shen et al. (2002) reported for different chelating agents (in decreasing

order) effectiveness for stimulating Pb accumulation in *Brassica oleracea* shoots: EDTA > HEDTA > DTPA >> citric acid.

**Table-2.** Effect of citric acid (CA) on Pb uptake, translocation and toxicity to plants.

| CA | Pb | Duration | Crops | Uptake | Trans. | Effects | References |
|---|---|---|---|---|---|---|---|
| mM | | days | | | | | |
| 1.2 | 2.4 mM | 7 | *Z. elegans* | NM | ↑ 1.2 | No effect on Root & shoot length | Cui et al., 2007 |
| 2.4 | | 7 | | | ↓ 1.2 | ↑shoot length | |
| 4.8 | | 7 | | | ↓ 1.1 | No effect on Root & shoot length | |
| 2.4 | 4.8 mM | 7 | | | ↑ 1.3 | ↓ Root length | |
| 4.8 | | 7 | | | ↓ 1.1 | ↓ Root length | |
| 9.6 | | 7 | | | ↓ 1.16 | ↓ Root length | |
| 5 | 254 µg/l | 4 | *B. juncea* | NM | ↑ 8 | No effect on shoot dry weight | Duquène. 2009 |
| | 35 µg/l | 4 | | | 1 | No effect on shoot dry weight | |
| | 254 µg/l | 4 | *L. perenne* | | 1 | ↓ Shoot dry weight | |
| | 35 µg/l | 4 | | | 1 | No effect on shoot dry weight | |
| 5 | 1015 mg/kg | 10 | *S. alfredii* | 2.4 | 1.4 | ↓ Shoot dry weight | Liu et al. 2008 |
| 5 | 1331 mg/kg | 7 | *B. juncea* | 1 | 1 | ↓ Plant dry weight | Quartacci et al. 2006 |
| 5 | 25 mg/kg | 14 | Radish | ↓ 2.4 | ↓ 1.3 | ↓ Pb toxicity | Chen et al. 2003 |
| 5 | 44.2 mg/kg | 7 | *Z. mays* | NM | ↑ 1.5 | ↓ Shoot and root dry weight | Luo et al. 2005 |
| | | 14 | | | ↓ 1.7 | ↓ Shoot and root dry weight | |
| | | 7 | *P. vulgaris* | | ↑ 3.5 | ↓ Shoot and root dry weight | |
| | | 14 | | | ↓ 1.1 | ↓ Shoot and root dry weight | |
| 5 | 58 mg/kg | 5 | *S. alfredii* | ↑ 1.1 | ↑ 1.1 | ↓ Shoot, root, leaf and stem DW | Sun et al., 2009 |
| 8 | | 5 | | ↑ 2.2 | ↑ 1.2 | ↓ Shoot, root, leaf and stem DW | |
| 1.25 | 7500 mg/kg | 14 | *S. drummondii* | ↑ 5 | ↑ 2.4 | ↓ shoot length, ↓ chlorophyll a | Ruley et al. 2006 |
| 2.5 | | 14 | | ↑ 13 | ↑ 2.8 | ↓ shoot length, ↓ chlorophyll a | |
| 5 | | 14 | | ↑ 4 | ↑ 2.1 | ↓ shoot length, ↓ chlorophyll a | |
| 10 | | 14 | | ↑ 3 | ↑ 1.7 | ↓ shoot length, ↓ chlorophyll a | |
| 62.2 | 50 mg/kg | 21 | *N. tabacum* | NM | ↑ 1.2 | ↓ Shoot dry weight | Evangelou et al. 2006 |
| 62.2 | 300 mg/kg | 21 | | | ↑ 1.2 | ↓ Shoot dry weight | |
| 62.2 | 600 mg/kg | 21 | | | ↑ 1.1 | ↓ Shoot dry weight | |

NM: Not mentioned, Trans: Translocation

### *3.3. Effect of LMWOAs on Pb-induced toxicity to plants*

First, LMWOA addition can differently influence plant development, depending on plant and LMWOA nature. A positive LMWOA (especially citric and oxalic acid) influence

on plant growth was reported by Duarte et al. (2007) for tobacco shoots. Luo et al. (2005) observed no significant effects of citric acid application on dry matter production of *Zea mays* and *Phaseolus vulgaris*. Evangelou et al. (2006) reported toxic effects of citric acid application on *Nicotiana tabacum* plants. Türkoğlu (2007) also reported significant decrease in mitotic index by citric acid in *Allium cepa* L. and Shahid et al. (2011a) reported that high citric acid concentration (> mM) induced genotoxicity to *Vicia faba*.

By chelating Pb in rhizosphere or apoplastic spaces, LMWOAs could change its phyto-toxicity and can prevent its entry into the symplast. In this way, Pb tolerant species maintain low shoot Pb level by restricting its uptake and translocation over a wide range of external concentrations. However, variation in the rate and composition of root exudates by different species is still under debate with respect to their role inside plant and in rhizosphere under Pb and other stress conditions. Chen et al (2003) concluded that Pb toxicity decrease to radish in presence of citric acid was due to change in Pb speciation in soil solution and inside the plant. Several authors concluded that as affinity of citric acid for Pb is low, only high concentrations of this chelate can modify phyto-toxicity (Ruley et al. 2004; Shahid et al. 2011a). LMWOAs effect on Pb-induced toxicity in some plant species is shown in Table 2: according to plant and LMWOAs types, contrasted effects were observed on Pb-induced phyto-toxicity.

**4. Humic substances (HSs)**

According to Evangelou et al. (2004 and 2006), natural organic molecules could be used as an alternative to potentially toxic and high-cost synthetic chelating agents. This is due to large specific surface area, high CEC, low cost and wide spread availability (Hamidpour et al. 2010). DOM consists of humic and non-humic substances; HSs consist of humic acids (HAs), fulvic acids (FAs) and humin. HA is the fraction of HSs that is not soluble in water under acidic conditions (pH < 2) but is soluble at higher pH, with average molecular weight of 2000 to 3000. FA is the fraction that is soluble at all pH values, with average molecular weight less than 1,000. These substances have similarities but vary greatly from one another (according to origin) with respect to molecular weight, molecular size, substructures and functionalities, depending on the nature and sources of organic matter, from age and functional groups. Functional groups include COOH, phenolic, enolic and alcoholic OH, quinone, hydroxyquinone and lactone. Chelation by neighboring carboxyl and phenolic groups give them an exceptional capacity for Pb complexation (Halim et al. 2003; Havelcová

et al. 2009; Botero et al. 2010; Soler-Rovira et al. 2010; Anirudhan and Suchithra, 2010; Kleber and Johnson, 2010; Güngör and Bekbölet 2010; Pédrot et al. 2010). Due to lower molecular weight, higher oxygen contents, number of functional group and exchange capacity, FAs are more reactive than HAs (Zeng et al. 2002; Wang et al. 2009). Figure-5 represents the general structural formula and functional groups present in fulvic and humic acids.

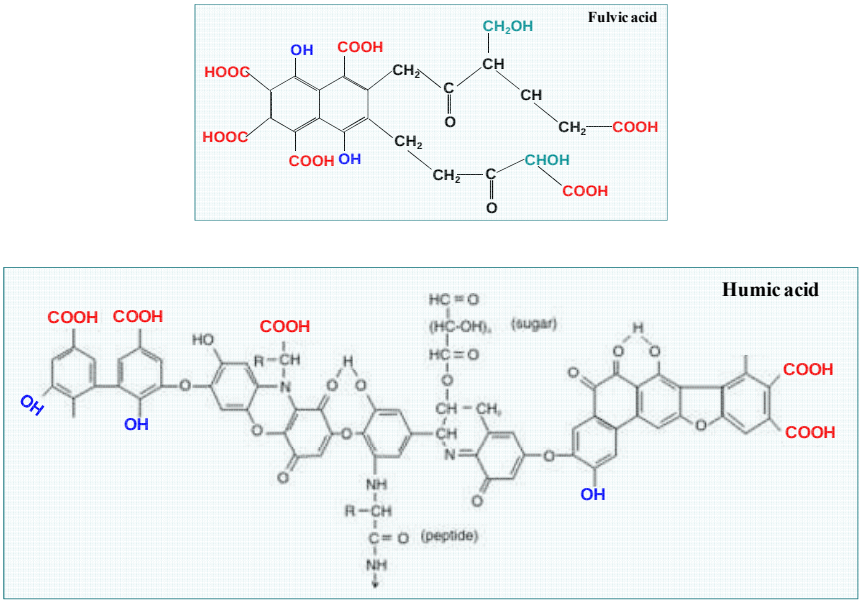


**Figure-5:** Model structural formula of fulvic and humic acid.

### *4.1. Effect of HSs on Pb speciation and behavior in soil*

Humic substances are involved in many soil processes such as solubilization / precipitation, sorption / desorption and reduction / oxidation. These processes affect soil chemistry by changing soil pH, CEC, redox status, buffering capacity, fixation/aging, chemical form of organic and inorganic ligands, mycorrhization and competing ion concentration, which in turn modify Pb mobility, bioavailability and toxicity (Feller et al. 2010; Heier et al. 2010; Janoš et al. 2010; Punamiya et al. 2010; Lawal et al. 2010; Vega et al. 2010; Arias et al. 2010; Bi et al. 2010; Cenkci et al. 2010; Sammut et al. 2010; Botero et al. 2010; Charriau et al. 2011). HSs react with Pb to form complexes, thus influence the concentrations of free $Pb^{2+}$ and labile Pb-complexes and their subsequent mobility and biotoxicity in soil and target organisms (Mager et al. 2008; Sánchez-Marín et al. 2010; Zeng et al. 2010; Heier et al. 2010).

Humic substances may exist in solid or aqueous phases (either dissolved or suspended form) depending on environmental conditions. In the dissolved form, HSs have the ability to form Pb-complexes. When present as an organic solid phase, HSs provide a surface for Pb adsorption from the aqueous system. However, inconsistency exists in the literature on the role of HSs towards Pb bioavailable fraction in soil (Zeng et al. 2010; Anirudhan and Suchithra, 2010): both a decrease in Pb bioavailable fraction (Guerrero et al. 2000; Slaveykova et al. 2003; Liu et al. 2009; Janoš et al. 2010) and an increased bioavailable fraction in the presence of HSs (Sánchez-Marín et al. 2007; McCauley et al. 2009; Zeng et al. 2010) were observed.

According to the free ion activity model and its recent extension biotic ligand model, DOM could decrease Pb bioavailable fraction in soil medium by binding $Pb^{2+}$ ions and thus reducing their available concentration (Worms et al. 2010; Zeng et al. 2010). Evangelou et al. (2004) observed that Pb bioavailable fraction and other metals increase in HAs amended soils due to metal-HS complexes formation, which are taken up by plants with more ease than free metal ions. Halim et al. (2003) reported that 2% HA addition reduced extractability of the soluble and exchangeable Pb forms. Farrell et al. (2010) observed that soil solution Pb concentration decreased in an acidic contaminated soil after the HSs (as compost) application. Botero et al. (2010) showed that HSs have high complexing affinity for Pb compare to essential nutrients.

On the other side, some reports indicate that HSs increase Pb bioavalable fraction in soil. Khan et al. (2002) reported increased extractable Pb with increasing HA concentrations. Laborda et al. (2008) also stated that HAs contribute to Pb mobilization. Dai et al. (2004) found that DTPA-extractable Pb content in contaminated soils correlates with organic matter contents in soils. However, the fraction of Pb solubilization by HSs varies considerably between soils. Clemente and Bernal (2006) reported that HSs addition to the acidic soil cause less solubilisation of Zn and Pb compare to calcareous soil. Zeng et al. (2010) described a positive correlation between HSs content and EDTA-extractable Pb content in a contaminated soil. Gharbi et al. (2010) observed increased mobility of organically-bound Pb in soil.

These contrasted results could be due in particular to various HSs nature and conformation changes in function of physico-chemical media conditions. HSs-induced Pb solubilization in soil also depends on chemical and structural characteristics of HSs (Kleber

and Johnson 2010). Key factors controlling metal solubilization efficiency of HSs includes reaction constant (Pinskii and Zolotareva, 2004), HSs co-application (Shi et al. 2008), different humification stages of HSs (Calace and Petronio, 2004) and molar mass or size of HSs (Cabaniss et al. 2000).

**Table-3.** Effect of humic substances on Pb uptake, translocation and toxicity to plants.

| HS | Pb | Duration | Crops | Uptake | Trans. | Effects | References |
|---|---|---|---|---|---|---|---|
| mg/kg | mg/kg | days | | | | | |
| 10 | 200 | 12 | *L. minor* | NM | ↑ 1.9 | ↑ Chlorophyll contents, growth rate | Kruatrachue et al. |
| 20 | 200 | 12 | | | ↑ 3.3 | ↑ Chlorophyll contents, growth rate | 2002 |
| 40 | 200 | 12 | | | ↑ 2.8 | ↑ Chlorophyll contents, growth rate | |
| 80 | 200 | 12 | | | ↑ 1.8 | ↑ Chlorophyll contents, growth rate | |
| 160 | 200 | 12 | | | ↓ 1.6 | ↑ Chlorophyll contents, growth rate | |
| 125 | 125 | 60 | Rape | ↓ 2.6 | ↓ 1.1 | NM | Shi et al., 2009 |
| 500 | 500 | 60 | | ↓ 1.7 | ↑ 1.1 | | |
| 1000 | 1000 | 60 | | ↓ slightly | ↓ 1.6 | | |
| 2000 | 2000 | 60 | | ↓ slightly | No effect | | |
| 140 | 150 | 28 | *T. aestivum* | ↑ 1.4 | ↑ 1.4 | ↑ Shoot and root dry weight | Khan et al., 2006 |
| 280 | 150 | 28 | | ↑ 1.7 | ↑ 1.7 | ↑ Shoot and root dry weight | |
| 560 | 150 | 28 | | ↑ 1.9 | ↑ 2.6 | ↑ Shoot and root dry weight | |
| 53000 | 122 | 55 | maize | NM | ↑ 1.6 | ↑ Dry weight | Salati et al., 2010 |
| 400000 | 6703 | 112 | *A. capillaris* | | ↑ 10 | | |
| 400000 | 6703 | 112 | | NM | ↑ 16 | ↑ Dry weight | Farrell et al., 2010 |

NM: Not mentioned, Trans: Translocation

### *4.2. Effect of HSs on Pb plant uptake and accumulation in different plant parts*

The role of HSs towards Pb plant uptake is very complex due to its heterogeneous structure and polymerization degree (Kleber and Johnson 2010). HSs form soluble Pb-HS complexes with free Pb ions (Antoniadis and Alloway, 2002; Laing et al. 2009; Zeng et al. 2010), which could be readily taken up by plant roots (Evangelou et al. 2004). Therefore, application of HSs can increase Pb plant uptake. Halim et al. (2003) stated that HAs soil amendment accelerates Pb phytoremediation. Bandiera et al. (2009) showed HA tendency towards enhanced translocation of Pb to the shoot. Salati et al. (2010) reported that Pb uptake was favored by HSs presence. Khan et al. (2002) also reported increased plant Pb concentrations with increasing concentrations of HA in solution. Higher Pb bio-uptake by the *Chlorella kesslerii*, *Chlorella vulgaris* and *Stephanodizcus hantzschii* has been reported in the

presence of the Suwannee River fulvic acid or riverine organic matter (Slaveykova et al. 2003). Ho et al. (2008) also confirmed higher accumulation of Pb in kenaf roots after the application of organic fertilizer using scanning transmission electron microscope X-ray microanalysis. Evangelou et al. (2004) reported HA addition increased metal concentration in shoots of *Nicotiana tabacum*. Bandiera et al. (2009) reported dose dependent increase in Pb concentration in the shoots of *Raphanus sativus* in response to HA application (0.1 and 1 g $kg^{-1}$ of soil). Table 3 presents Pb uptake by different plants species in the presence of HSs.

In contrast, Mager et al. (2009) reported strong protection against Pb accumulation by increased DOC in soil. Slaveykova et al. (2003) reported that Pb uptake by *Pimephales promelas* decreased in the presence of Suwannee River fulvic acid with respect to non complexed Pb. Kalis et al. (2006) stated that application of HA decreased Pb uptake by *Lolium Perenne* due to high DOC affinity. Kruatrachue et al. (2002) reported that application of HSs decreased Pb uptake by *Lemna minor* only at higher concentrations (> 100 mg/L). Addition of organic fertilizer is also reported to reduce the Pb mobility in soil and ultimately the uptake by plants (Pichtel and Bradway, 2008).

These contrasting effects of HSs on Pb uptake by plants depend on their efficiency to form Pb complexes. HSs are reported to form polymer in nutrient solution, which make them very complex and resistant to degradation by microorganisms and other physico-chemical factors of soil (Kleber and Johnson 2010). This polymerization phenomenon makes strong and large size complexes with Pb, which are not easily taken up by plants. However, the phenomenon of polymerisation varies with their concentration, in addition to physico-chemical factors of soil. Under conditions where this phenomenon doesn't occur (diluted conditions), HSs makes soluble and mobile complexes with Pb (Pb-HSs), which could enter plants more easily due to small size and thus increased Pb uptake (Evangelou et al. 2004; El-Ghamry, 2009).

### *4.3. Effect of HSs on Pb-induced phyto-toxicity*

Many studies provide evidence of HSs participation in the development of tolerance against Pb toxicity and several protection mechanisms were proposed. According to Babich et al. (2000) HSs could act as clay minerals, i.e., adsorption to the exchange complex and, therefore, limiting the availability of Pb for uptake by the living organisms. Guerrero et al. (2000) reported decreased Pb toxicity in the presence of HSs due to decreased uptake of free

metal ions. Khan et al. (2002) also stated increased plant biomass with increasing HAs concentrations in solution. Kruatrachue et al. (2002) reported that HSs application inhibited Pb-induced decreased total chlorophyll contents and growth rate of *Lemna minor*. Grosell et al. (2006) demonstrated protective of DOC as Aldrich humic acid. Mager et al. (2010) also reported that strong protection was afforded by DOC against acute Pb toxicity to *Pimephales promelas*, whereas milder protection was observed for *Ceriodaphnia dubia*. However, Bandiera et al. (2009) reported phytotoxic effects of higher levels of HA (10 $g.kg^{-1}$ of soil) to *Raphanus sativus*. Table 3 summarizes the effects of HSs on Pb-induced phytotoxicity, it can be noticed that HSs can variously modify both Pb transfer and phyto-toxicity. As previously observed for Pb fate in soil and phyto-availability, contrasted results were recorded and could be explained both by media parameters and HS characteristics.

## 5. Measurement of Pb speciation using speciation models

The above mentioned effects of different organic ligands revealed that the environmental impact of Pb varies with its speciation. Therefore, to know the potential risk of Pb to plants, animals and human beings, it is necessary to determine Pb speciation. In fact, the improvement of ecotoxicology tests need a better understanding of mechanisms involved for metals behavior in the polluted media and living organisms. In natural media, the direct measure of Pb speciation is often very complex and time consuming (Dumat et al. 2001). However, recent progress in the development of different speciation models, capable of predicting (often *in situ*) relative Pb species in defined medium, gave further impetus to the research in this area (Ge et al 2005; Gandois et al. 2010; Tipping et al. 2010; Wällstedt et al. 2010; Juang et al. 2010). Some of these speciation models includes:VISUAL MINTEQ, WHAM VI, PHREEQC, CHEAQS, ORCHESTRA, ECOSAT and CHESS. These models use the complexing ability of ligands to calculate the relative abundance of different metal species (Tipping et al. 2010; Gandois et al. 2010; Shahid et al. 2011a). The complexing ability of ligands is primarily related to amount and type of its functional groups. The choice of best suited speciation model depends, mainly, on the type of complexant ligand and purpose of measurement (Shahid et al. 2011a). Most of these models allow the user to modify or replace the data base by using database editor. Figure-6 indicates the input and output parameters of these organic ligands.

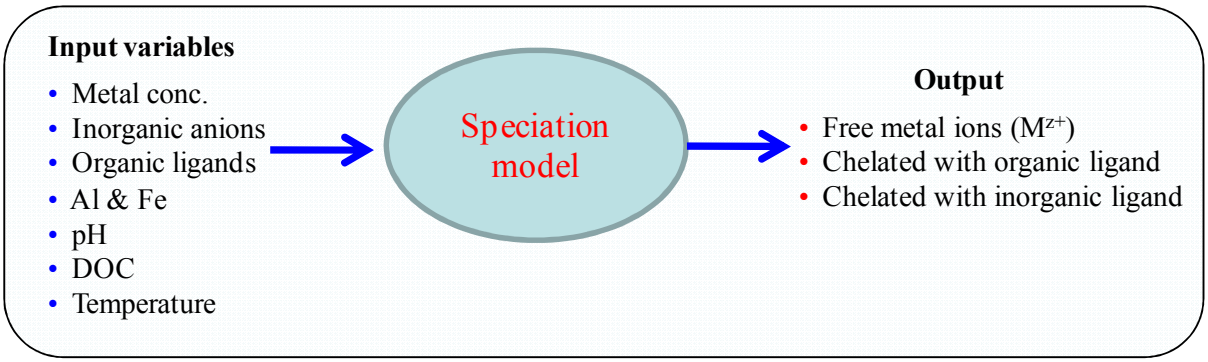


**Figure-6:** Input and output variables of a speciation model of a metal in the ecosystem.

## 6. Comparison of organic ligands influence on free cations quantities, using speciation models

In the present review, two different speciation models (Visual Minteq ver. 2.60 and Windermere Humic Aqueous Model VI**)** are used to calculate the Pb speciation in nutrient solution. These models vary with respect to their applications; Visual Minteq (Gustafsson, 2008) is the most widely used (cited in more than 400 research articles) and is best fitted to an environment with organic chelates like EDTA and LMWOAs, whereas WHAM (Tipping et al. 1998) takes into account the complexation of metals by HSs (HA and FA) and is therefore, best suited to organic matter rich environment.

Fig. 7 depicts the effect of pH on percent distribution of Pb in nutrient solution using WHAM VI and Visual Minteq ver. 2.60. This figure shows that $Pb^{2+}$ is the dominant Pb form (80-90 %) under acidic conditions, whereas Pb-OH (90-100 %) under alkaline condition in soil solution. Zhao et al. (2010) also reported similar pattern of Pb speciation while describing Pb adsorption of β-$MnO_2$. Yang et al. (2010) also reported a similar pattern of Pb speciation while describing Pb adsorption on Na-bentonite. Precipitates as Pb(OH) at alkaline pH. $Pb^{2+}$ is the main species at pH < 7, whereas $Pb(OH)^{+}$ and $Pb(OH)_2^{0}$ in the range of pH 7-10 (Weng, 2004; Wang et al. 2009; Zhao et al. 2010). Prediction of Pb speciation by two different speciation models was very much similar with respect to dominant form ($Pb^{2+}$ and Pb-OH) under acidic and alkaline conditions (Fig. 7). However, the binding of Pb by inorganic ligands varies. Indeed, WHAM VI does not take into account Pb binding with nitrate ions ($NO_3^{-}$) but over estimates its binding with sulphate ions ($SO_4^{2-}$). Therefore, the sum of Pb binding by nitrate and sulphate (14 %) between pH 2-7 by Visual Minteq was equal to sulphate alone (14 %) by WHAM VI at pH 2-7.

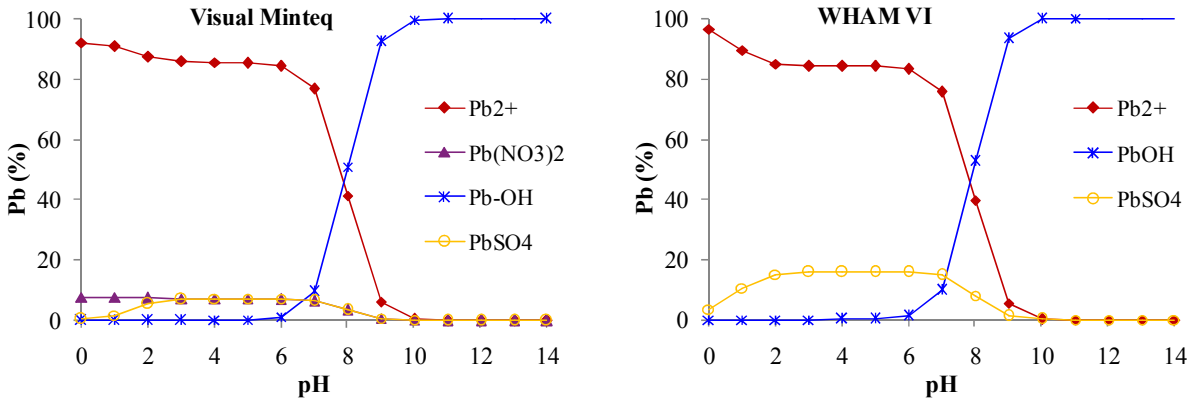


**Figure-7:** Variation in relative abundance of Pb in nutrient solution over pH. The speciation calculations are made by Visual Minteq and WHAM VI using elements concentration of Hoagland solution.

The soil solution pH is considered as the major parameter controlling metal speciation and in turn the uptake by plants (Kopittke et al. 2008; Lawal et al. 2010; Vega et al. 2010). In addition to chelation of metal, organic ligands also affect metal speciation by changing solution pH, particularly the LMWOAs. Therefore, it is necessary to predict the effect of organic ligands on solution pH. Figure-8 shows that the addition of organic ligands to Pb solution decrease the pH.

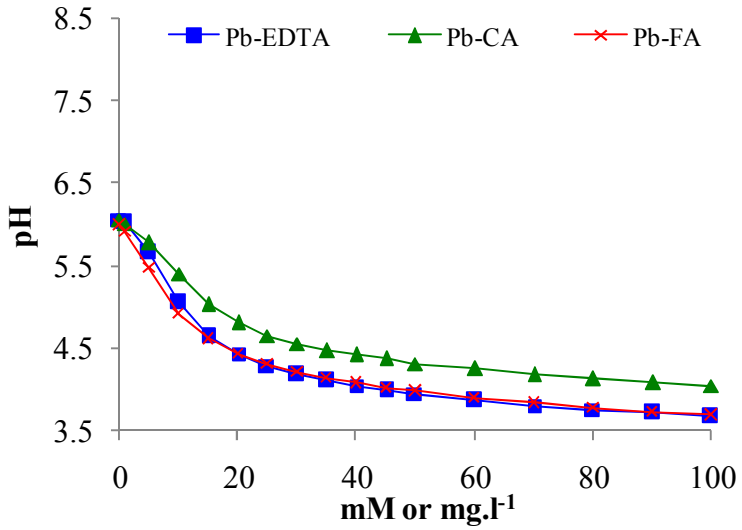


**Figure-8:** Comparison of the effect of organic ligands on solution pH using Visual Minteq ver. 2.60 (for EDTA and citric acid) and WHAM VI (for fulvic acid) in Hoagland solution. In the figure, the x-axis value is µM for EDTA and citric acid and mg.l$^{-1}$ for fulvic acid, which correspond to their common use in literature.

The comparison of the three organic ligands regarding their effect on solution pH shows that EDTA and fulvic acid have same effects on pH and decreased pH more strongly compare to citric acid. Therefore, in addition to their direct influence on metal solubility by chelation, these organic ligands increase Pb solubility indirectly by acidification of the solution. This is due to the high relative acidic strengths of EDTA and fulvic acid compare to citric acid.

Fig. 9 presents efficiency of organic ligands to complex Pb in nutrient solution at three different pH values (5, 7 and 9). The speciation models show the binding capacity in descending order of EDTA > FA > citric acid. Addition of only 5 µM EDTA can chelate more than 90 % of Pb in nutrient solution under acidic and neutral conditions. Chelation of Pb by EDTA was same at pH 5 and 7, which causes overlapping of line in Fig. 9A. However, under alkaline conditions, the Pb chelation capacity of EDTA decreases compare to acidic and neutral conditions. In the case of citric acid and FA, the quantities required for 90 % Pb chelation are many fold high compare to EDTA i.e. 1000 and 80 times, respectively for citric acid and FA (Fig. 9A and 9B). Like EDTA, CA also complexes more Pb at neutral and acidic pH compare to alkaline conditions. The decrease in Pb binding capacity of EDTA at alkaline pH is attributed to competing ions, particularly, Fe, Ca and Cu. In soils that have higher active $CaCO_3$ contents compared to the degree of Pb contamination, the efficiency of EDTA is decreased due to displacement of Pb from its EDTA complexes and the subsequent formation of Ca-EDTA and insoluble $Pb(CO_3)_2$ (Walker et al. 2003; Manouchehri et al. 2006). The reduction in contaminant removal efficiency of $Na_4$-EDTA was attributed to alkaline pH of the chelating agent solution, which decreased Pb dissolution (Saifullah et al. 2009). Therefore, in alkaline and neutral soils, EDTA-induced solubility and bioavailability of Pb to plants can be improved by lowering the pH by co-application of citric or acetic acid (Blaylock et al. 1997; Begonia et al. 2002, 2005; Saifullah et al. 2009), physiological acidic fertilizers (Puschenreiter et al. 2001), and elemental sulfur (Cui et al. 2004).

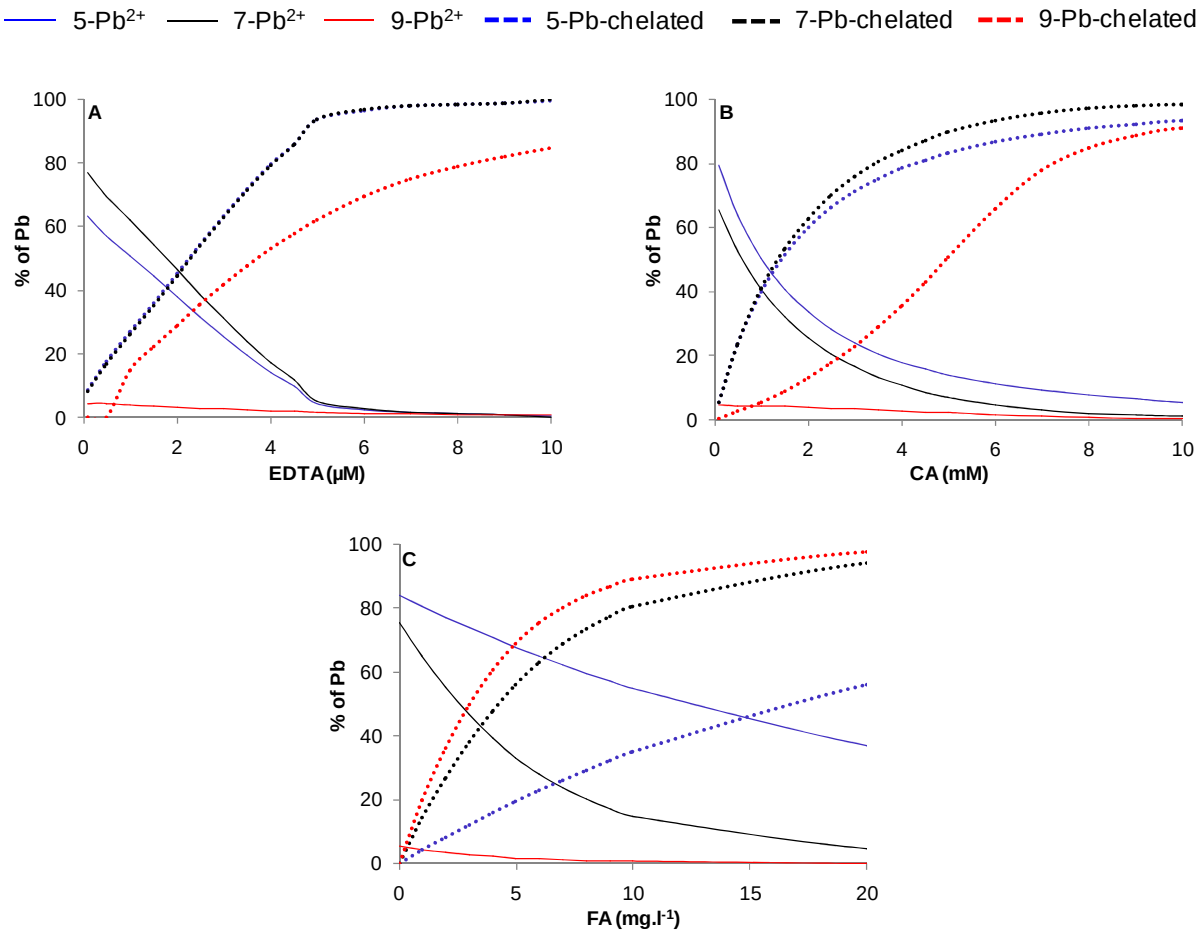


**Figure-9:** Comparison of organic ligands capacity to chelate/complex Pb in nutrient solution at varying pH (5, 7 and 9) in Hoagland solution using WHAM VI and Visual Minteq. The number 5, 7 and 9 in the figure legend represent the solution pH.

In the case of FA, Pb complexation is greater at alkaline pH than at acidic or neutral pH. Several previous studies also reported enhanced Pb complexation by HSs with increasing pH (Xu et al. 2006; Tan et al. 2008; Zhao et al. 2010; Sheng et al. 2010). HSs are macromolecules and contain a large number of binding sites but only a small fraction of the these binding sites are free to interact with $Pb^{2+}$ at low pH (Zhao et al. 2010). This is because most of these sites are positively charged at acidic pH due to the protonation reaction on the surfaces and electrostatic repulsion occurred between $Pb^{2+}$ ions and positively charged binding sites. Moreover, an excess of $H_3O^+$ at acidic pH competes with $Pb^{2+}$, resulting in $Pb^{2+}$ abundance in soil solution (Wang et al. 2010). At alkaline pH, the FA binding sites becomes negatively charged due to the deprotonation process and electrostatic repulsion decreases with increasing pH (Yang et al. 2010; Zhao et al. 2010; Heidmann et al. 2010). This

comparison further highlights the importance of solution pH regarding the application of organic ligands, especially in phytoremediation and risk assessment studies.

This comparison of organic ligands using speciation models also explained the variation of above mentioned behaviour towards Pb solubilisation, bio-uptake and toxicity. The increased solubilisation of Pb by EDTA is due to its higher binding capacity for Pb compare to citric acid and FA (Table 4). This efficiency also led to increased Pb uptake by EDTA due to formation of soluble Pb-EDTA complex. In the case of CA, the Pb-CA complex is very week with short half life and generally dissociates quickly in nutrient solution. Moreover, microbial activity is also reported in the degradation of Pb-CA complex. However, the role of FA towards Pb solubilisation and uptake by plants is very complex due to its heterogeneous structure and nature of polymerization.

## 7. Conclusions and perspectives

Pb behavior in the soil-plant system and its phytotoxicity is greatly influenced by chemical speciation. Organic ligands are capable to modify Pb speciation by forming organo-metallic complexes of varying stability, bioavailability and toxicity. Efficiency of organic ligands to modify Pb behavior and impact greatly depends on their metal binding capacity (Table 4). This binding capacity, in turn, depends on molecular structure, amount and type of functional groups of organic ligands.

**Table-4.** Binding constant of different organic ligands for Pb (obtained from default database of Visual Minteq version 2.60 and WHAM VI).

| **Complex** | **pK** |
|---|---|
| Pb-Citrate- | 5.7 |
| Pb-$(Citrate)_2^{-4}$ | 6.6 |
| $PbEDTA^{-2}$ | 19.7 |
| $PbHEDTA^{-}$ | 22.5 |
| Pb-HA ($pK_1$) | 4.1 |
| Pb-HA ($pK_2$) | 8.8 |
| Pb-FA ($pK_1$) | 3.2 |
| Pb-FA ($pK_2$) | 9.4 |
| $pK_1$ for COOH & $pK_2$ for OH group | |

Therefore contrasted influences were observed comparing the three different ligands. **EDTA** forms stable and bioavailable neutral Pb-EDTA chelates inducing: (1) enhanced Pb desorption/solubilisation in soils, (2) increased Pb translocation to shoots with disruption of Casparian strip (3) alleviation of Pb toxicity by binding toxic $Pb^{2+}$ cations. **LMOWAs** effect on Pb fate or impact is generally attributed to decrease in soil pH and increase in DOC or microbial activity. However, due to relatively low stability and soil microbial activity, Pb-LMWOAs complexes can rapidly dissociate before or after plant uptake and LMWOAs influence on Pb translocation to shoots tissues or phyto-toxicity is therefore relatively weak. Finally, the role of **HSs** towards Pb solubilisation, translocation and toxicity varies greatly due to their complex structure. In addition, physico-chemical properties of soil also influence the effect of organic ligands on Pb speciation. All these influencing parameters induce a wide range of changes in Pb fate and impact in soil-plant systems.

Based on the above mentioned link between Pb speciation and its behavior in ecosystems, it has been proposed that further researches on organic ligands influence should be carried out to optimize the best chelators under applied conditions such as physico-chemical properties of soil, plant type and the extent of Pb pollution and purpose of application. Indeed, complex structure of HSs induces strong difficulties to predict their influence on Pb behavior and use them for industrial applications. Pb leaching to groundwater, high stability in soil and toxicity to soil microbial community are the major concerns against practical EDTA utilization at field scale. Natural LMOWAs role towards Pb translocation or toxicity is not clear and largely depends on plant type; in consequence supplementary studies are needed in order to better understand and manage environmental Pb impact.

Further, different toxicity assessment tests could therefore be carried out under different Pb speciation conditions to optimize the most appropriate and best suited ecotoxicological test under applied conditions. These findings could be further used in genetic engineering studies to improve the techniques for desired results. Similarly some additional organic ligands or polymers could be discovered, which are environmental friendly and are capable to efficiently solubilize and transfer Pb. Most of the studies using organic ligands deal with hyper-accumulator plants, which in most cases have natural Pb and/or Pb-complex detoxification mechanisms. The toxic effects of Pb and/or its complexes and the role of plant detoxification mechanism can be better assessed using a metal-sensitive plant like *Vicia faba* or *Allium cepa*. Therefore, it's necessary to conduct some studies concerning the

role of organic ligands towards Pb toxicity and detoxification mechanisms in plant metabolisms using Pb sensitive plants to evaluate their role in risk assessment studies in relation with uptake and speciation. Finally, the role of LMWOAs and HSs has been mainly studied with respect to their effects on rhizosphere chemistry or as absorbent phase in remediation studies. There is very little data available regarding their effects on Pb phyto-toxicity. Therefore, it's necessary to study the toxic nature of different Pb-organic acids complexes and their role in Pb detoxification mechanisms such as antioxidant enzymes activities, phytochelatins and reactive oxygen species induction.

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# Chapter 2:

# Materials & Methods

1. Context

In order to respond to different above mentioned scientific objectives of the PhD, a series of experiments were performed in phytotron under controlled conditions at INP-ENSAT-Toulouse. This chapter presents all the techniques and protocols used in this study. These protocols are also presented in the respective chapter/article but this chapter deals mainly with the principles and mechanisms of protocols, which are not explained in detail in these sections/articles.

In this study, *Vicia faba* plants were used as toxicity biomarker to evaluate the effects of three organic ligands (EDTA, citric acid and fulvic acid) on Pb toxicity. Initially, the speciation modeling of Pb in nutrient solution was carried out using two speciation models (WHAM VI & Visual Minteq). After this the effect of Pb speciation on its uptake and toxicity to *Vicia faba* was assessed by comparing several stress biomarkers of differing sensitivities (Chapter 3, Section A) in a series of continuous and short-term experiments (Figure-1). After that, micronucleus *Vicia faba* test (recommended by AFNOR) was used to established a link between Pb speciation and Pb-induced genotoxicity in the presence of organic ligands (Figure-1) (Chapter 3, Section B). Finally, the link developed was applied to natural complexing substances (humic substances) using stress biomarkers of oxidative stress (Chapter 3, Section C).

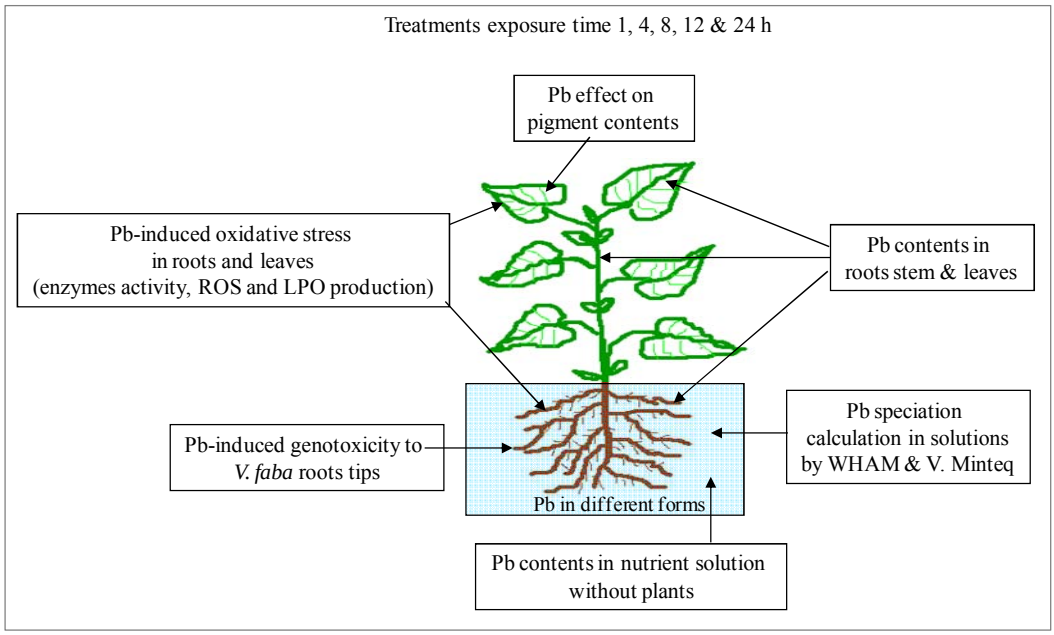


**Figure 2:** Different parameters evaluated in this study.

The parameters determined in these experiments include:

- Pb accumulation by roots, stem and leaves using ICP-OES
- Genotoxicity using *V. faba* micronucleus test
- Proteins content determination
- Measurement of antioxidant enzymes activities (SOD, CAT, GPOX, APX and GR)
- Lipid peroxidation (LPO) evaluation
- Reactive oxygen species (like $H_2O_2$) quantification
- Pigments contents (chlorophyll-a, chlorophyll-b, chlorophyll-a+b and carotinoids) evaluation.

## 2. Choice of the plant (Biological material)

The broad bean (botanical name, *Vicia faba;* cultivar, Primabel; type, aguadulce; family, Fabaceae) plants were chosen for present study. They are dicotyledonous higher plants with average length of 25 cm containing 7 to 8 seeds (Figure 2). With their seeds rich in protein and energy and their ability to grow in various climatic zones, *Vicia faba* production has a long history of numerous and valuable uses in feed and food (Créponen et al. 2010). The *Vicia faba* contributes to the sustainability of cropping systems *via*: (I) its ability to contribute nitrogen to the system *via* biological $N_2$ fixation, (II) diversification of systems Pbing to decreased disease, pest and weed build-up and potentially increased biodiversity, (III) reduced fossil energy consumption in plant production, and (IV) providing food and feed rich in protein (Jensen et al. 2010; Köpke and Nemecek, 2010).

**Figure 3:** *Vicia faba* seedlings at different stages of growth

Nevertheless, the plant is also commonly used in laboratories as a model plant for evaluating the toxicity of various micro pollutants such as heavy metals (Piechalak, 2002; El Hajjouji et al. 2007; Cecchi et al. 2008; Pourrut et al. 2008; Marcato-Romain et al. 2009a; Marcato-Romain et al. 2009b; Probst et al. 2009). The *Vicia faba* micronucleus test is recommended by the AFNOR NF T 90-327 for the assessment of genotoxicity. The increased use of this plant in literature as biomarker of toxicity is due to its fast growth, important biomass and high sensitivity to metals (Thesis Pourrut 2008; Probst et al. 2009). This plant responds very quickly to the toxicity induced by pollutant. Moreover, the cells and chromosomes are large, so one can easily identify the damage caused by pollutants. After staining cells, the structural defects are easily detected under a microscope (Chenon, 2001).

### 3. Germination and transplanting

Dry seeds of broad beans (*V. faba* L. stored in darkness at 4 C°) were soaked in tap water to break dormancy and to synchronize germination. After decoating, the seeds were surface-sterilized with 1 % calcium hypochlorite [$Ca(ClO)_2$] for 10 minutes to eliminate any fungal contamination followed by rinsing thoroughly with distilled water. The seeds were then germinated on moistened filter paper in a germination chamber under optimal conditions of germination, i.e. in darkness at 22 °C temperature and 100 % of moisture. After 5-7 days in germination chamber, when the primary roots were about 2–3 cm in length, the healthy and uniform seedlings were transplanted to PVC tanks (Figure 3) containing modified Hoagland solution (Table 1, Uzu et al. 2009; Arshad et al. 2008; Shahid et al. 2011).

**Table-1.** Composition of nutrient solution.

| Element | Concentrations | Elements | Concentrations |
|---|---|---|---|
| $KNO_3$ | 5 mM | $ZnSO_4.7H_2O$ | 1.53 µM |
| $Ca(NO_3)_2$, $4H_2O$ | 5 mM | $CuSO_4,5H_2O$ | 0.235 µM |
| $KH_2PO_4$ | 2 mM | $H_3BO_3$ | 24.05 µM |
| $MgSO_4$, $7H_2O$ | 1.5 mM | $Na_2MoO_4,2H_2O$ | 0.1 µM |
| $MnSO_4.H_2O$ | 9.11 µM | Fe/EDTA | 268.6 µM |

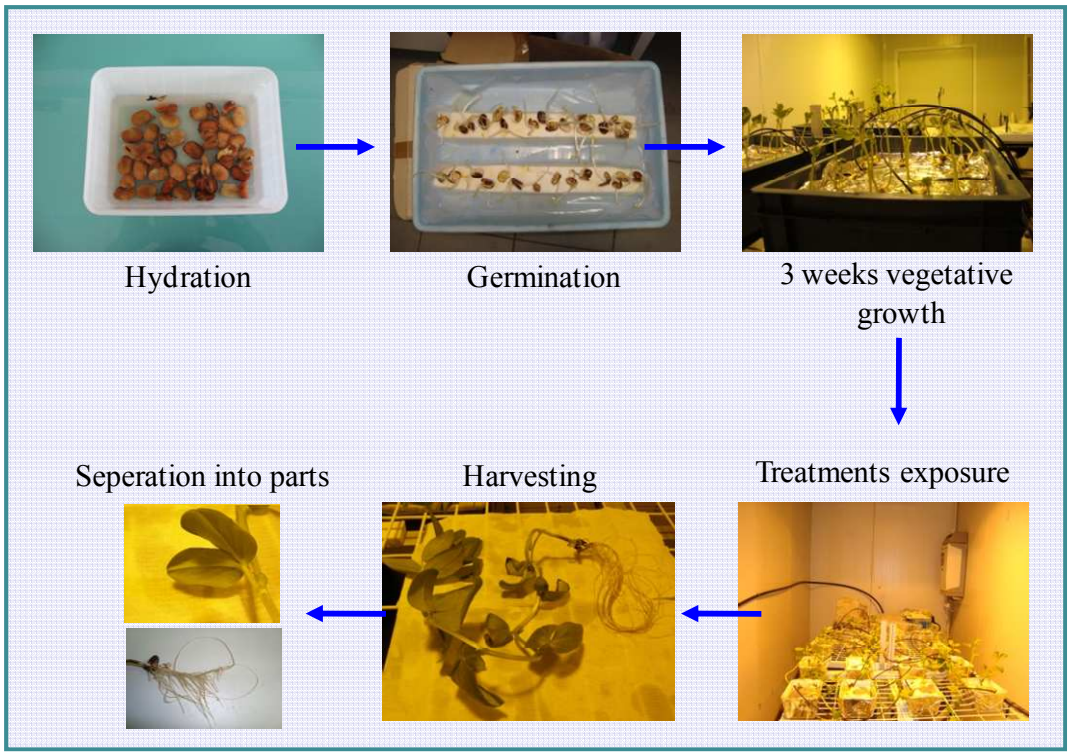


**Figure 4:** Steps of plant vegetative growth, treatment exposure and harvest.

## 4. Experimental conditions

Plants were grown under controlled conditions in a phytotron with 16 h photoperiod at 70 % relative humidity and day/night temperatures of 24/22 °C (Figure 4). Light was supplied by 600 W Osram Nav-T Super High Pressure Sodium Lamp providing a minimum photosynthetical photon flux density of 500 µmol.$m^{-2}$.$s^{-1}$ at the top of the plant. Nutrient solution was renewed on alternate days to keep the nutrient composition and pH constant.

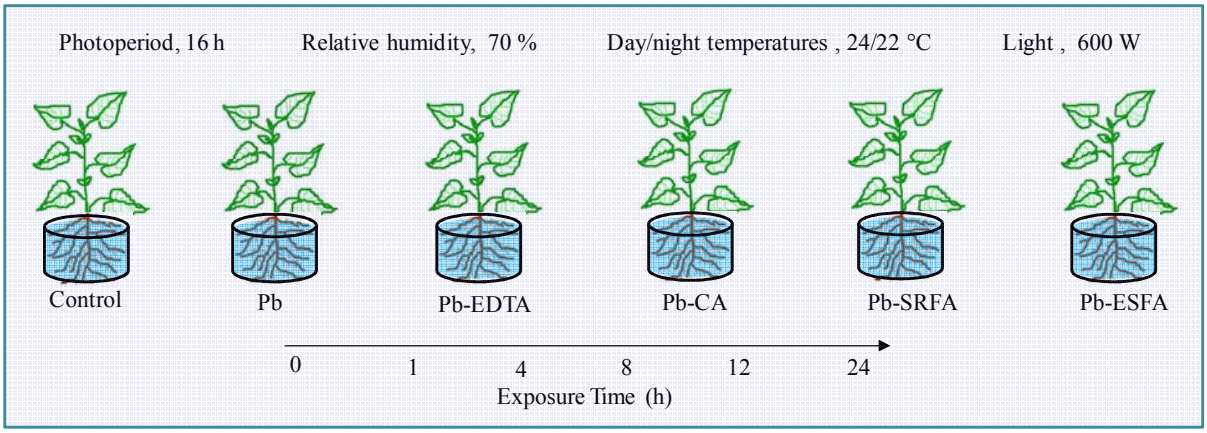


**Figure 5:** Experimental conditions (phytotron) and treatments used in this study.

## 5. Treatments exposure and sampling

After pre-culturing of 15 days (5th-6th foliar stage), plants were exposed to different treatments (Table 2) for 6 h in *V. faba* genotoxicity test and for 1, 4, 8, 12 and 24 h for all the other toxicity tests (Figure 4). The short exposure treatments times were chosen due to short-lived production of ROS before being scavenged rapidly by antioxidants. In all the experiment, each treatment was replicated 6 times. After respective time period of treatment exposure, the *Vicia faba* seedlings were harvested, stored and analysed according to the parameter to be analysed.

## 6. Calculation of Pb speciation in nutrient solution

First of all we calculated the effect of organic ligands on Pb speciation in soil solution. For this purpose, two speciation models, i.e. Visual Minteq ver. 2.60 (Gustafsson, 2008) and Windermere Humic Aqueous Model VI (Tipping, 1998) were used to calculate Pb speciation in soil solution in the presence of organic ligands (EDTA, citric acid and fulvic acids). These organic ligands were chosen based on their most frequent use in literature and variation in their structure, functional group and metal complexing ability. T

Many attempts to test the viability of speciation models to calculate metal speciation have already been conducted (Gandois et al. 2010; Stockdale et al. 2010; Feyte et al. 2010; Tipping et al. 2010; Wällstedt et al. 2010; Semião et al. 2010; Kaludjerovic-Radoicic and Raicevic, 2010; Shahid et al. 2011). These studies reported successful prediction of complexed and free metal ions concentrations by speciation models when compared with experimental measurements (Meylan et al. 2004; Parat et al. 2009). Christensen et al. (1999) reported that MINTEQ2 with default database, whereas WHAM with adjusted database offers a very useful prediction of Pb complexation by DOC. Guthrie et al. (2005) reported that Windermere Humic Aqueous Model (WHAM) can predict labile and free metal ion concentrations of Ni, Zn, and Cd. However, most of these studies employed the speciation model to predict metal speciation or compare it with calculated values (Qu et al. 2008; Parat et al. 2009; Gandois et al. 2010; Stockdale et al. 2010; Tipping et al. 2010). Recently, Shahid et al. (2011) showed that speciation models can also be employed with success to toxicological studies to make a link between metal speciation, transfer and toxicity in risk assessment studies.

In the present study, Pb speciation in the presence of EDTA and citric acid is calculated using equilibrium constants from the Visual Minteq database (Table 6, Chapter 1B) while for HS by WHAM VI database (Table 5, Chapter 1B). The concentration of the elements present in modified Hoagland solution (Table 1) along with applied levels of Pb, EDTA, citric acid or HS were used as input for both the models. These calculations were used for fitting the experimental design (Table 2).

### *6.1. Windermere Humic Aqueous Model VI (WHAM VI)*

Windermere Humic Aqueous Model VI (WHAM VI, Tipping et al. 1998, Centre for Ecology & Hydrology, Lancaster Environment Centre, UK) is designed to calculate equilibrium speciation of metals in complex systems such as surface and ground waters, sediments, and soils. It is reported to be very useful for systems in which HS, such as FA and HA, are the main complexants that control the speciation of metals. This is a composite model, which combines three sub models for ion binding chemistry. These models include; (1) Humic Ion Binding Models VI (Tipping et al. 1998), which is comprised of binding parameters of humic substances for 21 metals, (1) Surface Chemistry Assemblage Model for Particles (SCAMP, Lofts & Tipping, 1998) with binding constants of 20 metals to four types of surface (iron, manganese and aluminium oxides, and silica), (III) cation exchange model for clay particle. Table-5 shows the binding constants of humic substances and different inorganic ligands for Pb used in WHAM VI. In WHAM VI, HS are assumed to be rigid spheres of uniform size with ion binding groups on the surface. The proton binding groups on the humic substances are heterogeneous, having a range of intrinsic pKs values. Two types of acid groups, $pK_1$ and $pK_2$, which are represented by COOH and phenolic OH groups, respectively, are considered. Binding can take place at both monodentate (single), bidentate (consisting of two proton-dissociating groups) tridentate (consisting of three proton-dissociating groups) sites.

The chemical speciation problems, comprising a set of concentrations of substances in a system, are stored in input files of WHAM software. Equilibrium constants and related parameters are stored in a set of databases. The core WHAM program is used to write input files and to view output data. Input data include pH, concentrations of major cations (Ca, Na, K, Mg), concentrations of major acid anions (Cl, $SO_4$ and $NO_3$) and trace elements (Pb). The DOC was considered, as in previous studies (Tipping et al. 2003; Pampura et al. 2007) to be constituted by 65 % of “active” fulvic acids with respect to cation binding. The model has

been already used in different studies (Gandois et al. 2010; Stockdale et al. 2010; Feyte et al. 2010; Tipping et al. 2010).

**Table-2.** Binding constants and chemical reactions of fulvic acid used to measure the Pb speciation by WHAM VI.

| Reactions | Log K |
|---|---|
| $Pb^{2+} + OH = PbOH^{+}$ | 6.29 |
| $Pb^{2+} + 2OH = Pb(OH)_2$ | 10.88 |
| $Pb^{2+} + 3OH = Pb(OH)_3^{-}$ | 13.94 |
| $Pb^{2+} + Cl^{-} = PbCl^{+}$ | 1.59 |
| $Pb^{2+} + 2Cl^{-} = PbCl_2$ | 1.8 |
| $Pb^{2+} + SO_4^{2-} = PbSO_4$ | 2.75 |
| $Pb^{2+} + CO_3^{2-} = PbCO_3$ | 7.2 |
| $Pb^{2+} + 2CO_3^{2-} = Pb(CO_3)_2^{2-}$ | 10.5 |
| $Pb^{2+} + FA\ (COOH) = Pb\text{-}FA$ | 9.4 |
| $Pb^{2+} + FA\ (OH) = Pb\text{-}FA$ | 3.2 |
| $Pb^{2+} + HA\ (COOH) = Pb\text{-}HA$ | 8.8 |
| $Pb^{2+} + HA\ (OH) = Pb\text{-}HA$ | 4.1 |

### *6.2. Visual Minteq*

The geochemical equilibrium model Visual Minteq was compiled by Jon Petter Gustafsson, Division of Land and Water Resources, Stockholm, Sweden. The model is employed to estimate equilibrium of aqueous speciation, precipitation and dissolution of minerals, complexation, adsorption, solid phase saturation states, etc. This is a windows version of MINTEQA2 version 4.0 (Allison et al. 1991), which was released by the USEPA in 1999. The model uses an extensive thermodynamic database to solve the chemical equilibrium problems on the basis of parameters defined in an input file. These parameters include pH, ionic strength, initial concentration of ions, initial amounts of solids, etc. of the system to be simulated. Table-6 indicates the binding constants of citric acid, EDTA and inorganic ligands for Pb used in visual Minteq. Using this model, the overall objective is generally to calculate distribution coefficient, Kd (liters of solution/kg of solid), that

describes the amount of the contaminant on the solid phase versus the amount in the mobile solution phase. These measured Kd values are used to report output data. Visual MINTEQ has been used extensively in literature to simulate chemical speciation natural water and soil solution (Wällstedt et al. 2010; Juang et al. 2010; Semião et al. 2010; Kaludjerovic-Radoicic and Raicevic, 2010; Shahid et al. 2011).

**Table-3.** Binding constants and chemical reactions of EDTA and citric acid used to measure the Pb speciation by Visual Minteq.

| Reactions | Log K |
|---|---|
| $Pb^{2+} + H_2O = PbOH^+ + H^+$ | -7.6 |
| $Pb^{2+} + 2H_2O = Pb(OH)_2 + 2H^+$ | -17.09 |
| $Pb^{2+} + 3H_2O = Pb(OH)_3^- + 3H^+$ | -28.09 |
| $Pb^{2+} + Cl^- = PbCl^+$ | 1.55 |
| $Pb^{2+} + NO_3^- = PbNO_3^+$ | 1.17 |
| $Pb^{2+} + SO_4^{2-} = PbSO_4$ | 2.69 |
| $Pb^{2+} + 2SO_4^{2-} = Pb(SO_4)_2^{2-}$ | 3.47 |
| $Pb^{2+} + PO_4^{3-} + 2H^{+1} = PbH2PO_4^+$ | 21.07 |
| $Pb^{2+} + Citrate^{-3} = Pb\text{-}Citrate\text{-}$ | 5.67 |
| $Pb^{+2} + Citrate^{-3} + H^{+1} = PbH\text{-}Citrate$ | 10.29 |
| $Pb^{2+} + EDTA^{4-} = PbEDTA^{2-}$ | 19.71 |

### *6.3. Effect of pH on Pb speciation in nutrient solution*

Soil solution pH is a master variable controlling Pb speciation (Kopittke et al. 2008; Vega et al. 2010). Therefore, first of all, the effect of pH variation on Pb speciation (% distribution of Pb in different chemical forms) was calculated using above mentioned models in the presence and absence of organic ligands in Hoagland nutrient solution (Figure 5). In the absence of organic ligands, Pb exists mainly in free ionic form under acidic conditions while in hydroxide form at alkaline pH. The presence of EDTA mainly chelated Pb between pH 3 to 10, FA between pH 5 to 10 and citric acid between 5 and 7 (Figure 5). These results suggested that Pb speciation varies with pH, which can in turn affect its uptake and toxicity. Therefore, in order to eliminate/minimize the effect of pH variation on Pb uptake and

toxicity, the nutrient solution pH was kept constant (pH 5). This is because that our objective was to evaluate the effect of organic ligands (speciation) on Pb uptake and toxicity keeping the other parameters constant.

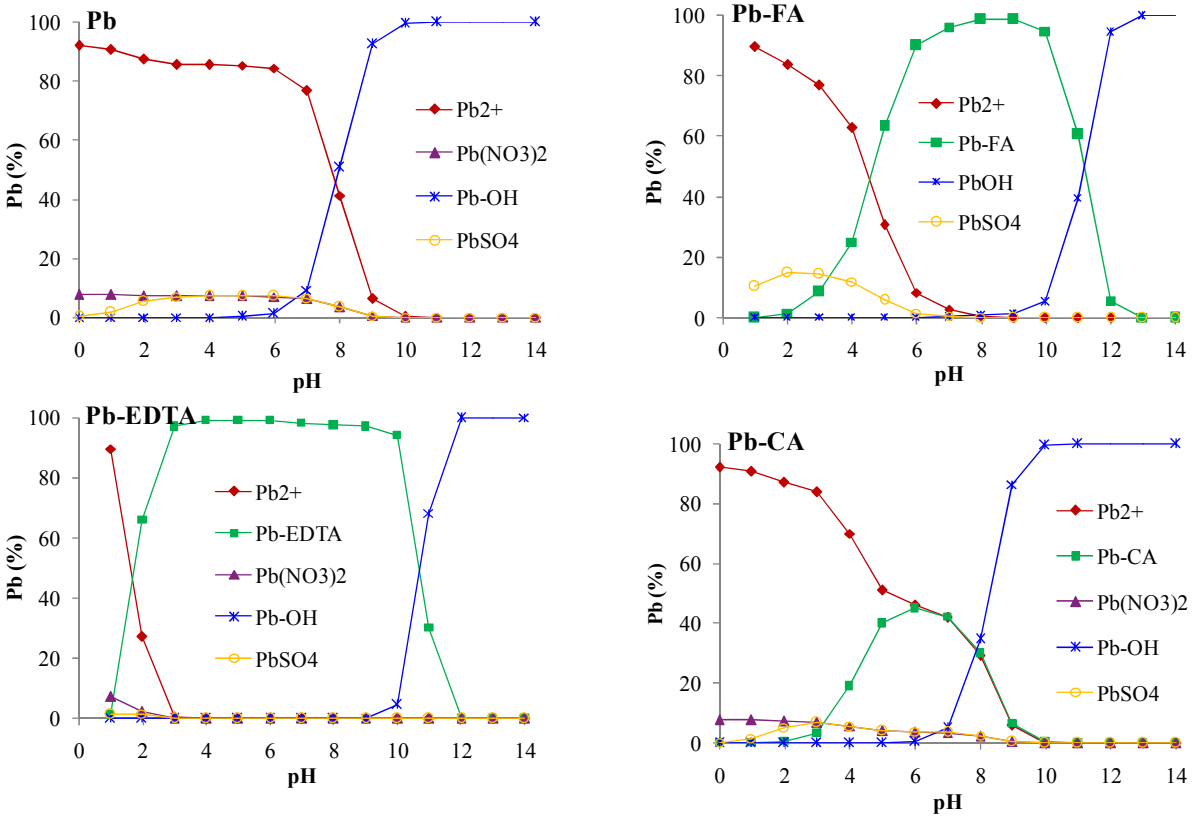


**Figure 6:** Effect of change of nutrient solution pH on Pb speciation in the presence and absence of organic ligands. Pb speciation is calculated at pH 5 with ionic strength 0.1 M in modified nutrient solution (concentration Table 1).

### *6.4. Calculation of Pb speciation in the presence and absence of organic ligands*

After evaluating and optimizing the effect of solution pH on Pb speciation, we calculated the Pb binding efficiency of organic ligands at pH 5. For this purpose, the % of Pb chelated was calculated in the presence of increasing concentrations of organic ligands (Figure 6). The applied concentration ranges of organic ligands used in Figure 6 were obtained from literature. These speciation calculations were used to design the experimental lay out.

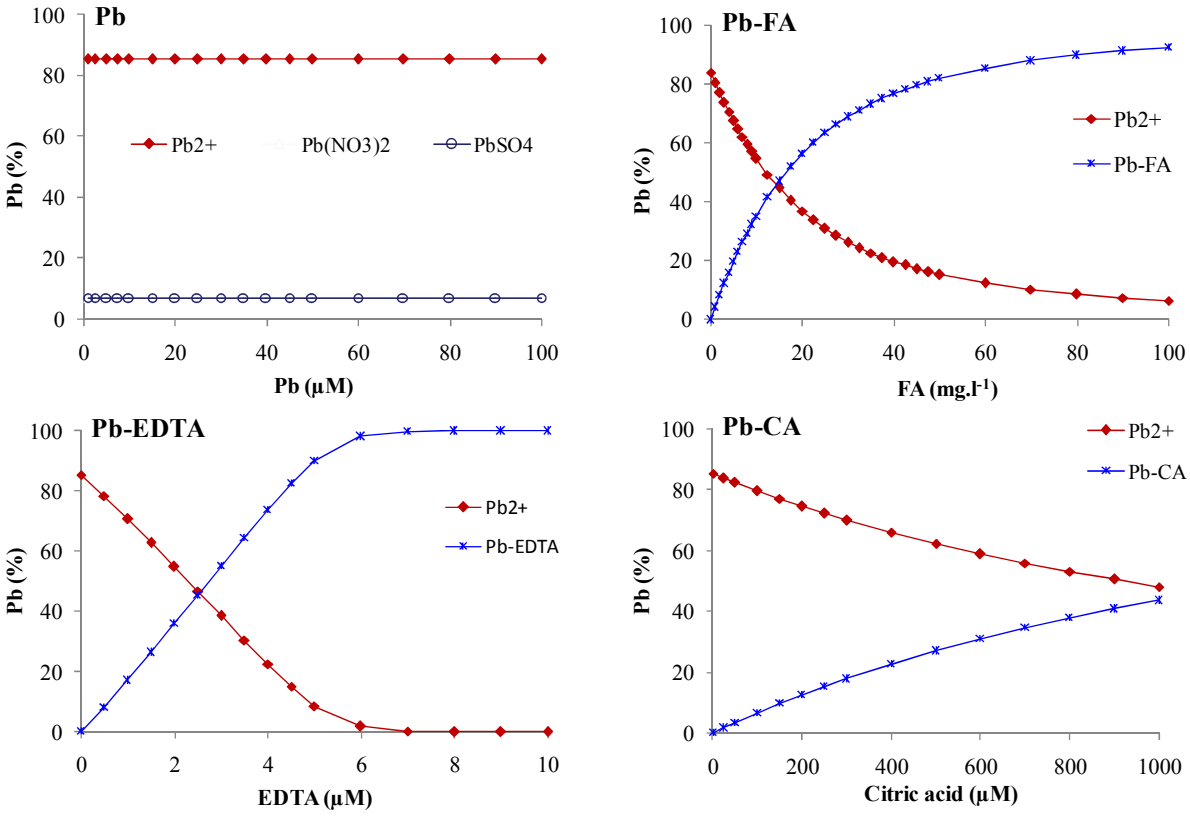


**Figure 7:** The Pb speciation in nutrient solution at pH 5 in the presence and absence of different levels of organic ligands calculated by Visual Minteq and WHAM.

### *6.5. Experimental treatments used*

Using the above mentioned Pb speciation calculations, different Pb treatments were designed in the presence and absence of organic ligands (Table-2). In the first study (Section 3A), Pb was chelated by EDTA and citric acid at two levels; these two levels of Pb chelation by EDTA were Pb-EDTA-40 (represented as Pb-EDTA-a) and Pb-EDTA-99 (Pb-EDTA-b), whereas for citric acid Pb-CA-25 (Pb-CA-a) and Pb-CA-40 (Pb-CA-b). In treatments notation, the numbers 25, 40, 75 and 99 represent % of Pb chelated by organic ligands. In the second study (Section 3B), Pb was chelated at four levels by EDTA (25, 40, 75 and 99 %), whereas at three levels by citric acid (15, 25 and 40 %) as shown in Table 2. In these studies, the toxic effect of higher concentrations of citric acid alone (>1000 µM) restricted the Pb chelation up to 40 % and limited its comparison with Pb-EDTA-75 and Pb-EDTA-99. In the last study (Section 3C), Pb was chelated at two levels by two different fulvic acids. This chelation was 12 and 36 % by ESFA, whereas 16 and 44 % by SRFA. These applied levels of organic ligands with respect to Pb speciation allowed us to compare Pb toxicity against free and chelated Pb % in solution.

**Table-4.** Composition of treatments and experimental design used in different experiments. The Pb speciation was calculated using Visual Minteq and WHAM.

| Treatments | Visual Minteq input values | Visual Minteq calculations | |
|---|---|---|---|
| **Notations** | **Treatments medium composition** | **Pb-chelated (%)** | **Pb-free (%)** |
| Pb | HS[a] + $Pb(NO_3)_2$ (5 µM) | 0 | 85 |
| Pb-EDTA-25 | HS + $Pb(NO_3)_2$ + EDTA (1.45 µM) | 25 | 64 |
| Pb-EDTA-40 | HS + $Pb(NO_3)_2$ + EDTA (2.25 µM) | 40 | 51 |
| Pb-EDTA-75 | HS + $Pb(NO_3)_2$ + EDTA (4.25 µM) | 75 | 21 |
| Pb-EDTA-99 | HS + $Pb(NO_3)_2$ + EDTA (10 µM) | 99 | 1 |
| Pb-CA-15 | HS + $Pb(NO_3)_2$ + citric acid (300 µM) | 15 | 73 |
| Pb-CA-25 | HS + $Pb(NO_3)_2$ + citric acid (550 µM) | 25 | 64 |
| Pb-CA-40 | HS + $Pb(NO_3)_2$ + citric acid (1000 µM) | 40 | 51 |
| | **Controls without Pb** | | |
| NC[b] | Hoagland solution (HS) | – | – |
| PC[c] | HS + Maleic hydrazeide (40 µM) | – | – |
| EDTA-75 | HS + EDTA (4.25 µM) | – | – |
| EDTA-99 | HS + EDTA (10 µM) | – | – |
| CA-25 | HS + citric acid (550 µM) | – | – |
| CA-40 | HS + citric acid (1000 µM) | – | – |

[a]Hoagland solution (concentrations in introduction), [b]Negative control, [c]Positive control.

| | WHAM VI input values | WHAM VI calculations | |
|---|---|---|---|
| **Treatments** | **Composition** | **Pb-chelated (%)** | **Pb-free (%)** |
| | **Controls** | | |
| Control | Hoagland solution (HS) | – | – |
| ESFA-25 | HS + 25 $mg.l^{-1}$ ESFA | – | – |
| SRFA-25 | HS + 25 $mg.l^{-1}$ SRFA | – | – |
| | **Pb treatmetns** | | |
| Pb | HS + 5 µM Pb | 0 | 84 |
| Pb-ESFA-5 | HS + 5 µM Pb + 5 $mg.l^{-1}$ ESFA | 12 | 74 |
| Pb-ESFA-25 | HS + 5 µM Pb + 25 $mg.l^{-1}$ ESFA | 36 | 57 |
| Pb-SRFA-5 | HS + 5 µM Pb + 5 $mg.l^{-1}$ SRFA | 16 | 70 |
| Pb-SRFA-25 | HS + 5 µM Pb + 25 $mg.l^{-1}$ SRFA | 44 | 47 |

## 7. Pb content analysis in nutrient solution

Before the start of experiments, Pb content analysis was carried out in the nutrient solution after 24 h without exposure to *V. faba* seedlings for all the treatments. The objective

of this analysis was to determine the possible precipitation of Pb. For this purpose, a 50 µM Pb solution was prepared from $Pb(NO_3)_2$ using mQ water (millipore). This concentrated solution was then filtered (0.22µm), acidified to pH 5.0 with diluted $HNO_3$ (15 M, suprapur 99.9 %) and placed under environmental conditions in phytotron. Pb contents analysis was carried out by inductively coupled plasma–atomic emission spectrometry (ICP-AES) with an IRIS Intrepid II XDL. The measured Pb concentration was 50.02 µM Pb. That concentrated Pb solution was then diluted ten times with mQ water or organic ligands (EDTA or CA) in order to obtain the various treatments solutions. The pH was adjusted to 5.0 with diluted $HNO_3$ (15 M, suprapur 99.9 %) and were kept under experimental conditions for 24 h. Pb concentration was checked on five replicates for each treatment. Without contact with plants, both pH and total Pb concentration measured for the various exposure solutions, in function of time, stayed constant after 24 hours. Virginia Tobacco leaves (CTA-VTL-2, polish certified reference material; ICHTJ) were used as a reference material for verifying the accuracy of the analytical procedure. Certified values for Pb in tobacco leaves were given for 22.1 ± 1.2 mg $Pb.kg^{-1}$ 146 dry weights. Measured values for the three replicates were 22.0 ± 0.9, 22.4 ± 0.8 and 21.9 ± 0.8 mg $Pb.kg^{-1}$ dry weight.

After treatment exposure, *V. faba* seedlings were harvested into root, stem and leaves. Roots were rapidly washed in distilled water and the Pb bound to the rhizoderm was removed by HCl according to Ferrand et al. (2006) followed by rinsing with distilled water. Plant samples were dried to a constant weight in an oven and were ground to fine powder (40 mesh) with mechanical grinder. The samples were then subjected to acid mineralization by wet process, which solubilise the metal elements after complete digestion of the organic matter by a mixture of nitric acid and hydrogen peroxide using DigiPREP Jr. (SCP Sciences) as described by Pourrut et al. (2008) (Figure 7).

This DigiPrep is a hot plat equipped with a temperature sensor, thus, making it possible to work at desired temperature. For mineralization, nitric acid was added to each sample and left to react and cool overnight. The next day the samples were heated at 80 ° C for 1 hour (with offset time of 30 min; the time necessary to reach the desired temperature). After a cooling time of approximately 1 hour (return to room temperature), $H_2O_2$ was added. The samples were then heated first at 55 °C for 25 min (15 min offset time) and after degassing, re-heated at 80 °C for 180 min (15 min offset time). Before analysing Pb contents by ICP-OES, the samples were filtered and diluted to lower acid content < 10 % (Figure 7).

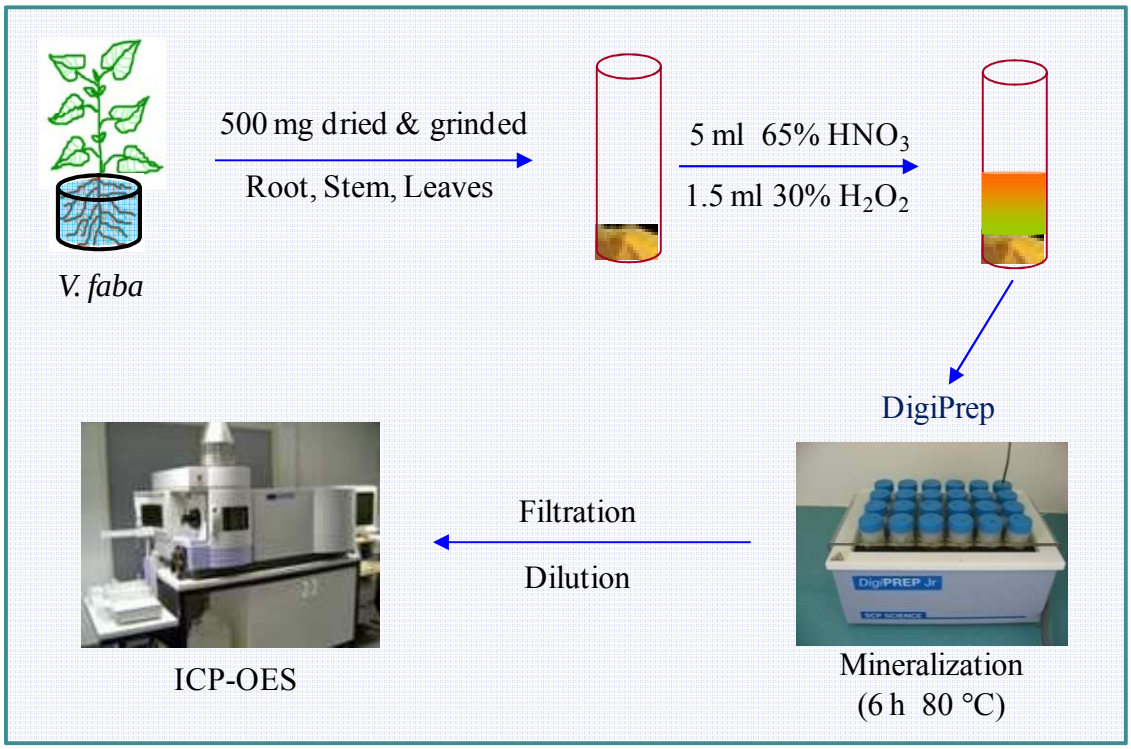


**Figure 8:** Plant sample digestion by DigiPrep and Pb contents analysis by Inductively Coupled Plasma-Optical Emission Spectrometers (ICP-OES).

## 8. Toxicity Parameters analysis

After harvest at same time, both root and shoot were immediately frozen in liquid nitrogen to conserve the physiological status. The response of the plants to stress varies with respect to age and stage of development. To avoid such variations between the leaves (old and young leaves) or roots (primary and secondary roots), the samples were homogenized by crushing manually. The samples were then preserved at -80 °C. This pre-crushing does not modify the quantity or profile of proteins or the activity of certain enzymes, even after two months of conservation (Saint-Martin and Pourrut, 2005).

## 9. Evaluation of the lipid peroxidation

Lipid peroxidation is the oxidative degradation of lipids. It is the process whereby free radicals produced by heavy metals oxidize lipids and their esters in cell membranes. It most often affects the polyunsaturated fatty acids, because they contain bis-allylic hydrogens, which are highly susceptible to oxidation. Nowadays, oxidative stress due to over production of ROS has been proposed as a possible mechanism of Pb toxicity (Pourrut et al. 2008)

Lipid peroxidation was calculated by measuring the thiobarbituric-acid-reactive-substances (TBARS) according to Hodges et al. (1999). Plant samples were homogenised in hydro-alcoholic solution (80/20: v/v) under liquid nitrogen at 4 °C in darkness. The homogenate is then incubated at 95 °C with the acid thiobarbituric in the presence of butyl hydroxytoluene (HTB) to avoid any oxidation of the mixture. The reaction involves the formation of a red colour complex between two molecules of acid thiobarbituric. After centrifugation, the absorbance of the supernatant was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of TBARS is calculated using the extinction coefficient 155 $mM^{-1}cm^{-1}$.The contents are calculated according to the equations of Hodges et al (1999):

$$[(Abs_{532+TBA} - Abs_{600+TBA}) - (Abs_{532-TBA} - Abs_{600-TBA})] = A$$

$$[(Abs_{440+TBA} - Abs_{600+TBA}) * 0.0571] = B$$

$$\text{TBARS equivalents } (nmol.ml^{-1}) = [(A - B)/157\,000] * 10^6$$

The amount of TBARS was finally expressed in nanomoles of TBARS per grams of fresh weight (TBARS $nmol.g^{-1}$ FW).

**10. Evaluation of the $H_2O_2$**

The reactive oxygen species (ROS) are basically unstable molecules that have an unpaired electron in their outer shell. They react with (oxidize) various cellular components including DNA, proteins, lipids / fatty acids and Pb to oxidative stress. The oxidative stress is generally the result of imbalance between the generation and the neutralization of ROS by antioxidant mechanisms. Among these ROS, $H_2O_2$ is a very strong oxidant and requires quick removal, which is achieved by the action of APX in the ascorbate-glutathione cycle or by GPOX and CAT in cytoplasm and other cell compartments (Mishra et al. 2006).

The measurement of $H_2O_2$ contents was carried out according to Islam et al. (2008). Each sample (500 mg frozen) was homogenized with 5mL 0.1% (w/v) trichloroacetic acid (TCA) under liquid nitrogen, and was centrifuged at 12 000 g for 20 min. The mixture assay contained 0.5 mL of the supernatant added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassiumiodide (KI). Absorbance was determined at 390 nm and the contents of $H_2O_2$ were evaluated using a standard curve under the same conditions (Figure 8).

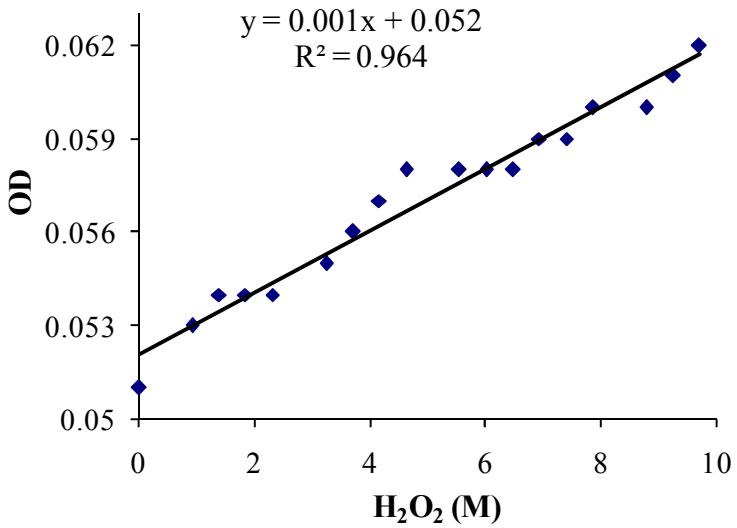


**Figure 9:** The standard curve used for $H_2O_2$ contents calculation.

**11. Extraction and proportioning of the foliar pigments**

The pigments contents are very sensible to environmental changes. Particularly, heavy metal toxicity reduces the pigment content in green plants. Therefore, pigment contents analysis is commonly used to monitor metal-stress in green plants. The pigments contents have been shown to correlate negatively with Pb uptake (Cenkci et al. 2010; Sing et al. 2010; Gupta et al. 2009). Frozen leaves were ground in a mortar with a pestle under liquid nitrogen. Pigments (chlorophyll-a, chlorophyll-b and carotinoids) were extracted by incubating with an extraction buffer hydro-acetone (80 % v/v) for 24 h at 4 °C in darkness. After centrifugation, the absorbance of the extract was recorded at 663.2, 646.8 and 470 nm against an extraction buffer control. Concentrations of chlorophyll a, b, total chlorophylls (a+b) and total carotenoids were calculated according to extinction coefficients and equations reported by Lichtenthaler (1987).

$$\text{Chlorophyll a} = 12.25 * Abs_{663.2} - 2.798 * Abs_{646.8}$$

$$\text{Chlorophyll b} = 21.5 * Abs_{646.8} - 5.1 * Abs_{663.2}$$

$$\text{Total chlorophyll} = 7.15 * Abs_{663.2} + 18.71 * Abs_{646.8}$$

$$\text{Carotenoids} = (1000 * Abs_{470} - 1.82 * \text{chlorophyll a} - 85.02 * \text{chlorophyll b})/198$$

The contents were expressed in µg of pigment per mg of leaves fresh weight ($\mu g.g^{-1}$ FW).

## 12. Extraction, proportioning of proteins

Induction of oxidative stress due to over production of ROS is an important feature of Pb toxicity (Pourrut et al. 2008; Mishra et al. 2006; Singh et al. 2010). One of the most efficient adjustment or detoxification mechanisms in plant cells against these free radicals is activation of antioxidants enzymes, which include superoxide dismutases (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPOX) and glutathione reductase (GR). The activity of these enzymes was determined against a protein extract -free blank.

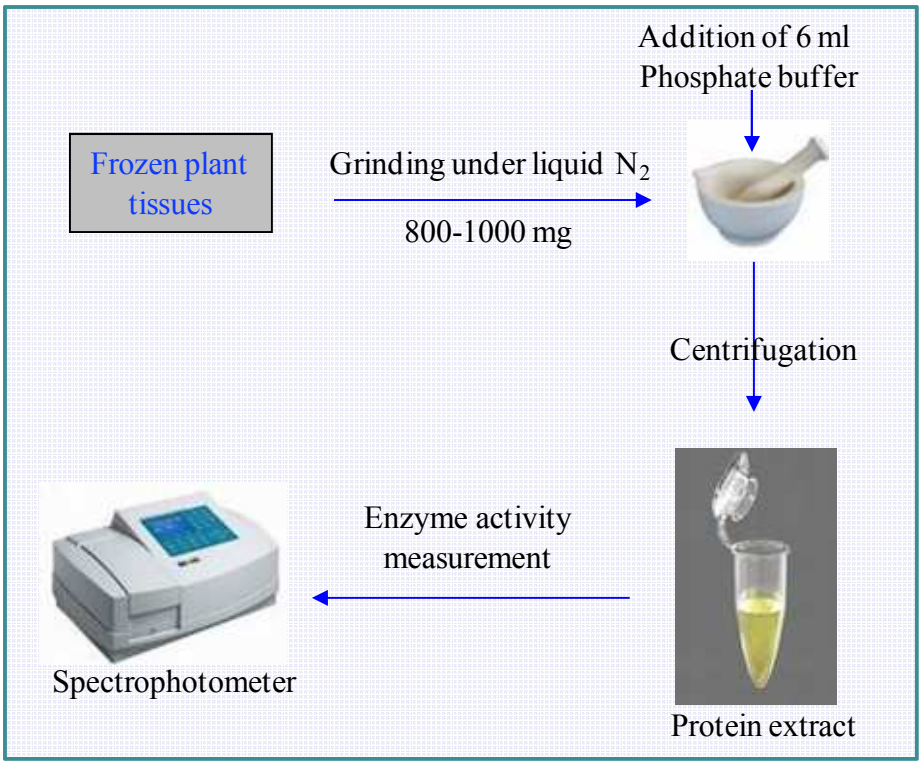


**Figure 10:** Schematic diagram of protein contents analysis by spectrophotometer using Bradford method.

The protein contents were determined according to Bradford (1976), using bovine serum albumin (BSA, Sigma) as standard with UV spectrophotometer (Helios Alfa, Thermo Electron Corporation, USA) (Figure 9). Frozen *V. faba* roots and leaves (1.0 g) were ground in a mortar with pestle in the presence of liquid nitrogen, and homogenized in 6 mL cold potassium phosphate buffer (100 mM pH 7, Sigma) containing EDTA (Sigma), polyvinylpyrrolidone, ß-mercaptoethanol, ascorbic acid (Sigma) and antiproteases (chymostatin and protease inhibitor cocktail). The suspension was centrifuged for 15 min at

15,000 g (4 °C) (Singh et al. 2010; Mishra et al. 2006) and supernatant was then assayed for antioxidant enzyme activity.

## 13. Calculation of enzymatic activities

The enzymatic activities are evaluated spectrophotometrically, while following the absorption of reaction medium due to the oxidation of their co-substrate (ascorbate for APX, guaiacol for guaiacol peroxidase and NADPH for GR.) or the reduction of substrate ($H_2O_2$ for CAT).

### *13.1. Catalase (CAT, EC 1.11.1.6)*

Catalase is an antioxidant enzyme ubiquitously present in aerobic cells. It catalyses the decomposition of $H_2O_2$ to water and oxygen (Figure 10). The activity of catalase was assayed according to Aebi, (1984). Assay mixture contained the enzyme extract (50 µg protein) in 50 mM phosphate buffer (pH 7.5). The decomposition of $H_2O_2$ was followed at 240 nm (extinction coefficient of 39.4 $M^{-1}$ $cm^{-1}$) for 1 min at 25 °C by decrease in absorbance. Enzyme specific activity is expressed as µmoles of $H_2O_2$ degraded.$min^{-1}$.$mg^{-1}$ protein.


**Figure 11:** Schematic reaction of $H_2O_2$ conversion to $H_2O$ by catalase.

### *13.2. Guaiacol peroxidase (GPOX, EC 1.11.1.7)*

Guaiacol peroxidase was assayed according to Hemeda and Klein, (1990). The reaction mixture contained 50 mM phosphate buffer (pH 7.5), 4 mM guaiacol (Sigma), $H_2O_2$ (15 mM) and enzyme extract (70 µg protein). Decrease in absorbance due to guaiacol oxidation was recorded at 470 nm (extinction coefficient of 26.6 $mM^{-1}$ $cm^{-1}$). Enzyme specific activity is expressed as µmoles of guaiacol oxidised (GO).$min^{-1}$.$mg^{-1}$ protein.

### *13.3. Ascorbate peroxidase (APX, EC 1.11.1.1)*

Under stress conditions, the antioxidants can work in co-operation, thus providing better defense. Ascorbate and glutathione remove $H_2O_2$ via the Halliwell-Asada pathway and

play important role in metal detoxification. Ascorbate peroxidise was assayed according to Nakano and Asada (1981) The reaction mixture contained 50 mM phosphate buffer (pH 7.5), 20 mM $H_2O_2$, 0.5 mM ascorbic acid and enzyme extract (45 µg protein). The disappearance of ascorbate was recorded at 290 nm (extinction coefficient of 2.6 $mM^{-1}$ $cm^{-1}$) using a spectrophotometer. The specific activity of enzyme is expressed as µmoles of $H_2O_2$ degraded.$min^{-1}$.$mg^{-1}$ protein.

### *13.4. Glutathione reductase (GR, EC 1.6.4.2)*

Glutathione reductase being the most important enzyme involved in the protection against Pb and the ROS was assayed according to Dringen and Gutterer (2002) using Glutathione Reductase Assay Kit (Cayman; Cat. 703002). The reaction mixture contained 50 mM phosphate buffer (pH 7.5), 0.5 mM EDTA, 1 mM GSSG (Sigma), 0.1 mM NADPH (Sigma) and enzyme extract (100 µg protein). The reaction was monitored by following the change in A340 as oxidized glutathione-dependent oxidation of NADPH for 1 min at 25 °C. The specific activity of enzyme is expressed as µmoles of NAPDH oxidised.$min^{-1}$.$mg^{-1}$ protein. Figure 11 shows the function of GR in GSH cycle.

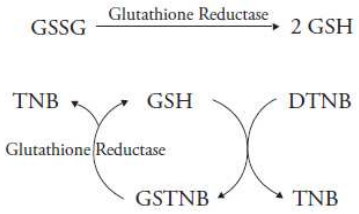


**Figure 12:** Role of glutathione reductase in GSH cycle.

### *13.5. Superoxide dismutase (SOD, EC 1.15.1.1)*

The evaluation of SOD activity is different compare to other enzymes. Superoxide dismutase activities were measured following its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) according to Giannopolitis and Ries (1977). The 3 ml reaction mixture contained 75 µM NBT (Sigma), 2 µM riboflavin (Sigma), 13 mM methionine (Sigma), 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), and 50 µg enzyme extract. Prior to measure absorbance at 560 nm, the test tubes containing the mixture were placed 30 cm below a light source (30 W fluorescent lamps) for 10 min. The activity of SOD

in the samples removes the superoxide anion and, thus, limits the formation of this blue formasan.

Riboflavin + Hν + $O_2$ → riboflavin. + $O_2$ .-

$O_2$.- + blue NBT → of formasan + $O_2$

One unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of formasan formation or NBT reduction.

**14. Micronucleus test**

*Principle*; Micronuclei are the result of chromosome breaks or mitotic anomalies that require a passage through mitosis to be recognisable. Indeed, during mitosis, fragments of chromosomes, which were not subjected to segregation do not join the principal core during the telophase, and can be observed under the microscope in the form of small spherical cores separated from the principal core, called micronuclei. The test is carried out according to standard AFNOR NF T90-327.

Before transplanting the *V. faba* seedlings to hydroponic conditions, the primary root tip was cut off (2 mm) to stimulate the emergence of secondary roots. Four days were necessary to obtain secondary roots of suitable length (1–2 cm) for the test. Exposure time was 30 h (6 h for the treated groups followed by a 24 h recovery period). In view of Pb toxicity after 30 h of exposure (blackening of the root tips and loss of mitosis), a 24 h recovery period was enough to develop micronuclei. Maleic hydrazide was used as positive control and aerated Hoagland's solution alone was used as negative control. After treatment, root tips were fixed during one night in a Carnoy solution, also called solution of Farmer (glacial acetic acid/ethanol 1:3 v/v), which allows to fix the cells in metaphase. Subsequently, the roots were hydrolyzed with HCl and were stained with an aqueous solution of aceto-orcein. The slides were observed under 1000 × magnification using an Olympus BX41 microscope coupled with a camera imaging FA1394-Q RETIGA Exi. The slides were observed under microscope to count the total number of cells, the number of cells in division (mitosis) and cells with micronucleus (Figure 12). This counting makes it possible to calculate: mitotic index (MI) and micronucleus (MN) frequency as below.

MI (%) = Number of cells in mitosis * 100 / total number of cells observed

MN (‰) = Number of micronuclei * 1000 / total number of cells observed

For each root tip, almost 5000 cells were observed. In order to avoid underestimation of micronucleus frequency due to impaired cell proliferation rate, the micronucleus test was performed only on root tips with a mitotic index of over 2 %. Moreover, the positive control must induce significantly higher number of micronuclei than the negative control.

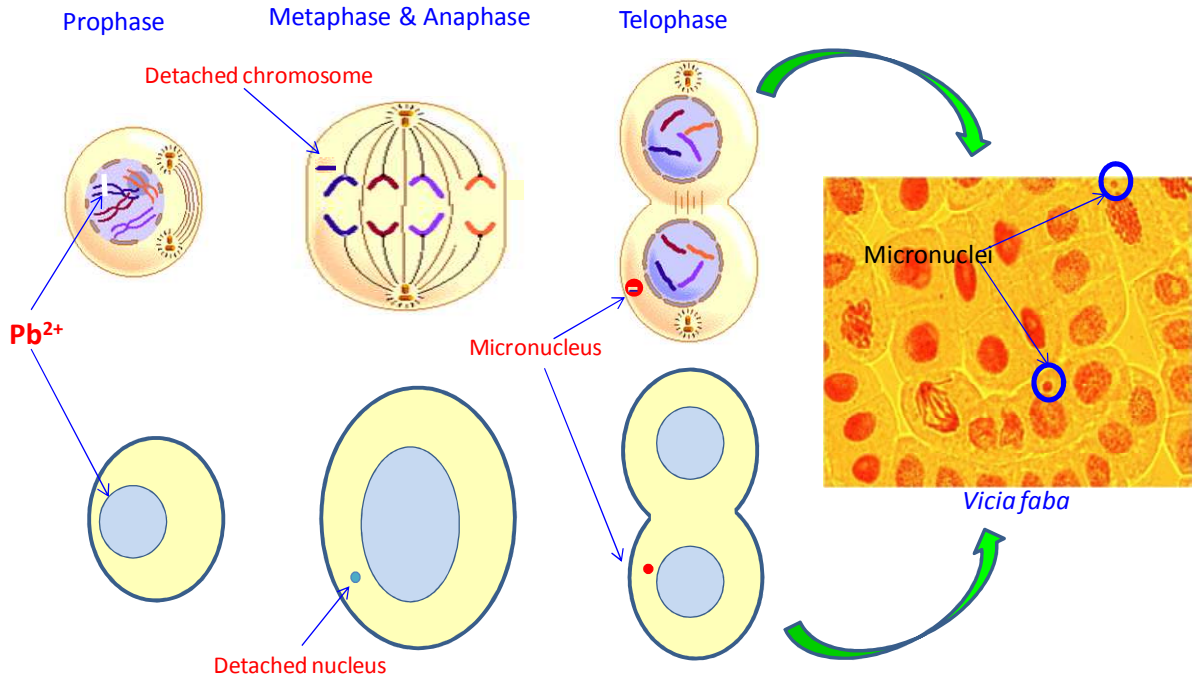


**Figure 13:** Mechanisms of Pb-induced micronuclei production in *Vicia faba* root cells tips.

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# Chapter 3:

# Results & Discussion

***Results & Discussion***

Using the above-mentioned experimental techniques, a series of short term experiments were carried out to investigate the effect of organic ligands on Pb uptake by plants and phytotoxicity. These experiments were carried out in a stepwise manner with the objective of a scientific publication. Therefore, the results and discussion are presented in the form of three articles as separate sections. Before each article, a short "*forewords*" has been presented to highlight the background and objective of the study. The three articles presented include;

- **Section 3A**: Comparison of *Vicia faba* metabolic responses in relation with Pb speciation and uptake (Ecotoxicology, ECTX1562).

- **Section 3B**: Pb-induced genotoxicity to *Vicia faba* L. roots in relation with metal cell uptake and initial speciation (Ecotoxicology and Environmental Safety, 74: 2011, 78–84).

- **Section 3C:** Effect of fulvic acids on Pb-induced oxidative stress to metal sensitive *Vicia faba* L. plant (Journal of Hazardous Materials, JHM-D-10-05879).

# Section 3A:

## Comparison of *Vicia faba* metabolic responses in relation with Pb speciation and uptake

*-Publication-*

M. Shahid, C. Dumat, C. Laplanche, J. Silvestre, E. Pinelli. Comparison of *Vicia faba* metabolic responses in relation with Pb speciation and uptake (Ecotoxicology, ECTX1562).

Forewords

Over the last thirty years, many studies have evaluated the toxic impact of Pb on plants (Gupta et al. 2009; Krzesłowska et al. 2010; Maestri et al. 2010; Sing et al. 2010) and several literature reviews have been devoted to this study (Seregin and Ivanov, 2001; Patra et al. 2004; Sharma and Dubey, 2005; Saifullah et al. 2009). These studies showed that Pb is a nonessential element in metabolic processes and can induce a large number of toxic effects to various morphological, physiological and biochemical process. However, it is unfortunate to note that majority field work performed deal almost exclusively on the basis of total metal contents in polluted soil. The potential effects of toxic elements in the environmental systems vary strongly by their physico-chemical behaviour, i.e. speciation (Uzu et al. 2009). Nowadays, it has become apparent that metal toxicity towards living organisms depends not only on their concentrations but also on the forms of their occurrence (Doig et al. 2007; Dumat et al. 2001). This makes total concentration, although relatively easier to measure, an unreliable indicator of toxicity. Predicting metal toxicity with respect to its applied form is, therefore, the pursuing of researchers.

The first objective of this preliminary study was to evaluate the effect of metal speciation on Pb uptake and toxicity to sensitive *V. faba* plants. For this purpose Pb was chelated by EDTA and citric acid at two different levels. EDTA, a strong organic chelator for metals, was chosen to act as a model compound for humic substances (Meers et al. 2005; Lai and Chen, 2005) and citric acid was chosen to model low-molecular-weight organic acids (Muhammad et al. 2009; Chen et al. 2003).

Secondly, plants respond to metal toxicity in a variety of different ways. The specific response of plant physiological status to environmental pollutant is used to asses acute toxicity of environmental hazardous matter through plant assay. The use of these short term plant assays/ toxicity parameters has gained special attention over last decades. These tests are highly sensitive, quite easy to conduct, inexpensive, rapid, and good predictors of metal toxicity (Panda et al. 2002). In the present study, the following metal toxicity indicators were used;

- ROS induction ($H_2O_2$), which indicate the metal toxicity
- Lipid peroxidation (TBARS contents) representing intensity of metal oxidative stress

- Antioxidant enzyme activities (SOD, CAT, GPOX, APX GR), which indirectly indicate the reduction of ROS in the organism
- Pigment contents (Chl-a, Chl-b, Carotinoids), which are negatively correlated with Pb contents in th plant

However, the sensitivity of these test vary under different experimental conditions in addition to metal/plant type. To address this variation in sensitivity, different metal toxicity test were compared with respect to applied Pb form (free $Pb^{2+}$ ions, chelated by EDTA or citric acid) using principal component analysis. In this study, this comparison was carried out to find the most sensitive oxidative stress parameters in relation with speciation.

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# Comparison of *Vicia faba* metabolic responses in relation with Pb speciation and uptake

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**Abstract**

Only few studies concern lead (Pb) phyto-toxicity in relation to its speciation, which can nevertheless influence metal uptake, translocation and mechanisms involved in phyto-toxicity. In this study, Pb-induced toxicity to the sensitive *Vicia faba* (*V. faba*) plant was assessed against metal speciation and uptake. Five antioxidant enzyme activities, lipid peroxidation (LPO) and photosynthetic pigment levels were measured to compare the efficiency of these ecotoxicological tests. *V. faba* seedlings were exposed (for 1, 4, 8, 12 and 24 h) in controlled hydroponic conditions to 5 µM of Pb nitrate alone or chelated at two levels by ethylenediaminetetraacetic acid (EDTA) or citric acid (CA). First, the specific responses of *V. faba* to Pb toxicity were found to depend on metal speciation and duration of exposure. Phyto-toxicity was inhibited dose dependently by EDTA indicating a protective role of that metal chelator. Especially that only EDTA significantly modified Pb transfer: it increased Pb uptake by *V. faba* roots dose-dependently, whereas decreased Pb translocation to shoot tissues. In contrast, CA had no significant effects on phyto-toxicity level but delayed the induction of the effects. The principal component analysis (PCA) of all the treatments highlighted two contrasting groups: toxic (Pb alone and Pb-CA) and non-toxic (controls and Pb-EDTA), demonstrating a strong influence of Pb speciation on phytotoxicity. In addition, PCA suggested that glutathione reductase activity in both roots and leaves must be measured in priority for Pb phyto-toxicity assessment.

**Key words:** lipid peroxidation, phytotoxicity, oxidative stress, reactive oxygen species, Pb, speciation.

## 1. ntroduction

Unnecessary for living organisms, Pb is the most toxic heavy metal widely distributed in the environment and highly persistent (Shahid et al. 2011a). Pb is known to induce adverse effects to morphological, physiological and biochemical processes in plants such as seed germination, plant growth, chlorophyll production, LPO, oxidative stress and DNA damage (Shahid et al. 2011b). Induction of oxidative stress due to overproduction of free radicals permits an upstream determination of Pb toxicity (Pourrut et al. 2008; Mishra et al. 2006). These free radicals such as superoxide radicals ($O_2^{\bullet-}$), hydroxyl radicals (·OH) and hydrogen peroxide ($H_2O_2$) are capable of causing cellular damage in plants (Yu et al. 2006). In plant cells, one of the most efficient adjustment or detoxification mechanisms against these free radicals is activation of antioxidant enzymes (Liu et al. 2009; Yu and Gu 2007; Yu et al. 2006). These enzymes include superoxide dismutases (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidise (APX, EC 1.11.1.1), guaiacol peroxidase (GPOX, EC 1.11.1.7) and glutathione reductase (GR, EC 1.6.4.2). To assess metal-induced toxicity in plants, measurement of antioxidant enzyme activities and LPO are routinely used (Mishra et al. 2006). Another method commonly used to monitor metal-stress in green plants is to measure chlorophyll activity which has been shown to correlate negatively with Pb uptake (Cenkci et al. 2010).

Literature indicates that speciation is strongly influent; not only in predicting metal mobility and bioavailability, but also in risk assessment to living organisms (Uzu et al. 2011 and 2010; Dumat et al. 2006). Therefore, in addition to knowing the total metal concentration, predicting the role of specific metal species is of utmost importance in improving the understanding of metal toxicity mechanisms. Organic chelating agents and low-molecular-weight organic acids excreted by plant roots and microorganisms have been found to change plant metal uptake and translocation to aerial parts through speciation (Kim et al. 2010; Ruley et al. 2006). Consequently, these organic ligands could modify metal toxicity, especially when metal bioavailability is low as in the case of Pb (Ruley et al. 2006; Ruley et al. 2004).

The role of organic ligands in metal phytoextraction by hyperaccumulators (which have natural detoxification mechanisms) has been extensively studied in the context of polluted soils. But, there is very scarce data on the toxic effects of metals on sensitive plants as a function of metal speciation (Shahid et al. 2011a). The influence of metal speciation on

phytotoxicity could be better assessed using a metal-sensitive plant. Therefore, the present study was carried out to identify the role of Pb speciation on its transfer and toxicity using Pb sensitive *V. faba* plants. EDTA forms strong complexes with metals (through its two amine and four carboxylate groups) and it was chosen, along with citric acid, as model compounds for humic substances and low weight organic acids, respectively. Moreover, different enzyme assays, pigment contents and LPO measures in relation with Pb uptake and speciation were compared to improve bio-indicator ecotoxicological tests in an environmental quality assessment context. It's the first time that influence of metal speciation was studied for sensitive plant using various oxidative stress and chlorophyll activity tests.

## 2. Materials and methods

### *2.1. Plant materials and growth conditions*

*V. faba* seedlings were cultured according to Marcato-Romain et al. (2009). Dry seeds of *V. faba* cultivar "aguadulce" (Tezier, France) were soaked for 6 h in deionized water. After decoating, the seeds were germinated between two layers of moist cotton in a germination chamber under optimal conditions, i.e. in the darkness at 22 °C and 100 % relative humidity. After 5 to 7 days, when the primary roots were about 2–3 cm in length, seedlings were transplanted to plastic tubs containing foam-plugged holes of thermo-pore sheets floating on continuously aerated Hoagland nutrition solution (Sigma) (Uzu et al. 2009; Pourrut et al. 2008) with the macro-elements: 5 mM $KNO_3$, 5 mM $Ca(NO_3)_2$, 2 mM $KH_2PO_4$ and 1.5 mM $MgSO_4$ and micro-elements: 9.11 µM $MnSO_4$, 1.53 µM $ZnSO_4$, 0.235 µM $CuSO_4$, 24.05 µM $H_3BO_3$, 0.1 µM $Na_2MoO_4$ and 268.6 µM Fe. The growth chamber conditions were: 16 h photoperiod at 70 % relative humidity and day/night temperatures of 24/22 °C. Light was supplied by 600 W Osram Nav-T Super High Pressure Sodium Lamps providing a minimum photosynthetic photon flux density of 500 $\mu mol.m^{-2}.s^{-1}$ at the top of the plant (Pourrut et al. 2008).

### *2.2. Treatments*

After a culture period of three weeks, *V. faba* seedlings were exposed to 5 µM Pb as $Pb(NO_3)_2$ in the presence and absence of two levels of EDTA or CA (Table 1). The higher levels of EDTA and CA were also applied alone as controls. All the treatments were applied for 1, 4, 8, 12 and 24 h keeping in view the short-lived production of reactive oxygen species

(ROS) before being scavenged rapidly by antioxidants. A $Pb(NO_3)_2$ concentration of 5 µM was chosen because, according to Pourrut et al. (2008), it induces genotoxicity in *V. faba* while remaining quite low and representative of the pollution levels often found in the environment. The concentration of $KH_2PO_4$ in the nutrient solution used for Pb treatment was reduced to 0.2 mM in order to prevent phosphate precipitation (Shahid et al. 2011a; Kopittke et al. 2008). Control plants (without Pb) were also cultured in the appropriate uncontaminated media with reduced $PO_4^{3-}$ concentration. The applied levels of EDTA (2.25 and 10 µM) and CA (550 and 1000 µM) were chosen using Visual Minteq (version 2.60) speciation model which showed 40 and 99 % of Pb chelation by EDTA, and 25 and 40 % by CA in nutrient solution, respectively. The toxic effect of higher concentrations of CA alone (>1000 µM) restricted Pb chelation to 40 % (data not shown). Using these applied levels of organic ligands, it was possible to compare Pb phyto-toxicity with respect to chelated and free Pb ion concentrations.

**Table-1.** Experimental design and composition of all the treatments. Pb measured (3rd column) indicates the Pb concentration determined by ICP-OES after 24h without plant exposure.

| **Treatments** | **Visual Minteq input** | **Visual Minteq output** | | **ICP-measured** |
|---|---|---|---|---|
| Notations | Composition | Pb-chelated (%) | Pb-free (%) | Pb (µM) |
| Control | Hoagland solution (HS) | – | – | 0 |
| EDTA-b | HS + 10 µM EDTA | – | – | – |
| CA-b | HS + 1000 µM CA | – | – | – |
| Pb | HS + 5 µM Pb | 0 | 85 | 4.97 ± 0.04 |
| Pb-EDTA-a | HS + 5 µM Pb + 2.25 µM EDTA | 40 | 51 | 5.02 ± 0.03 |
| Pb-EDTA-b | HS + 5 µM Pb + 10 µM EDTA | 99 | 1 | 5.01 ± 0.02 |
| Pb-CA-a | HS + 5 µM Pb + 550 µM CA | 25 | 64 | 4.99 ± 0.03 |
| Pb-CA-b | HS + 5 µM Pb + 1000 µM CA | 40 | 51 | 4.96 ± 0.02 |

### *2.3. Pb assay*

Before all the experiments, Pb content analysis was carried out in the nutrient solution after 24 h without exposure to plants. All the treatments solution (Table 1) were prepared from mother solutions of Pb (50 µM), EDTA (50 µM) and citric acid (10 mM) in milli-Q water. The pH of all the treatment solutions was adjusted to 5 ± 0.1 using distilled $HNO_3$ (15 M, suprapur 99.9 %, Sigma) and were kept under experimental conditions for 24 h. Inductively

coupled plasma-atomic emission spectrometry (ICP-AES, Jobin Yvon) with an IRIS Intrepid II XDL/ Thermo Electron Corporation was used to determine Pb contents after filtration (0.22 µm). Both the pH and total Pb concentration remained constant in all the treatments after 24 h (Table 1). Virginia Tobacco leaves (CTA-VTL-2, Polish certified reference material; ICHTJ) were used as a reference material to check the accuracy of the analytical procedure. Certified values for Pb in Tobacco leaves were given as 22.1 ± 1.2 µg $Pb.g^{-1}$ dry weight (D.W.). Measured values for the three replicates were 22.0 ± 0.9, 22.4 ± 0.8 and 21.9 ± 0.8 µg $Pb.g^{-1}$ D.W.

*V. faba* seedlings were harvested and divided into root, stem and leaves after the various periods of treatment application. Pb bound to the rhizoderm was removed by HCl as described by Uzu et al. (2009). Cell Pb was assayed as described by Pourrut et al. (2008). After drying at 80 °C for 48 h, plant samples were wet digested by a 1:1 mixture of 65 % $HNO_3$ (Sigma) and 30 % $H_2O_2$ (Sigma) at 80 °C over 6 h using DigiPrep Jr. Digested material was diluted with milli-Q water and metal content was determined using ICP-OES.

### *2.4. Enzyme assay*

Frozen *V. faba* roots and leaves (1.0 g) were homogenized in 6 mL cold potassium phosphate buffer (100 mM pH 7, Sigma) containing 0.1 mM EDTA (Sigma) and 1 % polyvinylpyrrolidone (w/v, Sigma) in an ice bath using a pre-chilled mortar and pestle. The suspension was centrifuged for 15 min at 15,000 g (4 °C) (Mishra et al. 2006) and the supernatant assayed for antioxidant enzyme activity. Each enzyme activity was measured against a blank without protein extract. The protein content was determined according to Bradford (1976), using bovine serum albumin (BSA, Sigma) as standard with a UV spectrophotometer (Helios Alfa, Thermo Electron Corporation, USA).

SOD activity was measured following its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT, Sigma) according to Giannopolitis and Ries (1977). One unit of SOD activity is defined as the amount of enzyme required to cause 50 % inhibition of NBT reduction. CAT activity was assayed by determining $H_2O_2$ consumption at 240 nm according to Aebi (1984) and the specific activity is expressed as µmoles of $H_2O_2$ $degraded.min^{-1}.mg^{-1}$ protein. GPOX was assayed according to Hemeda and Klein (1990). A decrease in absorbance due to guaiacol oxidation was recorded at 470 nm and specific activity was expressed as µmoles of guaiacol $oxidised.min^{-1}.mg^{-1}$ protein. APX was assayed

according to Nakano and Asada (1981) following the disappearance of ascorbate at 290 nm. The specific enzyme activity is expressed as µmoles of $H_2O_2$ degraded.$min^{-1}$.$mg^{-1}$ protein. GR being the enzyme playing the greatest role in the protection against Pb toxicity, it was assayed according to Dringen and Gutterer (2002) using Glutathione Reductase Assay Kit (Sigma). The GR activity was measured by following the change in absorbance at 340 nm caused by the oxidation of NADPH. The specific activity of enzyme is expressed as µmoles of NAPDH oxidised.$min^{-1}$.$mg^{-1}$ protein.

### *2.5. LPO and $H_2O_2$ assay*

LPO was assayed by determining the level of thiobarbituric acid reactive substances (TBARS) at 440 nm, 532 nm, and 600 nm according to the extinction coefficients and equations reported by Hodges et al. (1999). $H_2O_2$ was assayed according to Islam et al. (2008) at 390 nm using a standard curve under the same conditions.

### *2.6. Pigment content assay*

Samples of frozen leaves (600 mg) were ground with liquid nitrogen. Pigments were extracted by incubating leaves in 10 mL acetone 20:80 (v/v) for 24 h at 4 °C in darkness. After centrifugation at 1000 g for 10 min, the absorbance of the supernatant was recorded at 663, 645 and 480 nm. Concentrations of chlorophyll a, chlorophyll b and total carotenoids were calculated according to the extinction coefficients and equations reported by Lichtenthaler (1987).

### *2.7. Statistical analysis*

The variables were tested for differences between treatments using Analysis of Variance (one-way ANOVA) followed by Tukey's honestly significant difference (HSD) test. This statistical analysis was performed using Statistica software, Edition '98 (StatSoft Inc., Tulsa, OK, USA). For each bioassay, bold mean values with an asterisk (*) represent a significant difference ($p < 0.05$) as measured by Tukey's HSD test. Chemical variables were also subjected to Principal Component Analysis (PCA) in order to simplify the interpretation, compare all the treatments and techniques and to find the main trends in the data. Spad (version 7.0) statistical software was used for all PCA.

## 3. Results

### *3.1. Effect of EDTA and CA alone on $H_2O_2$ contents, LPO and enzymatic activities*

In order to determine the effect of EDTA and CA alone, the higher levels of these ligands were applied alone without Pb. No significant effect was observed on pigment contents, LPO and antioxidant enzyme activities as compared to controls for all treatment times. Therefore, the results obtained for EDTA and CA alone were only used in PCA and are not presented in Tables 2, 3, 4 and 5.

### *3.2. Pb uptake by V. faba in the presence of organic ligands*

Fig. 1 illustrates the tendency of *V. faba* roots, stem and leaves to accumulate Pb over time when grown in the presence and absence of EDTA and CA. Pb uptake by *V. faba* roots started immediately after exposure (33 ± 7 $\mu g.g^{-1}$ after 1 h) and reached an average value of 111 ± 18 $\mu g.g^{-1}$ after 24 h. Pb translocation from root to aerial parts was very low: only 4.4 ± 0.8 and 3.7 ± 0.5 $\mu g.g^{-1}$ Pb was transferred, respectively, to stem and leaves at 24 h.

Additions of EDTA increased Pb concentration significantly and dose-dependently in *V. faba* roots; 26 and 72 % respectively for Pb-EDTA-a and Pb-EDTA-b at 24 h compared to Pb alone (Fig. 1). In contrast, Pb translocation from roots to aerial parts was significantly reduced in the presence of EDTA at both levels. Application of Pb-EDTA-a decreased Pb in stem and leaves by 40 and 43 % respectively compared to Pb alone at 24 h. This Pb translocation restriction phenomenon was more intense for Pb-EDTA-b with 51 and 79 % decrease in Pb contents in stem and leaves respectively. The effect of EDTA at both levels of application on Pb translocation was significant for all exposure times in stem and only after 24 h in leaves.

Application of CA at both levels increased Pb uptake by *V. faba* roots and translocation to shoot tissues slightly. The increase in Pb contents in roots, stem and leaves was respectively 7, 5 and 3 % for Pb-CA-a while 13, 7 and 8 % for Pb-CA-b (Fig. 1). However, neither of the treatments reached statistical significance compared to Pb alone.

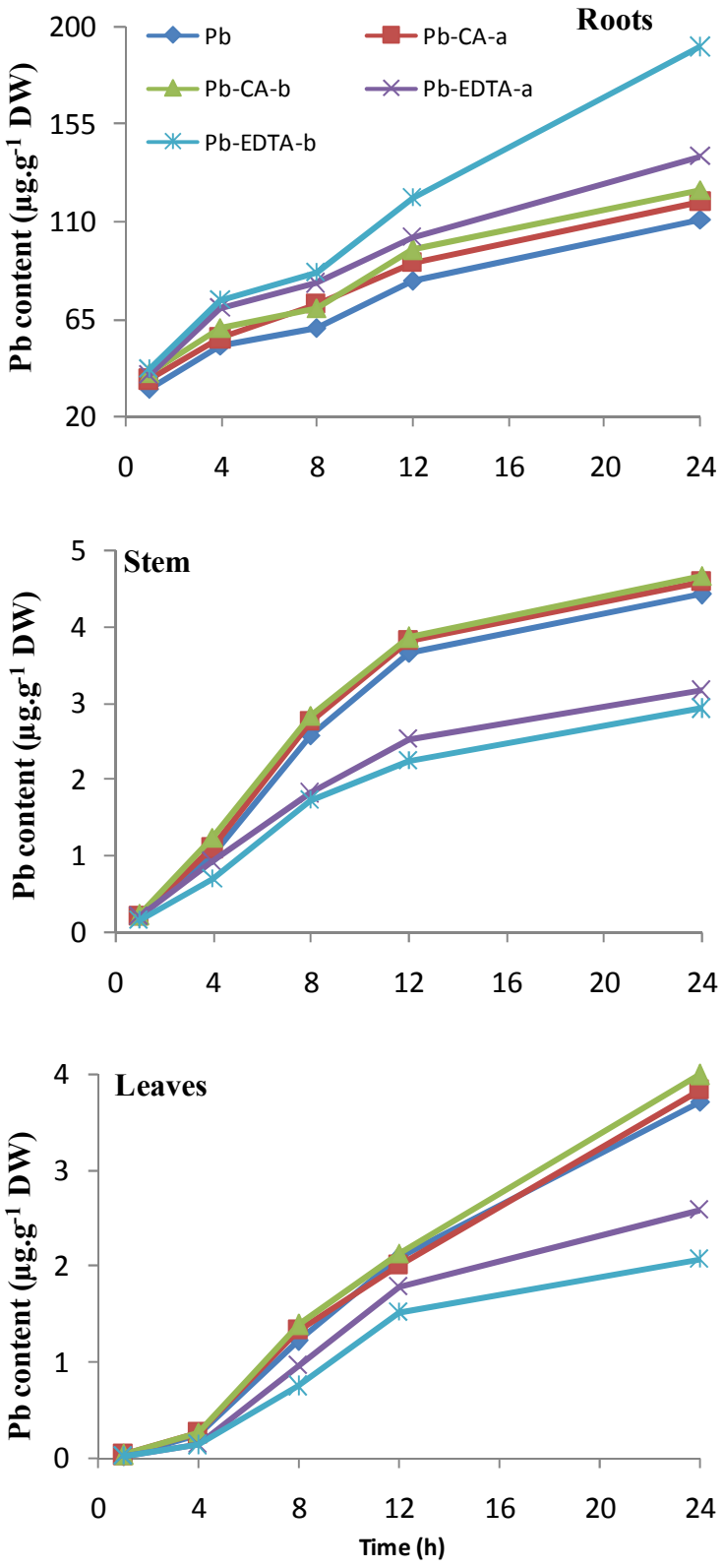


**Fig 1:** Effect of EDTA and CA on Pb concentration (µg.g$^{-1}$ D.W.) in *V. faba* roots, stem and leaves. Values are means of two separate experiments each replicated six times.

### *3.3. Effect of organic ligands on chlorophyll contents*

Chlorophyll and carotenoid contents of *V. faba* leaves are presented in Table 2. Pigment composition was not affected significantly until 8 h by either of the treatments. However, at 12 h, the chlorophyll and carotenoid contents decreased significantly by Pb alone. At 24 h, Pb reduced Chl-a, Chl-b and carotinoid contents by 11, 19 and 33 % respectively. Addition of EDTA significantly inhibited Pb-induced reduction of chlorophyll and carotenoid contents, the effect being dose dependent. The chlorophyll and carotenoid contents are comparable to controls for Pb-EDTA-b under all treatment times. Addition of CA to Pb caused a further slight decrease in chlorophyll and carotenoid contents compared to Pb alone. The effect of CA on Pb toxicity was concentration dependent, Pb-CA-b complex being more toxic.

### *3.4. Effect of organic ligands on LPO and $H_2O_2$ induction*

Table 3 presents the effect of Pb alone and combined with EDTA or CA on LPO and $H_2O_2$ induction in roots and leaves of *V. faba* seedlings. Application of Pb alone induced two significant bursts of LPO in roots after 1 and 12 h by increasing TBARS contents by 42 and 73 % respectively compared to the controls. These two bursts of LPO coincide with $H_2O_2$ production in the presence of Pb alone. When Pb was chelated with EDTA, LPO and $H_2O_2$ production was dose dependently inhibited in the roots of *V. faba* seedlings. TBARS and $H_2O_2$ contents remained close to those of the controls under Pb-EDTA-b application for all treatment times. Application of Pb-EDTA-a decreased TBARS and $H_2O_2$ production compared to Pb alone but the values were still significantly higher than the controls at 4 h for $H_2O_2$ and TBARS. When Pb was applied in combination with CA at both levels, Pb-induced TBARS induction remained continuously and significantly higher between 1 and 12 h. In case of $H_2O_2$, the first induction was delayed to 4 h compared to Pb alone at 1 h. The second induction of $H_2O_2$ at 12 h remained unchanged in the presence of CA at both levels.

In the leaves, TBARS contents remained close to control levels until 4 h for all treatments. Application of Pb increased the induction of LPO and $H_2O_2$ by 115 and 61 % respectively compared to the controls at 8 h. This increase in TBARS contents continued till the end of the experiment with 74 and 62 % higher TBARS content compare to control respectively at 12 and 24 h. For $H_2O_2$, induction decreased to basal levels at 12 h and increased again by 80 % compare to control at 24 h. The effect of organic ligands on LPO

and $H_2O_2$ production was similar to roots; EDTA reduced dose dependently whereas CA delayed LPO and $H_2O_2$ production.

**Table-2.** Effect of ligands on Pb-induced reduction of pigment contents ($\mu g.g^{-1}$) in *V. faba* leaves. Values are means of 6 replicates. Significant differences at $P < 0.05$ are indicated with an asterisk (*).

| Treatments | Time (h) | Chl-a Mean±S.D. | Chl-b Mean±S.D. | Carotinoids Mean±S.D. |
|---|---|---|---|---|
| **Control** | 1 | 805 ± 29 | 387 ± 37 | 204 ± 9 |
| **Pb** | | 792 ± 14 | 380 ± 22 | 210 ± 13 |
| **Pb-EDTA-a** | | 798 ± 45 | 398 ± 29 | 210 ± 13 |
| **Pb-EDTA-b** | | 805 ± 47 | 384 ± 21 | 195 ± 20 |
| **Pb-CA-a** | | 805 ± 28 | 402 ± 38 | 195 ± 12 |
| **Pb-CA-b** | | 781 ± 40 | 391 ± 31 | 193 ± 9 |
| **Control** | 4 | 813 ± 41 | 379 ± 23 | 208 ± 13 |
| **Pb** | | 795 ± 39 | 373 ± 44 | 197 ± 7 |
| **Pb-EDTA-a** | | 812 ± 34 | 382 ± 16 | 194 ± 12 |
| **Pb-EDTA-b** | | 813 ± 33 | 386 ± 32 | 200 ± 11 |
| **Pb-CA-a** | | 791 ± 54 | 394 ± 28 | 195 ± 19 |
| **Pb-CA-b** | | 771 ± 67 | 384 ± 30 | 198 ± 6 |
| **Control** | 8 | 805 ± 46 | 392 ± 22 | 205 ± 18 |
| **Pb** | | 786 ± 54 | 364 ± 23 | 182 ± 13 |
| **Pb-EDTA-a** | | 789 ± 33 | 389 ± 30 | 189 ± 21 |
| **Pb-EDTA-b** | | 813 ± 87 | 372 ± 47 | 203 ± 21 |
| **Pb-CA-a** | | 774 ± 71 | 373 ± 22 | 198 ± 34 |
| **Pb-CA-b** | | 763 ± 50 | 360 ± 34 | 187 ± 25 |
| **Control** | 12 | 815 ± 27 | 389 ± 32 | 219 ± 30 |
| **Pb** | | 783 ± 19 | **322** ± 29 * | **162** ± 14 * |
| **Pb-EDTA-a** | | 796 ± 24 | 372 ± 18 | 185 ± 26 |
| **Pb-EDTA-b** | | 800 ± 91 | 379 ± 24 | 203 ± 24 |
| **Pb-CA-a** | | 776 ± 38 | **328** ± 26 * | **160** ± 27 * |
| **Pb-CA-b** | | 748 ± 59 | **320** ± 17 * | **161** ± 34 * |
| **Control** | 24 | 808 ± 46 | 380 ± 41 | 213 ± 20 |
| **Pb** | | **721** ± 45 * | **307** ± 16 * | **144** ± 21 * |
| **Pb-EDTA-a** | | 781 ± 59 | 369 ± 29 | **175** ± 19 * |
| **Pb-EDTA-b** | | 812 ± 76 | 376 ± 32 | 200 ± 24 |
| **Pb-CA-a** | | **718** ± 66 * | **313** ± 19 * | **139** ± 22 * |
| **Pb-CA-b** | | **713** ± 53 * | **298** ± 24 * | **129** ± 23 * |

**Table-3.** Effect of ligands on Pb-induced $H_2O_2$ (mol.g$^{-1}$ FW) and lipid peroxidation (TBARS in nmol.g$^{-1}$ FW) productions in roots and leaves. Values are means of 6 replicates. Significant differences at $P < 0.05$ are indicated with an asterisk (*).

| Treatments | Time | Roots | | Leaves | |
|---|---|---|---|---|---|
| | (h) | LPO | $H_2O_2$ | LPO | $H_2O_2$ |
| | | Mean±S.D. | Mean±S.D. | Mean±S.D. | Mean±S.D. |
| **Control** | 1 | 5.0 ± 0.4 | 6.8 ± 0.9 | 3.3 ± 0.3 | 39 ± 4 |
| **Pb** | | **7.1** ± 0.3 * | **9.7** ± 1.2 * | 3.6 ± 0.5 | 40 ± 6 |
| **Pb-EDTA-a** | | 5.5 ± 1.8 | 6.6 ± 0.6 | 3.4 ± 0.4 | 40 ± 3 |
| **Pb-EDTA-b** | | 5.5 ± 0.7 | 6.7 ± 0.8 | 3.6 ± 0.3 | 40 ± 2 |
| **Pb-CA-a** | | **6.1** ± 0.4 * | 6.9 ± 0.4 | 3.8 ± 0.3 | 39 ± 3 |
| **Pb-CA-b** | | **6.3** ± 0.6 * | 6.6 ± 0.5 | 3.7 ± 0.5 | 41 ± 3 |
| **Control** | 4 | 5.1 ± 1.0 | 7.6 ± 0.7 | 3.1 ± 0.7 | 42 ± 4 |
| **Pb** | | 6.2 ± 0.3 | 8.4 ± 0.9 | 3.3 ± 0.4 | 42 ± 2 |
| **Pb-EDTA-a** | | **6.7** ± 0.4 * | **8.4** ± 0.5 * | 3.2 ± 0.5 | 41 ± 2 |
| **Pb-EDTA-b** | | 4.7 ± 0.6 | 8.0 ± 0.6 | 3.4 ± 0.5 | 37 ± 5 |
| **Pb-CA-a** | | **7.7** ± 0.2 * | **9.8** ± 0.6 * | 3.7 ± 0.4 | 41 ± 4 |
| **Pb-CA-b** | | **8.5** ± 0.5 * | **10.2** ± 0.9 * | 2.9 ± 1.4 | 45 ± 5 |
| **Control** | 8 | 5.4 ± 0.4 | 7.5 ± 1.0 | 4.0 ± 1.0 | 46 ± 5 |
| **Pb** | | 6.3 ± 0.7 | 7.6 ± 1.2 | **8.6** ± 2.1 * | **73** ± 4 * |
| **Pb-EDTA-a** | | 5.9 ± 1.2 | 8.2 ± 0.9 | 4.7 ± 0.5 | 43 ± 4 |
| **Pb-EDTA-b** | | 5.2 ± 0.3 | 7.3 ± 0.9 | 4.7 ± 0.3 | 44 ± 4 |
| **Pb-CA-a** | | **7.6** ± 0.4 * | 7.7 ± 1.0 | 4.6 ± 0.6 | 42 ± 4 |
| **Pb-CA-b** | | **6.8** ± 0.9 * | 8.4 ± 0.6 | 4.7 ± 0.8 | 48 ± 5 |
| **Control** | 12 | 4.8 ± 0.7 | 7.7 ± 1.1 | 3.4 ± 0.2 | 42 ± 3 |
| **Pb** | | **8.3** ± 1.3 * | **10.0** ± 0.5 * | **5.9** ± 1.3 * | 39 ± 3 |
| **Pb-EDTA-a** | | 5.5 ± 0.8 | 8.7 ± 0.9 | **4.4** ± 0.3 * | 49 ± 7 |
| **Pb-EDTA-b** | | 5.7 ± 0.4 | 8.0 ± 0.3 | 3.1 ± 0.4 | 44 ± 6 |
| **Pb-CA-a** | | 6.4 ± 1.5 | **9.8** ± 0.4 * | **6.5** ± 0.3 * | **64** ± 5 * |
| **Pb-CA-b** | | **6.8** ± 0.9 | **10.7** ± 1.5 * | **8.2** ± 0.2 * | **76** ± 7 * |
| **Control** | 24 | 5.0 ± 0.8 | 7.5 ± 0.6 | 3.4 ± 0.3 | 41 ± 3 |
| **Pb** | | 5.2 ± 0.2 | 7.8 ± 1.3 | **5.5** ± 0.7 * | **74** ± 8 * |
| **Pb-EDTA-a** | | 4.6 ± 0.3 | 8.1 ± 1.0 | 4.5 ± 0.8 | **50** ± 5 * |
| **Pb-EDTA-b** | | 5.5 ± 0.2 | 7.8 ± 0.7 | 3.7 ± 1.7 | 39 ± 2 |
| **Pb-CA-a** | | 6.1 ± 1.2 | 8.1 ± 1.4 | **5.3** ± 1.2 * | **51** ± 6 * |
| **Pb-CA-b** | | 6.3 ± 1.3 | 8.3 ± 1.1 | **9.1** ± 1.9 * | **66** ± 5 * |

### *3.5. Antioxidant enzyme activities*

Antioxidant activities of *V. faba* roots grown in the presence of chelated and non-chelated Pb varied in roots non-linearly with time (Table 4). Pb-induced activation of SOD, GPOX and GR in *V. faba* roots started at 1 h whereas that of APX started at 4 h. The activation of SOD continued upto 8 whereas for GR till the end of experiment at 24 h. In case of GPOX, induction decreased to levels at 4 h followed by a second induction at 8 h which continued till 24 h. However, the activity of APX decreased to basal levels at 8 h followed by a second induction at 12 h which continued till 24 h. In contrast to all other enzymes, CAT was inhibited throughout the experiment, the effect started after 4 h and continued till 24 h.

Pb chelation by EDTA dose dependently inhibited Pb-induced variations in enzyme activities in roots (Table 4). All enzymes activities remained close to control for Pb-EDTA-b except for GR after 1 and 4 h where activity was significantly higher compared to controls. Although Pb-EDTA-a decreased antioxidant activities, the activities of SOD at 1 h, GPOX at 4 h and GR at 1, 4 and 24 h were significantly higher compare to control. Addition of CA at both levels delayed the Pb-induced variation of antioxidant enzymes (Table 4). The reduction of CAT activity and major first induction of SOD, GPOX and GR were delayed to next treatment exposure time compared to Pb alone. Moreover, CA prolonged the activation of SOD to 24 h. APX showed no effect of CA at either level of application.

In leaves, no significant effect was observed on enzyme activities before 8 h for all treatments (Table 5). At 8 h, when Pb reached a significant level in the leaves, the activities of SOD, GPOX and APX increased significantly compared to control. This increase in activity dropped to basal levels at 24 h for GPOX whereas it remained significantly higher for APX and SOD up to 24 h. GR induction started at 12 h and continued up to 24 h. The activity of CAT was inhibited after 12 h but the effect was significant only at 24 h.

Like roots, addition of EDTA at both levels inhibited the Pb-induced modification in antioxidant enzymes activities. However, the activities of GPOX at 8 h, APX at 12 h and CAT at 8 h were still significantly different for Pb-EDTA-a compare to control. The addition of CA caused a very clear delay in the increase of enzymatic activities in leaves compared to roots. The activities of SOD, GPOX, APX and GR were delayed to the next treatment exposure time while that of CAT followed the same trend as for Pb alone.

**Table-4.** Effect of ligands on Pb-induced modification of enzyme activity ($U.g^{-1}$ protein) in roots. Values are means of 6 replicates. Significant differences at $P < 0.05$ are indicated with an asterisk (*).

| Treatments | Time | SOD | CAT | GPOX | APX | GR |
|---|---|---|---|---|---|---|
| | (h) | Mean±S.D. | Mean±S.D. | Mean±S.D. | Mean±S.D. | Mean±S.D. |
| **Control** | 1 | 26 ± 3 | 3.0 ± 0.6 | 2.6 ± 0.2 | 0.50 ± 0.06 | 0.046 ± 0.012 |
| **Pb** | | **56** ± 4 * | 2.6 ± 0.4 | **6.7** ± 0.3 * | 0.44 ± 0.05 | **0.144** ± 0.009 * |
| **Pb-EDTA-a** | | **40** ± 3 * | 3.2 ± 0.6 | 3.2 ± 0.9 | 0.49 ± 0.10 | **0.083** ± 0.019 * |
| **Pb-EDTA-b** | | 33 ± 4 | 2.9 ± 0.7 | 3.1 ± 0.7 | 0.52 ± 0.05 | **0.075** ± 0.001 * |
| **Pb-CA-a** | | 32 ± 5 | 2.5 ± 0.3 | 2.8 ± 0.5 | 0.52 ± 0.01 | **0.098** ± 0.011 * |
| **Pb-CA-b** | | **36** ± 3 * | 2.8 ± 0.1 | 3.2 ± 0.5 | 0.45 ± 0.02 | **0.078** ± 0.005 * |
| **Control** | 4 | 28 ± 7 | 2.9 ± 0.4 | 3.1 ± 0.4 | 0.55 ± 0.11 | 0.053 ± 0.004 |
| **Pb** | | **45** ± 4 * | **2.2** ± 0.2 * | 3.9 ± 0.1 | **0.83** ± 0.14 * | **0.075** ± 0.016 * |
| **Pb-EDTA-a** | | 32 ± 8 | 2.9 ± 0.4 | **4.4** ± 0.2 * | 0.45 ± 0.01 | **0.093** ± 0.012 * |
| **Pb-EDTA-b** | | 28 ± 4 | 3.0 ± 0.2 | 3.3 ± 0.3 | 0.52 ± 0.07 | **0.070** ± 0.007 * |
| **Pb-CA-a** | | **48** ± 3 * | 2.5 ± 0.3 | **8.3** ± 1.1 * | **0.77** ± 0.04 * | **0.127** ± 0.014 * |
| **Pb-CA-b** | | **52** ± 9 * | 2.6 ± 0.3 | **7.7** ± 2.0 * | **0.73** ± 0.14 * | **0.154** ± 0.019 * |
| **Control** | 8 | 27 ± 2 | 3.0 ± 0.4 | 3.3 ± 0.2 | 0.56 ± 0.04 | 0.064 ± 0.007 |
| **Pb** | | **38** ± 2 * | **1.9** ± 0.4 * | **4.5** ± 0.8 * | 0.49 ± 0.12 | **0.083** ± 0.001 * |
| **Pb-EDTA-a** | | 28 ± 3 | 2.5 ± 0.7 | 3.1 ± 0.5 | 0.51 ± 0.08 | 0.070 ± 0.002 |
| **Pb-EDTA-b** | | 34 ± 3 | 2.8 ± 0.6 | 2.9 ± 0.5 | 0.53 ± 0.08 | 0.078 ± 0.009 |
| **Pb-CA-a** | | **41** ± 10 * | **1.8** ± 0.2 * | **5.1** ± 0.6 * | **0.42** ± 0.07 * | **0.081** ± 0.006 * |
| **Pb-CA-b** | | **44** ± 2 * | **1.7** ± 0.3 * | **5.3** ± 0.4 * | 0.58 ± 0.03 | **0.092** ± 0.018 * |
| **Control** | 12 | 25 ± 5 | 2.8 ± 0.5 | 4.1 ± 0.7 | 0.46 ± 0.08 | 0.061 ± 0.011 |
| **Pb** | | 31 ± 3 | **2.2** ± 0.4 * | **6.3** ± 0.8 * | **0.59** ± 0.02 * | **0.124** ± 0.015 * |
| **Pb-EDTA-a** | | 27 ± 4 | 2.3 ± 0.6 | 4.0 ± 0.5 | 0.56 ± 0.10 | 0.072 ± 0.008 |
| **Pb-EDTA-b** | | 29 ± 2 | 2.8 ± 0.6 | 3.6 ± 0.5 | 0.52 ± 0.09 | 0.065 ± 0.002 |
| **Pb-CA-a** | | 34 ± 9 | **1.7** ± 0.7 * | **2.8** ± 0.3 * | **0.59** ± 0.03 * | **0.092** ± 0.007 * |
| **Pb-CA-b** | | **38** ± 6 * | **1.7** ± 0.4 * | 3.7 ± 1.1 | **0.55** ± 0.04 * | **0.082** ± 0.008 * |
| **Control** | 24 | 28 ± 4 | 2.8 ± 0.2 | 3.2 ± 0.5 | 0.38 ± 0.24 | 0.049 ± 0.008 |
| **Pb** | | 35 ± 4 | **1.3** ± 0.3 * | **4.5** ± 0.5 | **0.72** ± 0.24 * | **0.199** ± 0.024 * |
| **Pb-EDTA-a** | | 33 ± 6 | 2.5 ± 0.4 | 3.0 ± 0.2 | 0.48 ± 0.09 | **0.074** ± 0.005 |
| **Pb-EDTA-b** | | 26 ± 3 | 2.8 ± 0.6 | 3.6 ± 0.4 | 0.52 ± 0.05 | 0.054 ± 0.017 |
| **Pb-CA-a** | | **39** ± 3 * | **1.1** ± 0.2 * | **7.3** ± 0.8 | **0.87** ± 0.06 * | **0.167** ± 0.034 * |
| **Pb-CA-b** | | **36** ± 4 * | **1.1** ± 0.4 * | **6.3** ± 0.9 | **0.75** ± 0.01 * | **0.179** ± 0.025 * |

**Table-5.** Effect of ligands on Pb-induced modification of enzyme activity ($U.g^{-1}$ protein) in leaves. Values are means of 6 replicates. Significant differences at $P < 0.05$ are indicated with an asterisk (*).

| Treatments | Time | SOD | CAT | GPOX | APX | GR |
|---|---|---|---|---|---|---|
| | (h) | Mean±S.D. | Mean±S.D. | Mean±S.D. | Mean±S.D. | Mean±S.D. |
| **Control** | 1 | 48 ± 2 | 2.09 ± 0.06 | 0.22 ± 0.04 | 0.13 ± 0.01 | 0.060 ± 0.004 |
| **Pb** | | 55 ± 4 | 2.04 ± 0.18 | 0.26 ± 0.04 | 0.13 ± 0.03 | 0.059 ± 0.011 |
| **Pb-EDTA-a** | | 53 ± 9 | 2.09 ± 0.46 | 0.27 ± 0.03 | 0.15 ± 0.04 | 0.064 ± 0.007 |
| **Pb-EDTA-b** | | 51 ± 12 | 2.13 ± 0.44 | 0.20 ± 0.01 | 0.11 ± 0.02 | 0.060 ± 0.002 |
| **Pb-CA-a** | | 49 ± 10 | 2.10 ± 0.61 | 0.24 ± 0.03 | 0.14 ± 0.02 | 0.055 ± 0.003 |
| **Pb-CA-b** | | 50 ± 4 | 2.06 ± 0.42 | 0.23 ± 0.01 | 0.13 ± 0.02 | 0.062 ± 0.002 |
| **Control** | 4 | 54 ± 5 | 2.02 ± 0.18 | 0.23 ± 0.05 | 0.15 ± 0.05 | 0.064 ± 0.010 |
| **Pb** | | 48 ± 2 | 2.00 ± 0.18 | 0.25 ± 0.01 | 0.14 ± 0.03 | 0.077 ± 0.005 |
| **Pb-EDTA-a** | | 47 ± 2 | 1.91 ± 0.39 | 0.25 ± 0.05 | 0.13 ± 0.02 | 0.078 ± 0.009 |
| **Pb-EDTA-b** | | 47 ± 6 | 1.88 ± 0.11 | 0.23 ± 0.03 | 0.14 ± 0.05 | 0.068 ± 0.012 |
| **Pb-CA-a** | | 53 ± 15 | 1.90 ± 0.16 | 0.22 ± 0.02 | 0.14 ± 0.01 | 0.064 ± 0.014 |
| **Pb-CA-b** | | 57 ± 10 | 1.88 ± 0.09 | 0.23 ± 0.04 | 0.13 ± 0.01 | 0.067 ± 0.005 |
| **Control** | 8 | 46 ± 9 | 1.95 ± 0.16 | 0.25 ± 0.06 | 0.15 ± 0.03 | 0.067 ± 0.021 |
| **Pb** | | **77** ± 4 * | 1.92 ± 0.38 | **0.44** ± 0.01 * | **0.19** ± 0.03 * | 0.065 ± 0.005 |
| **Pb-EDTA-a** | | 49 ± 10 | 1.84 ± 0.32 | **0.33** ± 0.01 | 0.16 ± 0.05 | 0.056 ± 0.010 |
| **Pb-EDTA-b** | | 46 ± 11 | 2.03 ± 0.06 | 0.26 ± 0.03 | 0.15 ± 0.01 | 0.065 ± 0.010 |
| **Pb-CA-a** | | 53 ± 10 | 1.93 ± 0.62 | 0.27 ± 0.00 | 0.16 ± 0.04 | 0.077 ± 0.006 |
| **Pb-CA-b** | | 56 ± 11 | 1.83 ± 0.06 | 0.29 ± 0.10 | 0.14 ± 0.02 | 0.072 ± 0.008 |
| **Control** | 12 | 49 ± 8 | 1.88 ± 0.16 | 0.24 ± 0.06 | 0.13 ± 0.01 | 0.064 ± 0.003 |
| **Pb** | | **80** ± 8 * | 1.60 ± 0.23 | **0.42** ± 0.04 * | **0.19** ± 0.06 * | **0.094** ± 0.009 * |
| **Pb-EDTA-a** | | 53 ± 9 | 1.84 ± 0.13 | 0.28 ± 0.08 | **0.18** ± 0.04 * | 0.079 ± 0.019 |
| **Pb-EDTA-b** | | 47 ± 7 | 1.91 ± 0.38 | 0.22 ± 0.03 | 0.16 ± 0.03 | 0.069 ± 0.006 |
| **Pb-CA-a** | | **78** ± 13 * | 1.62 ± 0.52 | **0.36** ± 0.05 * | **0.21** ± 0.04 * | 0.074 ± 0.002 |
| **Pb-CA-b** | | **76** ± 10 * | 1.66 ± 0.33 | **0.42** ± 0.06 * | **0.23** ± 0.06 * | 0.078 ± 0.011 |
| **Control** | 24 | 48 ± 3 | 2.06 ± 0.17 | 0.24 ± 0.03 | 0.15 ± 0.04 | 0.069 ± 0.013 |
| **Pb** | | **63** ± 6 * | **1.35** ± 0.11 * | 0.25 ± 0.01 | **0.21** ± 0.02 * | **0.110** ± 0.004 * |
| **Pb-EDTA-a** | | 55 ± 8 | **1.51** ± 0.22 * | 0.27 ± 0.04 | 0.18 ± 0.02 | 0.086 ± 0.013 |
| **Pb-EDTA-b** | | 50 ± 8 | 1.91 ± 0.11 | 0.24 ± 0.05 | 0.17 ± 0.05 | 0.074 ± 0.005 |
| **Pb-CA-a** | | **70** ± 8 * | **1.44** ± 0.13 * | **0.42** ± 0.05 * | **0.24** ± 0.02 * | **0.117** ± 0.014 * |
| **Pb-CA-b** | | **78** ± 22 * | **1.56** ± 0.05 * | **0.50** ± 0.03 * | **0.24** ± 0.05 * | **0.112** ± 0.016 * |

### *3.5. Principle components analysis*

In PCA, the large numbers of independent variables are transformed to an orthogonal set (common or principal components called PCs) which have a mutual correlation for all the independent variables. These PCs have a mutual correlation for all the independent variables (Fig. 2).

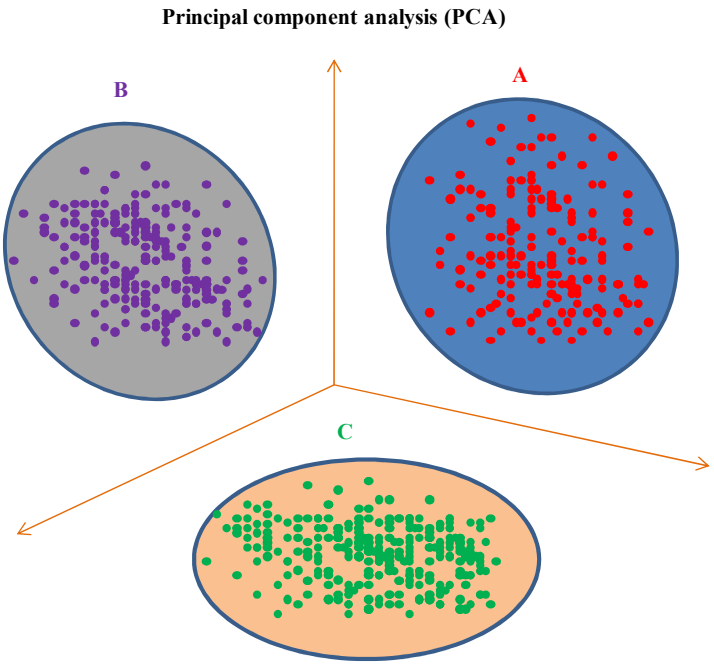


**Fig 2:** Representative diagrame of PCA.

Fig. 3 showed that the first two factors (PC1 and PC2) explained 69 and 86 % of the variation in the chemical trait data respectively for roots and leaves. PCA divided the treatment into two separate groups (indicated by circles) on the left and right side of the y-axis both in roots and leaves. The left side group includes Pb, Pb-CA-a and Pb-CA-b whereas the right side group includes the control, EDTA-b, CA-b, Pb-EDTA-a and Pb-EDTA-b. Moreover the right side group was further divided into controls and Pb-EDTA below and above x-axis, respectively.

On the other hand, toxicity parameters were divided into three different groups (indicated by rectangles) (Fig. 3). GR was grouped with Pb contents on the left side above the x-axis for both leaves and roots whereas APX was grouped with them for roots only. SOD, GPOX, $H_2O_2$ and LPO were grouped together on the left side below the x-axis for both leaves and roots. In contrast, CAT was separated from all other enzymes on the right side of

the y-axis for both roots and leaves. Pigment contents in leaves were also grouped together with CAT.

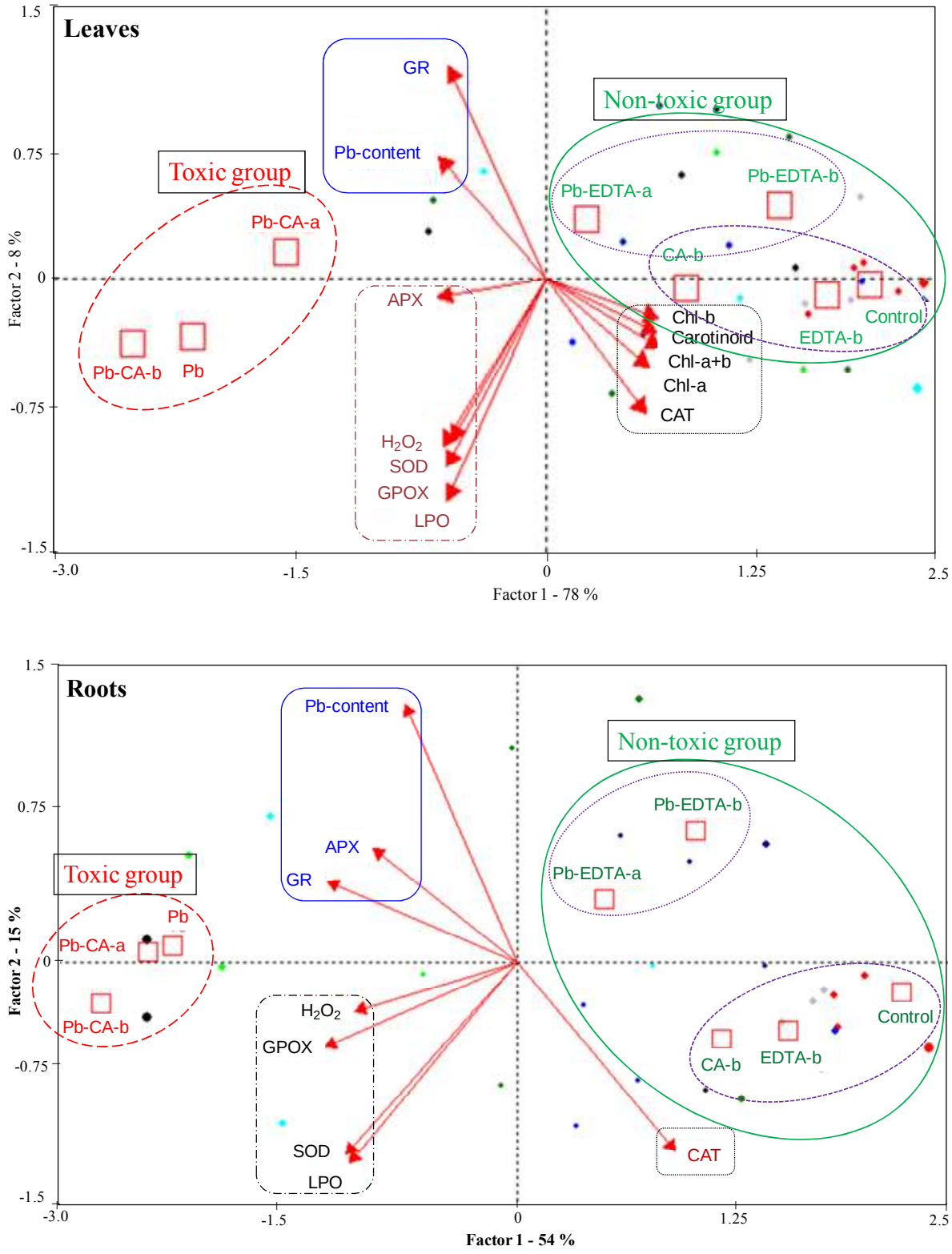


**Fig 3:** Comparison of all the treatments and techniques using principal component analysis in roots and leaves.

## 4. Discussion

### *4.1. Pb uptake by V. faba in the presence of organic ligands*

The results show that the majority of Pb absorbed (approximately 90-95 %) is stored in the roots and only a small fraction is translocated to above-ground parts after 24 h of Pb incubation (Fig. 1). The metal translocation restriction phenomenon illustrates the well-known ability of Pb to link to biological molecules such as sugars, pectins, celluloses and hemicelluloses and has been documented earlier in *V. faba* by Piechalak et al. (2002) and in *Allium sativum* by Liu et al. (2009). Moreover, two phases of maximum Pb uptake (after 1 and 12 h) by *V. faba* roots were observed. Pb uptake was maximum after 1 h (33 $\mu g.g^{-1}$ DW $h^{-1}$) and decreased continuously up to 8 h (2 $\mu g.g^{-1}$ DW $h^{-1}$) then again increased at 12 h (5 $\mu g.g^{-1}$ DW $h^{-1}$) followed by another decrease at 24 h (2 $\mu g.g^{-1}$ DW $h^{-1}$). In stem and leaves, Pb translocation rate increased till 8 h (0.39 and 0.24 $\mu g.g^{-1}$ DW $h^{-1}$, respectively) and then decreased at 24 h (0.06 and 0.14 $\mu g.g^{-1}$ DW $h^{-1}$, respectively). The contrasting trends in the rate of Pb uptake by *V. faba* roots and translocation to aerial parts show that Pb movement does not occur freely from root to shoot. Pb translocation from root to shoot needs more than 8 hours to become significant. In addition, movement of Pb from stem to leaves occurred freely with the same kinetics.

The EDTA-induced increase in Pb uptake by plants roots has been documented in several previous studies since last decade (Ruley et al. 2006; Piechalak et al. 2003; Huang et al. 1997). However, in our experimental conditions, a decrease in Pb translocation in the presence of EDTA might be due to shorter exposure times than in the literature (24 h *vs* more than 1 week). In addition, the nature of the plant studied (sensitive to Pb) and/or the absence of Pb transporter proteins (Schaider et al. 2006) could be the possible reasons of these results. The data suggests two possible pathways of Pb transfer: (i) an ionic form, using ion channels and interacting with cell components and/or transporters which store or transfer Pb to stem and shoots, (ii) a non-ionic form (chelated form), able to enter and be quickly accumulated in the roots, and stored for example in the vacuoles or cell walls.

With CA, the uptake and translocation of Pb to aerial parts increased slightly but no treatment led to a significant difference (Fig. 1). The effect of CA on metal uptake is plant dependent and is generally attributed to increases in exchangeable metal contents (Kim et al. 2010; Quartacci et al. 2006), and a decrease in the pH of culture media (Quartacci et al. 2006;

Chen et al. 2003). Kim et al. (2010) reported that the non-significant effect of CA on Pb uptake by *Brassica juncea* was due to both stable soil pH and unchanged soluble Pb contents. Acidification after CA application did not happen in our experimental condition, because the pH value was kept constant at 5. This could the reason of non significant effect of CA on Pb uptake by *V. faba*.

### *4.2. Effect of organic ligands on chlorophyll contents*

The decrease in pigment contents is a well-known aspect of Pb toxicity and has been widely documented in the literature. Recently, Cenkci et al. (2010) reported a negative correlation between Pb concentration and chlorophyll content in *Brassica rapa*. In our experimental conditions, Pb also decreased Chl-a, Chl-b and carotinoid contents by 11, 19 and 33 % respectively at 24 hours (Table 2).

Addition of EDTA decreased Pb-induced reduction of pigment contents in a dose dependent manner (Table 2). Recently, Saifullah et al. (2010) reported a significantly increased photosynthetic rate in *Triticum aestivumn* after EDTA application in Pb-contaminated loamy sand soil, along with increased uptake and translocation of Pb to aerial parts. Jamil et al. (2009) reported that increased uptake of essential elements might be responsible for increased chlorophyll synthesis on addition of EDTA which can overcome Pb-induced reduction of pigment level. In our experiments, decreased translocation of Pb to aerial parts (43 % after 24 h, Fig. 1) in the presence of EDTA could explain the absence of Pb toxicity on pigment contents.

In the presence of CA, Pb toxicity to pigment contents increased slightly at both levels of application (Table 2). These results indicate the inefficiency of CA against Pb toxicity. The slight increase in Pb toxicity might be due to a slight increase in Pb uptake and translocation in the presence of CA. Ruley et al. (2006) reported reduced toxic effects of Pb to Chl-a fluorescence kinetics and growth of *Sesbania drummondii* with high concentrations of CA that can effectively bind Pb.

### *4.3. Effect of organic ligands on $H_2O_2$ induction and LPO*

Pb-induced overproduction of ROS resulting in membrane damage has been reported in a number of previous studies (Zhou et al. 2010; Wang et al. 2010; Liu et al. 2009; Pourrut et al. 2008; Islam et al. 2008). In this study, the time course experiment showed that Pb-induced

LPO occurred after the Pb concentration reached significant levels in roots (after 1 h) and leaves (after 8 h) (Table 3). This suggests that Pb-induced production of $H_2O_2$ and LPO is a very rapid phenomenon, highlighting the importance of time dependent short-duration oxidative studies. Moreover, the oxidative bursts in roots and leaves coincided with periods of high Pb entrance rates to these tissues (1 and 12 h). Therefore, although cause of sequential Pb uptake is still unknown, the consequence is increased generation of ROS which exceed antioxidant capacities and generated LPO. The results also showed longer and intense LPO in leaves compared to roots under a very low concentration of Pb (almost 35 times lower compared to roots) showing high sensitivity of *V. faba* leaves to Pb.

Application of Pb-EDTA treatments to *V. faba* seedlings decreased Pb-induced LPO significantly by reducing ROS production (Table 3). The results are in line with those of Liu et al. (2008) where the presence of EDTA reduced $H_2O_2$ and TBARS production in non-accumulating *Sedum alfredii* Hance both in leaves and roots**.** Similar results were also reported by Huange et al. (2008) who found that the Pb stress increased the ROS content significantly, whereas Pb-EDTA decreased the $H_2O_2$ burst. Indeed, EDTA masks the toxic effect of Pb by forming soluble, very stable (due to the high stability constant, log $K$ = 17.88) and non-toxic complexes with free $Pb^{2+}$ in solution.

Application of CA in general had no significant effects on ROS production or LPO in *V. faba* seedlings except that their induction was delayed both in roots and leaves (Table 3). There is no data in the literature concerning the effect of CA on $H_2O_2$ induction and LPO. Based on the results presented, a possible explanation for this delay could be the degradation rate of the Pb-CA complex. The Pb-CA complex dissociates just before or after uptake due to a lower stability constant (log $K$ = 5.67) thus delaying the production of the toxic form of Pb ($Pb^{2+}$) in *V. faba* roots and ultimately $H_2O_2$ production and LPO. These results underline the toxicity of the $Pb^{2+}$ free cations.

### *4.4. Effect of organic ligands on enzyme activities*

The stimulation of APX, GPOX, SOD and GR by Pb both in roots and leaves confirmed the Pb-induced production of ROS during the first 24 h of Pb incubation (Table 4, 5). Recently, Wang et al. (2010) also reported Pb-induced activation of antioxidant enzymes due to over-production of ROS in *V. faba* grown under increasing concentrations of Pb-added soils (0–2000 $mg.kg^{-1}$). SOD is considered as the first defense against oxidative stress (Liu et al. 2009;

Mishra et al. 2006). This enzyme dismutes the anion superoxide radical to $H_2O_2$ and oxygen and thus maintains superoxide radicals in steady state levels (Islam et al. 2008). $H_2O_2$ is a very strong oxidant and requires quick removal. This is achieved by the action of APX in ascorbate-glutathione cycle or by GPOX and CAT in the cytoplasm and other cellular compartments (Mishra et al. 2006). The role of reduced glutathione (GSH) and glutathione reductase (GR) in the $H_2O_2$-scavenging mechanism in plant cells is well established in the Halliwell-Asada enzyme pathway (Foyer and Noctor, 1998). Moreover, antioxidant enzymes might be activated due to an increase in the concentration of their substrates instead of direct interaction with Pb (Islam et al. 2008). The decline observed for CAT in the presence of Pb could be due to direct interaction between Pb and catalase or to a change in the assembly of its subunits (Mishra et al. 2006). Pb is also known to inhibit activities of enzymes at the cellular level by directly binding to the active center of the enzyme (Shahid et al. 2011b; Mishra et al. 2006) or indirectly by induction of $H_2O_2$ (Qureshi et al. 2007).

Pb-induced activation of enzymes was prevented in the presence of EDTA (Tables 4 and 5). The results are in line with those of Piechalak et al. (2003) where application of EDTA inhibited Pb-induced activation of glutathione, homoglutathione and phytochelatins up to 24 h in *Pisum sativum*. Inhibition of Pb-induced activation of antioxidant enzymes by EDTA is also reported by Ruley et al. (2004) in *Sesbania drummondii* and by Huange et al. (2008) in *Sedum alfredii* Hance. Like $H_2O_2$ and TBARS production, antioxidant enzyme activities were also delayed by CA application (Tables 4 and 5). The reason could be delayed induction of ROS due to late production of $Pb^{2+}$ ions by degradation of the Pb-CA complex.

### *4.5. Comparison of treatments and toxicity techniques by principle component analysis*

PCA divided the treatments in two separate groups (Fig. 3) based on associated toxicity; the left side group with toxic treatments (Pb alone, Pb-CA-a and Pb-CA-b), whereas the right side contained non-toxic treatments (control, EDTA-b, CA-b, Pb-EDTA-a and Pb-EDTA-b). The PCA approach confirmed the protective role of EDTA and the inefficiency of CA against Pb toxicity. Sub-division of the right side into two groups of treatment: control, EDTA alone, CA alone (below x-axis) and Pb-EDTAa and Pb-EDTAb (above x-axis), indicates (i) the absence of CA and EDTA toxicity when they are used alone and (ii) the presence of Pb to the plants even if slight toxicity was recorded.

The division of toxicity parameters into three groups indicated their different roles against Pb toxicity in relation with speciation. The grouping of $H_2O_2$, LPO, SOD and GPOX in the same cluster in roots and leaves indicated the induction of oxidative stress. Separation of CAT from other anti-oxidant enzymes both in roots and leaves was due to the inhibition of its activity compared to other enzymes. In leaves, pigment contents were clustered with CAT, due to Pb-induced degradation of pigment contents as previously described (Fig. 3).

In roots, the presence of GR, APX and Pb contents in the same group indicated that these two enzymes function in accordance with Pb content. In leaves, GR alone was grouped with Pb content (Fig. 3). Indeed, these two enzymes are involved in plant detoxification mechanisms as described by the Halliwell-Asada enzyme pathway (Foyer and Noctor 1998). GR contributes to metal detoxification either by its role in the ascorbate–glutathione cycle by maintaining a high GSH/GSSG ratio (Foyer and Noctor 1998) or direct binding of Pb to sulphur atoms of cysteine and to oxygen atoms of glycine carboxylate (Cruz et al. 2001). GR might independently detoxify ROS via GSH under low Pb contents in leaves compared to roots (35-fold lower in leaves). Moreover, different forms of Pb in leaves and roots may also be responsible for separation of APX and GR in leaves. In addition, GR and APX in leaves could interact with biochemical processes independently of oxidative stress. Mishra et al. (2006) reported increased activity of GR despite low GSH/GSSG ratios which might be due to high rates of GSH consumption or to some other detoxification roles of GR under Pb stress.

## 5. Conclusion

The present study first highlighted the influence of Pb speciation on its uptake and phytotoxicity by sensitive *V. faba plant*. EDTA increased Pb uptake and accumulation by the roots while it strongly reduced Pb translocation to aerial parts. In contrast, CA had no effect on Pb uptake or translocation. EDTA reduced Pb-induced oxidative stress by forming stable and non-toxic complexes; CA had no influence on Pb-induced toxicity to *V. faba* roots, due to week complexation with Pb. Induction of phytotoxicity by free $Pb^{2+}$ cations was demonstrated by these results.

Moreover, the relationship between ROS induction, LPO and enzyme activation in response to Pb uptake by plants was highlighted. The results suggest that Pb-induced oxidative stress in *V. faba* coincides with $Pb^{2+}$ ions entrance in the tissue. These results

confirm that *V. faba* is a sensitive plant model in accordance with Pb speciation for short term ecotoxicological test. The principal component analysis shows that GR and APX are the main enzymes reacting against Pb toxicity in relation with Pb uptake in *V. faba* roots while GR reacts alone in leaves. GR activity in both roots and leaves could, therefore, be a good indicator of Pb-induced stress in Pb-sensitive plants.

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# Section 3B:

## Pb-induced genotoxicity to *Vicia faba* L. roots in relation with metal cell uptake and initial speciation

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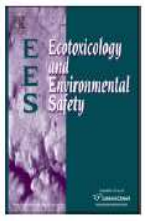


### Lead-induced genotoxicity to *Vicia faba* L. roots in relation with metal cell uptake and initial speciation

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**Forewords**

The previous study showed that the specific responses of *V. faba* physiological status to Pb toxicity are the function of metal speciation and duration of exposure. The uptake of Pb by *V. faba* roots, translocation to arial parts and phyto-toxicity depend on its speciation. Toxicity of Pb significantly reduced pigment contents induced lipid peroxidation and activated antioxidant enzymes activities (SOD, GPOX, APX and GR) both in roots and leaves. In contrast to other enzymes, CAT activity was depressed in the presence of Pb. The EDTA alleviated Pb-induced toxicity in dose dependent manner, whereas CA was inefficient towards Pb uptake and Phyto-toxicity.

Litrature alos showed that Pb induces genotoxicity to *V. faba* seedlings (Pourrut et al. 2008). However, no study deals with the mechanism behind this genetic toxicity of Pb in living organisms. Moreover, no literature data is available on Pb induced genotoxicity in relation with speciation. Therefore, the present study was performed to identify the effect of Pb speciation on Pb-induced genotoxicity to *V. faba* seedlings. Moreover, in order to make a relationship between Pb speciation and genotoxicity, Pb was exposed to *V. faba* seedlings alone and chelated at different levels by EDTA and CA. Pb was chelated 25, 40, 75 and 99 % by EDTA whereas 15, 25, 40 % by CA. Indeed, these chelated and free ion cnoncentrations of Pb were used to compare Pb toxicity with respect to chelated and free Pb ion concentration along with applied levels. The Visual Minteq software version 2.60 (Gustafsson, 2008) was used to calculate the speciation of Pb in nutrient solution in the presence of EDTA and CA. These calculations were used as experimental design. However, CA was found to be toxic at concentrations >1000 µM, which restricted its use upto 40 % of Pb chelation. *Vicia faba* micronucleus test was used to evaluate Pb-induced genotoxicity as described earlier (Marcato-Romain et al. 2009; El Hajjouji et al. 2007; Duan et al. 2000). This test is recommended by the AFNOR NF T 90-327 for the assessment of genotoxicity due to high sensitivity of this method. The cells and chromosomes of *Vicia faba* are large, so one can easily identify the damage caused by pollutants.

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# Pb-induced genotoxicity to *Vicia faba* L. roots in relation with metal cell uptake and initial speciation

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**Abstract**

Formation of organometallic complexes in soil solution, strongly influence metals phytoavailability. However, only few studies deal with the influence of metal speciation both on plant uptake and genotoxicity. In the present study, *V. faba* seedlings were exposed for 6 h in controlled hydroponic conditions to 5µM of Pb nitrate alone and chelated to varying degrees by different organic ligands. Ethylenediaminetetraacetic acid and citric acid were respectively chosen as models of humic substances and low weight organic acids present in natural soil solutions. Visual Minteq software was used to estimate free Pb cations concentration and ultimately to design the experimental layout. For all experimental conditions, both micronucleus test and measure of Pb uptake by plants were finally performed. Chelation of Pb by EDTA, a strong chelator, dose-dependently increased the uptake in *V. faba* roots while its genotoxicity was significantly reduced, suggesting a protective role of EDTA. A weak correlation was observed between total Pb concentration absorbed by roots and genotoxicity ($r^2$ = 0.65). In contrast, a strong relationship ($r^2$ = 0.93) exists between $Pb^{2+}$ concentration in exposure media and genotoxicity in the experiment performed with EDTA. Citric acid induced labile organometallic complexes and did not demonstrate any significant changes in Pb genotoxicity or uptake. These results demonstrate that metal speciation knowledge could improve the interpretation of *V. faba* genotoxicity test performed to test soil quality.

***Key words***: Phyto-availability, organometallic complex, micronucleus, citric acid, EDTA, Visual Minteq, soil solution.

## 1. Introduction

Pb is one of the most useful and toxic metals present in the environment on a global scale (Sharma and Dubey, 2005; Arshad et al., 2008; Uzu et al., 2009). When exposed to this metal, even at low concentrations, plants usually experience harmful effects such as micronucleus induction (National Toxicology Program, 2003), mitosis disturbance (Wierzbicka, 1999), DNA damage (Gichner et al., 2008) alterations in membrane permeability (Sharma and Dubey, 2005) and disturbance (inhibition or activation) of enzymatic activities (Reddy et al., 2005) along with various physiological impacts.

Recent literature indicates that the toxicity and/or bioavailability of trace metals, in addition to their mobility, show marked dependence on their speciation (Uzu et al., 2009) and these responses often correlate best with the activity of free metal ion (Dumat et al., 2001; Doig and Liber, 2007). Interactions between organic compounds and metals in natural media have been particularly studied due to their strong effects on metal behaviour (Quenea et al., 2009). These organic compounds, when present in growth medium, can adsorb or complex metals; affecting their mobility, uptake and even cytotoxicity. Moreover, it was observed that Pb can be accumulated and bound within the polysaccharides of cell walls (Sharma and Dubey, 2005). Therefore, in addition to knowing the total metal concentration, predicting the relevant species of metals is of utmost importance in improving our understanding of the mechanisms of metal uptake, accumulation and cytotoxicity.

The use of short-term bioassays, especially genetic toxicity bioassays, to assess potent environmental pollutants has gained special attention over last decades. These assays are capable of predicting the genotoxic potential of the pollutant under investigation by measuring gene mutations and damage to chromosomes and DNA. Plant assays are quite easy to conduct, inexpensive, rapid, and good predictors of genotoxicity (Panda and Panda, 2002). In particular, the *V. faba* micronucleus test is a very sensitive and useful method for the detection of both clastogenic and aneugenic effects (Duan et al., 2000; El Hajjouji et al., 2007; Marcato-Romain et al., 2009).

Pb-induced genotoxicity to *V. faba* was studied using current contact with aqueous extract or direct contact with soil by Marcato-Romain et al. (2009). Due to lower genotoxicity observed in the case of direct contact with soil, these authors concluded to a potential influence of soil organic matters. As soil solution represents the main available compartment for metals uptake by plants (Degryse et al., 2009; Gandois et al., 2009), one scientific question asked is the relation between aqueous extract of soil used in current genotoxicity *V. faba* test and soil solution. Therefore, the objectives of the present study were to identify the correlation between Pb speciation, phyto-availability and genotoxicity to *V. faba* as a function of organic chelates nature and levels.

## 2. Materials and methods

### *2.1. Plant materials and growth conditions*

Dry seeds of broad beans (*V. faba* L. stored at 4 C°) were germinated on moistened filter paper in a germination chamber under optimal conditions of germination, i.e. in darkness at 22°C temperature and 100% humidity. After 5-7 days, when the primary roots were about 2–3 cm in length, the seedlings were transplanted to a PVC tank (3 plants per tank) containing continuously aerated modified Hoagland nutrient solution (see Uzu et al., 2009 for more detail) with the macro-elements: 5 mM $KNO_3$, 5 mM $Ca(NO_3)_2$, 2mM $KH_2PO_4$ and 1.5 mM $MgSO_4$ and micro-elements: 9.11 µM $MnSO_4$, 1.53 µM $ZnSO_4$, 0.235 µM $CuSO_4$, 24.05 µM $H_3BO_3$, 0.1 µM $Na_2MoO_4$ and 268.6 µM Fe/EDTA. Nutrient solution was renewed on alternate days to keep its composition and pH constant.

After pre-culturing for 15 days, plants were exposed for 6 h to 5 µM Pb alone or chelated by EDTA or citric acid (Table 1). EDTA, a strong organic chelator for metals, was chosen to act as a model compound for humic substances (Lai and Chen, 2005; Meers et al., 2005) and citric acid was chosen to model low-molecular-weight organic acids (Chen et al., 2003; Muhammad et al., 2009). The concentration of 5 µM $Pb(NO_3)_2$ was chosen because according to Pourrut et al. (2008), it induces genotoxicity for *V. faba* while remaining quite low and representative of the pollution of the environment. The concentration of $KH_2PO_4$ in the nutrient solution used for Pb treatment was reduced to 0.2 mM in order to prevent phosphate precipitation (Kopittke et al., 2008; Waranusantigul et al., 2008). Control plants were also cultured in the appropriate uncontaminated media with reduced $PO_4^{3-}$ concentration. All plants were grown under controlled conditions in a 16-h photoperiod at 70 % relative humidity and day/night temperatures of 24/22 °C. Light was supplied by 600 W Osram Nav-T Super High Pressure Sodium Lamps providing a minimum photosynthetic photon flux density of 500 $\mu mol.m^{-2}.s^{-1}$ at the top of the plant (Pourrut et al., 2008).

### *2.2. Determination of Pb concentration and speciation in solution with Visual Minteq*

A 50 µM Pb solution was first prepared from $Pb(NO_3)_2$ and mQ water. That concentrated solution was then filtered (0.22 µm), acidified to pH 5.0 with distilled $HNO_3$ (15 M, suprapur 99.9 %) and stored at 4°C before Pb analysis by inductively coupled plasma – atomic emission spectrometry (ICP-AES) with an IRIS Intrepid II XDL. The measured Pb concentration was 50.02 µM Pb. That concentrated Pb solution was then diluted ($^1/_{10}$) by

mixture with mQ water or ethylenediaminetetraacetic acid (EDTA) or citric acid (CA) in order to obtain the various exposure solutions (Pb alone or with organic ligands). The pH was adjusted to 5 with distilled $HNO_3$ (15 M, suprapur 99.9 %) and Pb concentration was checked on ten replicates: 5. 01 ± 0.02 µM Pb. Without contact with plants, both pH and total Pb concentration measured for the various exposure solutions, in function of time, stayed constant after 6 h. Therefore, for the various estimations of Pb chemical speciation with Visual Minteq software a total Pb concentration of 5 µM was used.

**Table-1.** Experimental design and speciation of Pb in modified Hoagland solution under EDTA and citric acid calculated using Visual Minteq 2.60 (Gustafsson, 2008). In treatments notation, the numbers 15, 25, 40, 75 and 99 represent % of Pb chelated by EDTA and citric acid. Pb concentration was kept constant at 5 µM in all treatments except controls. The treatments without Pb (PC, EDTA-75, EDTA-99, CA-25, CA-40) were only used in the micronucleus test as controls.

| Treatments | Visual Minteq input values | Visual Minteq calculations | |
|---|---|---|---|
| notations | Treatments medium composition | Pb-chelated (%) | Pb-free (%) |
| Pb | HS[a] + $Pb(NO_3)_2$ (5 µM) | 0 | 85 |
| Pb-EDTA-25 | HS + $Pb(NO_3)_2$ + EDTA (1.45 µM) | 25 | 64 |
| Pb-EDTA-40 | HS + $Pb(NO_3)_2$ + EDTA (2.25 µM) | 40 | 51 |
| Pb-EDTA-75 | HS + $Pb(NO_3)_2$ + EDTA (4.25 µM) | 75 | 21 |
| Pb-EDTA-99 | HS + $Pb(NO_3)_2$ + EDTA (10 µM) | 99 | 1 |
| Pb-CA-15 | HS + $Pb(NO_3)_2$ + citric acid (300 µM) | 15 | 73 |
| Pb-CA-25 | HS + $Pb(NO_3)_2$ + citric acid (550 µM) | 25 | 64 |
| Pb-CA-40 | HS + $Pb(NO_3)_2$ + citric acid (1000 µM) | 40 | 51 |
| **Controls without Pb** | | | |
| NC[b] | Hoagland solution (HS) | – | – |
| PC[c] | HS + Maleic hydrazeide (40 µM) | – | – |
| EDTA-75 | HS + EDTA (4.25 µM) | – | – |
| EDTA-99 | HS + EDTA (10 µM) | – | – |
| CA-25 | HS + citric acid (550 µM) | – | – |
| CA-40 | HS + citric acid (1000 µM) | – | – |

[a]Hoagland solution (concentrations in introduction), [b]Negative control, [c]Positive control.

The Visual Minteq software version 2.60 (Gustafsson, 2008) was used to calculate the concentration of EDTA and citric acid required for the chelation of 15, 25, 40, 75 and 99 % of the Pb contained in the nutrient solution. Visual Minteq is a chemical equilibrium model extensively used in the literature (Ge et al., 2005; Doig and Liber, 2007) for the accurate calculation of metal speciation, precipitation and dissolution. Metal ion speciation is calculated using equilibrium constants from the Minteq database (Table 2). The concentration of the elements present in modified Hoagland solution, 5 µM Pb and EDTA or citric acid at different concentrations were used as input for the Visual Minteq model, at pH 5.0, 25 °C and an ionic strength 0.1 M (Table 1).

**Table-2.** Equilibrium constants used for the prediction of Pb speciation in nutrient solution. Equilibrium constants are the default values of the Minteq database from Visual Minteq 2.60 (Gustafsson, 2008).

| **Reactions** | **Log K** |
|---|---|
| $Pb^{2+} + H_2O \rightleftharpoons PbOH^+ + H^+$ | -7.6 |
| $Pb^{2+} + 2H_2O \rightleftharpoons Pb(OH)_2 + 2H^+$ | -17.09 |
| $Pb^{2+} + 3H_2O \rightleftharpoons Pb(OH)_3^- + 3H^+$ | -28.09 |
| $Pb^{2+} + Cl^- \rightleftharpoons PbCl^+$ | 1.55 |
| $Pb^{2+} + NO_3^- \rightleftharpoons PbNO_3^+$ | 1.17 |
| $Pb^{2+} + SO_4^{2-} \rightleftharpoons PbSO_4$ | 2.69 |
| $Pb^{2+} + 2SO_4^{2-} \rightleftharpoons Pb(SO_4)_2^{2-}$ | 3.47 |
| $Pb^{2+} + PO_4^{3-} + 2H^{+1} \rightleftharpoons PbH_2PO_4^+$ | 21.07 |
| $Pb^{2+} + Citrate^{-3} \rightleftharpoons Pb\text{-}Citrate^-$ | 5.67 |
| $Pb^{2+} + Citrate^{-3} + H^{+1} \rightleftharpoons PbH\text{-}Citrate$ | 10.29 |
| $Pb^{2+} + EDTA^{4-} \rightleftharpoons PbEDTA^{2-}$ | 19.71 |

### *2.3. Pb content analysis*

Cell Pb was assayed as described by Pourrut et al. (2008). After 6 h exposure, *V. faba* seedlings were harvested, roots were rapidly washed in distilled water and the Pb bound to the rhizoderm removed by 0.01 M HCl according to Ferrand et al. (2006). The roots were washed by shaking for another 5 min in distilled water. After harvest, each plant sample was dried at 50°C for 48h, before digestion in a 1:1 mixture of $HNO_3$ and $H_2O_2$ at 80 °C for 4 h and in hot aqua regia, respectively. After filtration, Pb concentration was measured by inductively coupled plasma – atomic emission spectrometry (ICP-AES) with an IRIS Intrepid II XDL. The accuracy of the acidic digestion and analytical procedures was checked using the reference material Virginia tobacco leaves, CTA-VTL-2, ICHTJ. Certified values for Pb in Tobacco leaves were given for 22.1 ± 1.2 mg $Pb.kg^{-1}$ dry weights. Measured values for the three replicates were 22.0 ± 0.9, 22.4 ± 0.8 and 21.9 ± 0.8 $mgPb.kg^{-1}$ dry weight.

### *2.4. Vicia faba micronucleus test*

The micronucleus test was carried out according to Ma et al. (1995) and El Hajjouji et al. (2007). Before the test, the primary root tip was cut off (2 mm) to stimulate the emergence of secondary roots before transplanting into hydroponic conditions. Four days were necessary to obtain secondary roots of suitable length (1–2 cm) for the test. Exposure time was 30 h (6 h for the treated groups followed by a 24-h recovery period). In view of Pb toxicity after 24 hours of exposure (blackening of the root tips and loss of mitosis), a 24-h recovery period was enough to develop micronuclei (AFNOR, 2004; El Hajjouji et al., 2007; Marcato-Romain et al., 2009). Maleic hydrazide (MH, Sigma Chemical Co., St. Louis, MO, USA) at the concentration of 40 µM was used as positive control and aerated Hoagland's solution alone was used as negative control (Table 1). For each treatment and controls, five plants were used.

After harvest, root tips were fixed in Conroy's solution (glacial acetic acid/ethanol 1:3 v/v) at 4 °C for 24 h, and kept in 70 % alcohol at 4 °C. Subsequently, the roots were washed with distilled water for 10 min and hydrolyzed with 1 M HCl at 60 °C for 6–7 min. For each replicate, five slides were prepared. Finally, the root tips were stained with an aqueous solution containing 1 % aceto-orcein. The slides were observed under 1000 × magnification using an Olympus BX41 microscope coupled with a camera imaging FA1394-Q RETIGA Exi. Pb-induced genotoxicity was recorded as permillage (number of micronuclei per 1000

cells) micronuclei (MN) frequency. For each root tip, 5000 cells were observed. In order to avoid underestimation of micronucleus frequency due to impaired cell proliferation rate, the micronucleus test was performed only on root tips with a mitotic index of over 2 % (AFNOR, 2004).

### *2.5. Statistical analysis*

The data obtained was subjected to analysis of variance (ANOVA) using the software Statistica, ver. 8 (StatSoft-France). A Tukey's honestly significant difference (HSD) test was used to determine the level of significance against the negative control or Pb alone values.

## 3. Results

### *3.1. Pb uptake by Vicia faba roots in the presence of organic ligands*

The uptake of Pb by *V. faba* roots in the presence of EDTA and citric acid is depicted in Table 3.

**Table-3.** Effect of EDTA and citric acid on Pb accumulation in whole plant *V. faba* roots. Values are means of three separate experiments each replicated five times. Different letters among treatments indicate significant differences at $P < 0.05$.

| **Treatments** | **mg kg$^{-1}$** |
|---|---|
| Pb | 61 ± 5.9 a |
| Pb-EDTA-25 | 73 ± 6.0 bd |
| Pb-EDTA-40 | 77 ± 4.3 cd |
| Pb-EDTA-75 | 82 ± 5.6 c |
| Pb-EDTA-99 | 81 ± 4.9 c |
| Pb-CA-15 | 63 ± 5.1 a |
| Pb-CA-25 | 67 ± 3.9 ab |
| Pb-CA-40 | 66 ± 5.2 ab |

The Pb concentrations in roots exposed to Pb alone, reached an average value of 61±5.9 mg $kg^{-1}$ DW after 6 h incubation. Addition of EDTA, led to a significant increase in root Pb concentration (18, 25, 34, 33 % increase for Pb-EDTA-25, Pb-EDTA-40, Pb-EDTA-75 and Pb-EDTA-99, respectively), as compared to Pb alone. The effect of EDTA on Pb uptake by *V. faba* roots was concentration-dependent, except for Pb-EDTA-99. In this experimental condition, the Pb concentration in Pb-EDTA-99 roots was no longer different to Pb-EDTA-75. With citric acid, only a slight but non significant increase of Pb uptake in *V. faba* roots was observed in comparison with uptake of Pb alone (3, 9 and 7 % for Pb-CA-15, Pb-CA-25 and Pb-CA-40, respectively).

### *3.2. Pb-induced genotoxicity in the presence of organic ligands*

When EDTA or citric acid alone (as control) were added to the nutrient solution, no significant effect was observed on micronucleus frequency or mitotic index compared to the negative control (Fig. 2A and 2B). However, a toxic effect was observed for high concentrations of citric acid (>1mM; results not shown) and limited its use to 40 % chelation of Pb (Pb-CA-40). Türkoğlu (2007) also reported a significant decrease in mitotic index with citric acid alone in *Allium cepa* L. A digital picture of the micronucleus induced by Pb in *V. faba* root tips is presented in Fig. 1.

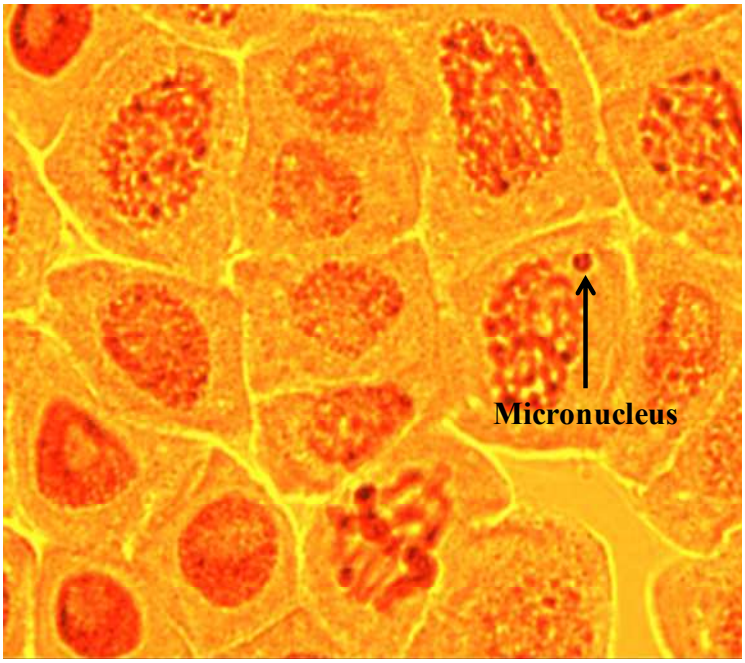


**Fig 1.** Micronucleus induced by Pb in *V. faba* root cells. Micronucleus is marked with an arrow.

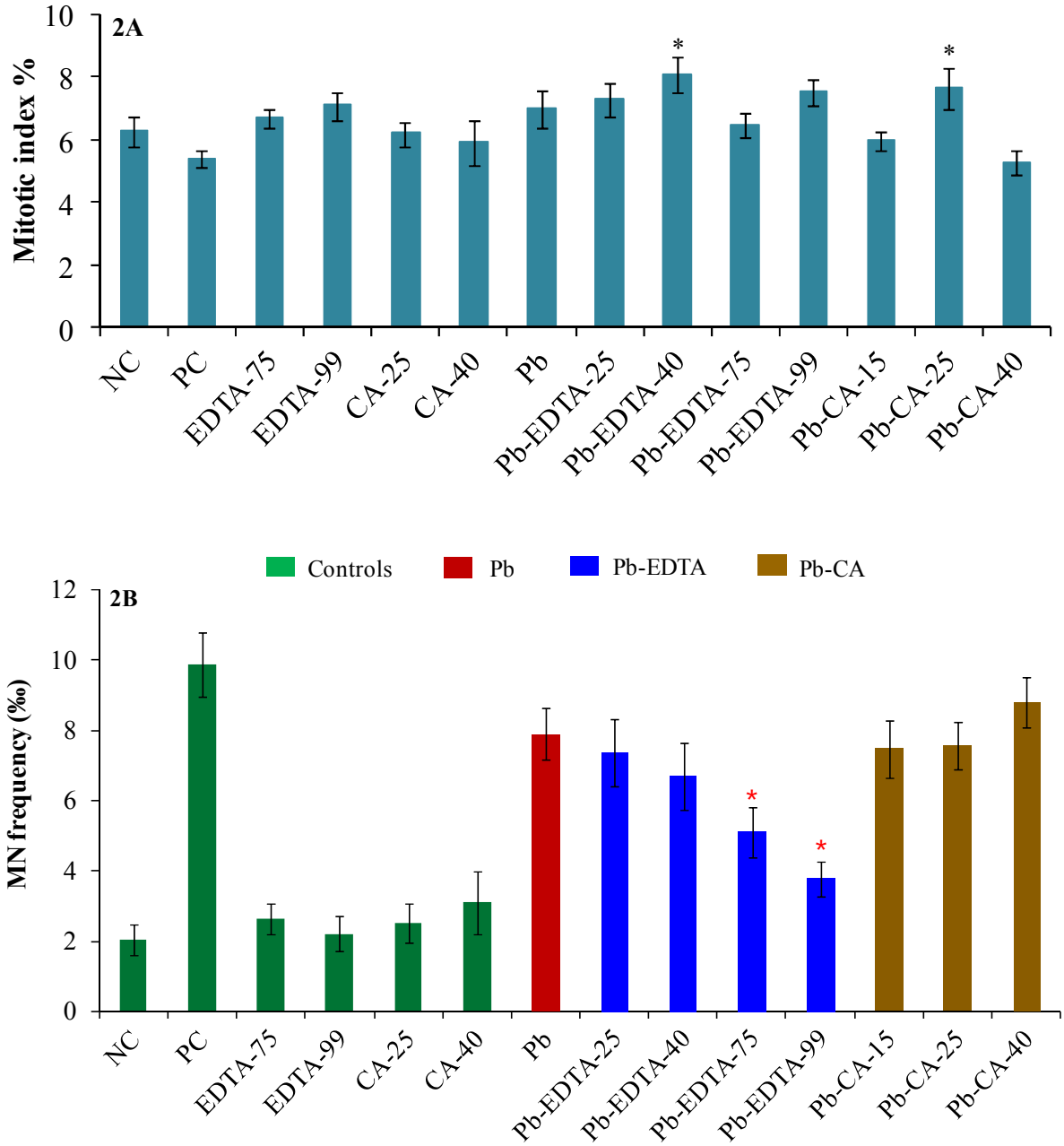


**Fig 2.** Mitotic index (a) and micronucleus frequency (b) values in *V. faba* root tips exposed to Pb alone and Pb chelated with EDTA and citric acid. In Fig 2B, the black and grey bars represent treatments with and without Pb, respectively, in order to compare their MN frequencies separately. Values are means of three separate experiments each replicated five times. The symbol (*) among treatments indicates significant differences at $P < 0.05$. (NC, negative control; PC, positive control).

When *V. faba* seedlings were exposed to Pb alone, the micronucleus frequency increased significantly (Fig. 2B). A four-fold increase in micronucleus frequency compared to the negative control was observed for Pb alone (Fig. 2B). Addition of EDTA did not affect the mitotic index, except Pb-EDTA-40 which caused a significant increase compared to Pb alone. However, in the presence of EDTA, Pb-induced micronucleus frequency (indicative of genotoxicity) decreased significantly and dose dependently. Chelation of 25, 40, 75, and 99 % of the Pb by EDTA reduced Pb-induced micronucleus frequency by 6, 15, 35 and 52 %, respectively.

Application of citric acid decreased mitotic index at the concentrations Pb-CA-15 and Pb-CA-40 but the effect was non significant (Fig. 2A). Addition of citric acid, however, had no significant effect on Pb genotoxicity (Fig. 2B). In these experimental conditions, the micronucleus frequency induced by Pb in the presence of citric acid remained close to that with Pb alone, except for Pb-CA-40, which increased slightly (16 %).

### *3.3. Relationship between genotoxicity and Pb concentrations*

No correlation was found between genotoxicity and total Pb concentration in *V. faba* roots tips under both EDTA and citric acid ($r^2 = 0.65$) as shown in Fig. 3. The results were similar when correlation was calculated separately for EDTA ($r^2 = 0.61$) and citric acid ($r^2 = 0.06$) treatments (data not shown). However, a linear curve was obtained for EDTA ($r^2 = 0.93$) when genotoxicity was plotted against the $Pb^{2+}$ concentration in solution (Fig. 4). Such a relation ($r^2 = 0.41$) was not found between micronucleus frequency and $Pb^{2+}$ concentration in solution for citric acid (data not shown).

## 4. Discussion

### *4.1. Pb uptake by V. faba roots in the presence of organic ligands*

As expected, the addition of EDTA significantly increased Pb uptake by *V. faba* roots (Table 3). This result is in agreement with several previous studies (Lai and Chen, 2005; Luo et al., 2005; Meers et al., 2005) which reported many fold increase in Pb uptake by various plants in the presence of EDTA. Chen et al. (2004) observed that the addition of EDTA (2.5 and 5 mmol $kg^{-1}$ EDTA) to a Pb-contaminated soil (2400 mg $kg^{-1}$ of total soil Pb) increased shoot Pb concentrations of ten plant species by 24-104 fold, with the greatest increases in dicotyledonous species. It has been demonstrated that Pb uptake is correlated with the

formation of Pb-EDTA in the hydroponics solution. Due to its high complexation constant value (log $K$ = 17.88), Pb-EDTA is the principal form of Pb to be taken up and translocated in the plants (Ruley et al., 2006). However, data presented in Table 3 indicated that no difference in Pb uptake was observed between Pb-EDTA-75 and Pb-EDTA-99, suggesting a possible saturation of Pb accumulation after 6 hours of incubation.

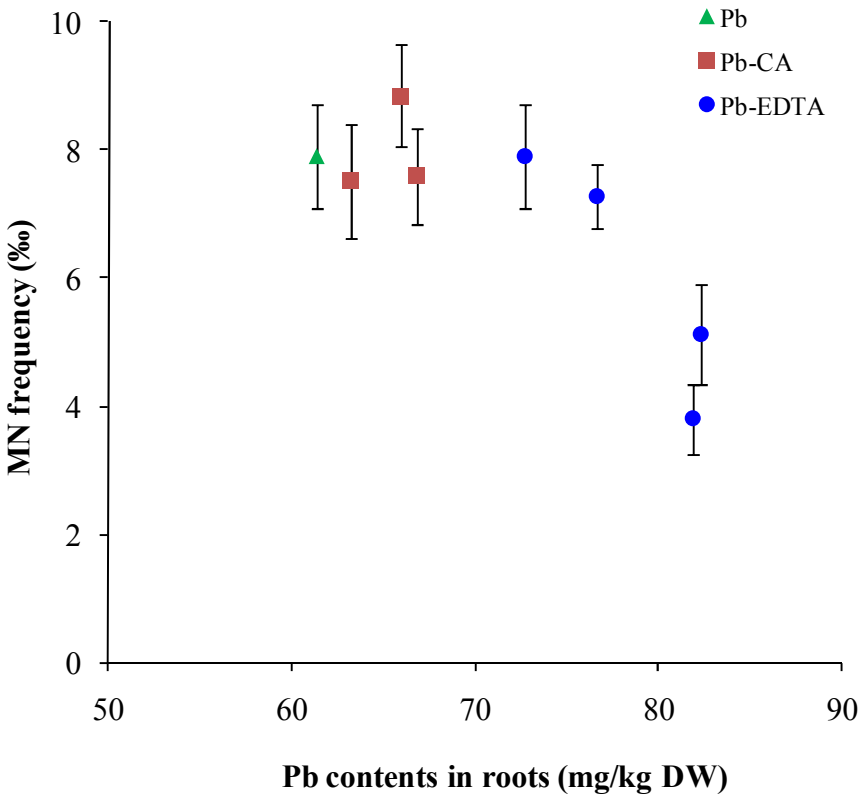


**Fig 3.** Micronucleus (MN) frequency plotted against total Pb concentration in *V. faba* roots in the presence of EDTA and citric acid. Values are means of three separate experiments each replicated five times.

In the Pb-CA treatments, however, Pb accumulation in *V. faba* roots was slightly higher than with Pb alone, but no treatment reached a significantly different concentration after 6 hours of incubation (Table 3). Similar results were observed by Quartacci et al. (2006) who reported that citric acid applied at 5 mmol $kg^{-1}$ to a metal-contaminated soil did not induce any significant change in metal uptake by *Brassica juncea*. However, some authors (Chen et al., 2003; Muhammad et al., 2009) also indicated increased absorption of Pb by application of citric acid. The increase in metal uptake by citric acid is plant-dependent and could be attributed to a decrease in the pH of culture media. Acidification did not occur in our experimental condition, because the pH was kept adjusted at 5. Moreover, the slight decrease

in Pb uptake by Pb-CA-40 than Pb-CA-25 might be due to the acute cytotoxicity associated with CA. Infact, at higher concentrations CA alone reduced mitotic index and increased genotoxicity, the effects being non significant (Fig. 2A, 2B).

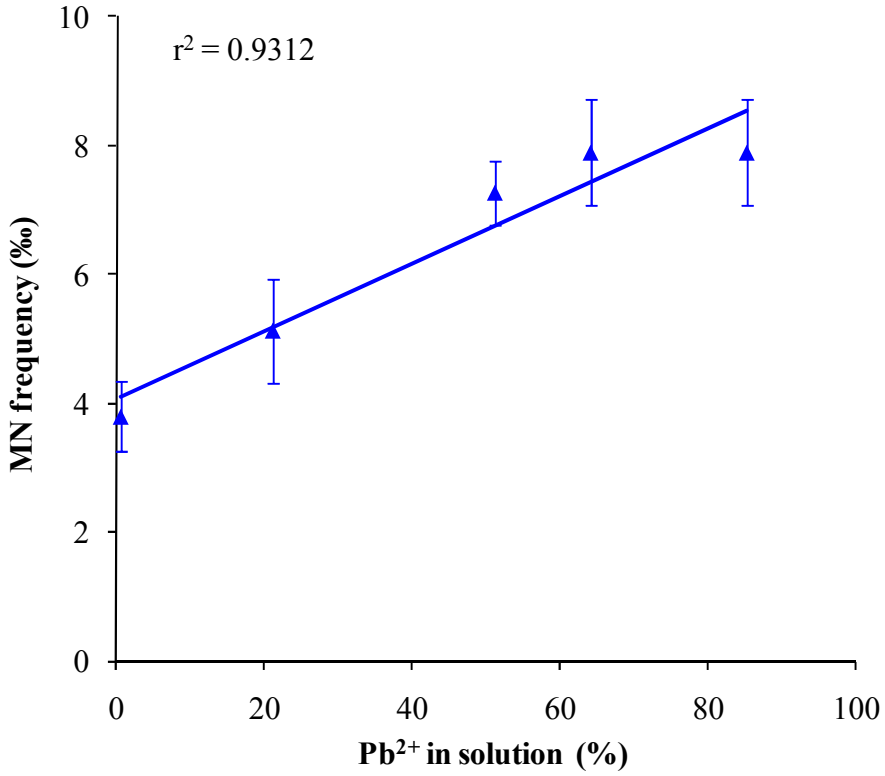


**Fig 4.** Micronucleus (MN) frequency plotted against free $Pb^{2+}$ ion concentration in solution in the presence of EDTA. Values are means of three separate experiments each replicated five times.

### *4.2. Micronucleus test and mechanism of Pb-induced genotoxicity*

Four-fold increases in micronucleus frequency in *V. faba* root tips by Pb alone (Fig. 2B) clearly illustrated Pb genotoxicity, analysed with the *V. faba* micronucleus test. The molecular mechanism of DNA breakdown in the presence of metal stress is not yet clearly understood. Micronuclei are the result of chromosome breaks or mitotic anomalies that require a passage through mitosis to be recognisable (Al Sabti and Metcalfe, 1995). According to Johnson (1998) Pb and its compounds are capable of interfering with the spindle apparatus of dividing cells thus Pbing to genotoxicity. Bonacker et al. (2005) thought that the micronuclei induced by Pb ions are most often associated with processes involving reactive oxygen generated by redox shuttling. Oxidative stress as a result of metal toxicity is

also described to play a major role in DNA-damage induction (Halliwel, 1990). In this experiment, a blackening of root tips grown in the presence of Pb nitrate alone or Pb-CA complexes were observed. Recently Probst et al., (2009) also found dark coloured *V. faba* roots grown on mine tailings contaminated with Pb. This root colouration is possibly due to Pb-induced oxidative stress (Pourrut et al., 2008).

### *4.3. Relationship between Pb uptake and genotoxicity in the presence of organic ligands*

Cytotoxicity and bioavailability/uptake are generally considered synonymously when one speaks of metal species. In our experimental conditions, a non-linear correlation was observed between total Pb concentration in *V. faba* roots and genotoxicity (Fig. 3) under applied organic ligands. Application of Pb-EDTA treatments to *V. faba* seedlings significantly increased Pb uptake, but concomitantly reduced Pb genotoxicity (Table 3, Fig. 2B). These results are in line with those of Liu et al. (2008) in *Sedum alfredii* or Ruley et al. (2006) who studied *Sesbania drummondii* a Pb hyper-accumulator. Hernández -Allica et al. (2007) also reported that proper management of the EDTA concentration can increase the uptake of metals with low phytoavailability like Pb and reduce their phytotoxicity. Recently, Marcato-Romain et al. (2009) presented that MN frequencies in *V. faba* root tips were more than ten fold higher in hydroponic exposure compare to direct contact in soil under same range of pollutant concentrations. These observations could be related to Pb speciation (different between soil and solution), with a particular protecting effect of soil organic matter. However, citric acid treatments increased, although not significantly, Pb-induced genotoxicity with increase in Pb uptake. This contrary behaviour of EDTA and citric acid towards Pb genotoxicity suggests the lack of a simple and linear relationship between genotoxicity and phytoavailability and/or Pb uptake in the presence of organic ligands. Indeed, Pb genotoxicity varies with its form/speciation rather than with its total uptake and hence concentration in the presence of organic ligands.

### *4.4. Correlation between Pb-induced genotoxicity and free $Pb^{2+}$ available in nutritive solution*

In the present study, the linear correlation ($r^2 = 0.94$) between Pb-induced genotoxicity and $Pb^{2+}$ concentration (Fig. 4) in nutrient solution under EDTA clearly demonstrated acute genotoxicity associated with this ionic form of Pb. In a previous work Pourrut et al. (2008) demonstrated that Pb-induced oxidative stress was antagonized by the calcium entry blocker

$LaCl_3$ or high concentrations of $Ca^{2+}$, suggesting an important role for the free ion $Pb^{2+}$. Sauvé et al. (1998) also found that the toxicological impact of $Cu^{2+}$ and $Pb^{2+}$ upon a variety of crop plants, soil organisms and soil microbial processes can be explained to a greater degree by free metal ion activity than the total soil metal concentration.

The mechanism behind this linear relationship between $Pb^{2+}$ and genotoxicity could be associated with Pb-induced oxidative stress. Pourrut et al. (2008) demonstrated a linear dose effect of Pb in NADPH-oxidase activation and reactive oxygen species (ROS) production, between 1 and 10 µM. This ROS production could in turn increases DNA alterations and particularly DNA breakdowns at the origin of micronucleus (Yang et al., 1999). Another hypothesis is a possible direct interaction between $Pb^{2+}$ and DNA (Valverde et al., 2001) and/or inhibition of microtubules by interaction of Pb with -SH group of nuclear proteins (Eun et al., 2000).

However, this correlation between Pb-induced genotoxicity and $Pb^{2+}$ concentration is not valid in the presence of citric acid. The contrary behaviour of EDTA and citric acid toward Pb-induced genotoxicity is due to differences in their complexing constants. Indeed, EDTA masks the genotoxic effect of Pb by forming stable and non-toxic complexes with free $Pb^{2+}$ in solution due to the high stability constant (log $K$ = 17.88). In contrast, Pb-CA complex most probably dissociates just before or after uptake due to the lower stability constant (log $K$ = 5.67), thus, slightly increasing $Pb^{2+}$ levels in *V. faba* roots and ultimately genotoxicity. Based on these results it is proposed the Pb-induced genotoxicity is directly associated with the uptake of free $Pb^{2+}$ ions.

Under the application of Pb-EDTA-99, where 99 % of the $Pb^{2+}$ ions were chelated by EDTA, genotoxicity decreased significantly in comparison with Pb alone, but still it was higher with respect to the negative control (NC, Fig. 2B). This was surprising as Pb is assumed to be taken up in the form of Pb-EDTA and Pb induced genotoxicity is activated by free metal ions ($Pb^{2+}$) only. This implies that the presence of 99 % chelated Pb should not cause micronucleus induction and the values should be close to those of the NC. Another hypothesis could be a different behaviour of Pb at a more dilute scale. In a previous study Sarret et al. (2001) reported that in the case of Pb-EDTA for *Phaseolus vulgaris*, dissociation after uptake is not possible due to strong chelation; however, local rhizosphere acidification could possibly result in the dissociation of the Pb-EDTA complex near the absorption sites of the roots. This production of $Pb^{2+}$ ions might be responsible for increased micronucleus

frequency under our conditions in the presence of Pb-EDTA-99. Moreover, Pb is toxic even at very low concentrations i.e. 0.5 µM (Kopittke et al., 2007) and *V. faba* is highly sensitive to Pb, therefore, micronucleus production by the remaining 1 % of free $Pb^{2+}$ in the Pb-EDTA-99 condition also cannot be ignored.

## 5. Conclusions

The present study is based on the hypothesis that organic ligands could modify both metal uptake and phyto-toxicity by changing free metal ions concentration through speciation. The results showed that uptake of Pb by *V. faba* roots varied according to the type of chelate used. EDTA is capable of dose-dependently increasing Pb uptake by *V. faba* roots but citric acid was unable to enhance Pb accumulation by *V. faba* roots following 6 h of Pb exposure. The *Vicia faba* micronucleus test demonstrates that Pb speciation plays a significant role towards its genotoxicity. The results suggest that Pb genotoxicity is directly correlated with its free ionic form. EDTA could alleviate Pb-induced genotoxicity in *V. faba* roots by forming soluble, stable and non-toxic complexes with Pb ions that have a high toxic potential when free. These results also underline that citric acid has no influence on Pb-induced genotoxicity to *V. faba* roots due to weak complexation with Pb which possibly undergoes dissociation producing $Pb^{2+}$ before or after cell uptake. Finally, our results demonstrate that metal speciation knowledge could improve the interpretation of *V. faba* genotoxicity test performed to test soil quality. Further work could be performed to validate the relationship between free Pb cations and genotoxicity and highlight the mechanisms at the cellular scale.

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# Section 3C:

## Effect of fulvic acids on Pb-induced oxidative stress to metal sensitive *Vicia faba* L. plant

*-Publication-*

M. Shahid, C. Dumat, J. Silvestre, E.Pinelli. Effect of fulvic acids on Pb-induced oxidative stress to metal sensitive *Vicia faba* plant (Journal of Hazardous Materials, HAZMAT-D-10-05879).

Forewords

The previous two studies showed that Pb toxicity is the function of its speciation and is related to free ion concentration in the nutrient solution. Moreover, the binding strength of chelating agent is the most important factor controlling metal speciation and ultimately uptake and toxicity. Due to its high complexation constant value (log $K$ 17.88), EDTA can modify Pb uptake and toxicity whereas CA is inefficient in changing Pb uptake and toxicity due to low complexation constant value (log $K$ 5.67). These studies were performed using synthetic chelator (EDTA) and low molecular weight organic acid (citric acid).

In order to apply our model of Pb toxicity in natural conditions, in this study we used two different kinds of humic substances with known chemical properties. These include; Suwannee River fulvic acid (SRFA) and Elliott Soil fulvic acid (ESFA). These HSs represent the natural organic matter present in the soil and the result obtained can be compared to natural condition.

Moreover, the treatments were applied for only 1, 12 and 24 h keeping in mind the production of ROS and induction of lipid peroxidation and antioxidant enzymes at these times in roots and leaves as described by our first study.

# Effect of fulvic acids on Pb-induced oxidative stress to metal sensitive *Vicia faba* L. plant

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**Abstract**

Pb is a ubiquitous environmental pollutant that can directly or indirectly induce various morphological, physiological and biochemical dysfunctions in plants. Only few publications focus on the influence of Pb speciation both on its phytoavailability and phytotoxicity. Therefore, Pb toxicity (in terms of lipid peroxidation, $H_2O_2$ induction and photosynthetic pigments contents) was studied to *Vicia faba* plants in relation with Pb uptake and speciation. *Vicia faba* seedlings were exposed to Pb alone or complexed at two levels by two types of fulvic acids for 1, 12 and 24 h in controlled hydroponic conditions. For both type of fulvic acids, Pb uptake and translocation by *Vicia faba* increased at low level (5 $mg.L^{-1}$), whereas decreased at higher level of application (25 $mg.L^{-1}$). Despite increased Pb uptake fulvic acids at low concentrations did not influence significantly Pb toxicity. However, at higher concentrations, fulvic acids reduced Pb toxicity by reducing its uptake. These results highlighted the role of dilution factor for fulvic acids reactivity in relation with structure. Suwannee River fulvic acid was more effective than Elliott Soil fulvic acid in reducing Pb uptake and alleviating its toxicity to *Vicia faba* due to comparatively strong binding affinity for Pb.

*Keywords:* Speciation; Pigment contents; Metal toxicity; Reactive oxygen species (ROS)

*Abbreviations:* Chl-a, chlorophyll a; Chl-b, chlorophyll b; EDTA, ethylenediaminetetraacetic acid; ESFA, elliott soil fulvic acid; FAs, fulvic acids; GSH, glutathione; HSs, humic substances; PCs, phytochelatins; ROS, reactive oxygen species; *V. faba, Vicia faba*; SRFA, suwannee river fulvic acid; TBARS, thiobarbituric acid reactive substances; WHAM VI , windermere humic aqueous model VI.

## 1. Introduction

Pb is widely used in many industrial processes, which led to its toxic levels in all the environmental compartments: soil, water, air and biosphere [1]. This metal is known to interfere with morphological, physiological and biochemical functioning of exposed plants or humans and could induce a broad range of noxious effects [2–5].

Over the last decades, more than 200 short term bioassays utilizing plants, micro-organisms or insects have been developed and used in the context of risk assessment [6,7]. Plant assays are highly sensitive, quite easy to conduct, inexpensive, and good predictors of carcinogenicity. Nowadays, oxidative stress due to over production of reactive oxygen species (ROS) has been proposed as a possible mechanism involved in Pb toxicity [8]. The potential effect of Pb in the environmental systems is the function of its speciation [9-11]. However the influence of Pb speciation on its phytotoxicity is poorly studied. Therefore, nowadays, to improve the biotests, there is a growing interest regarding the influence of Pb speciation concerning its biogeochemical behaviour and risk assessment in the ecosystem.

Humic substances influence the speciation, the bioavailability and the acute toxicity of metals in soils and natural waters [12-14]. These substances are a complex mixture of partially “decomposed” and otherwise transformed organic materials and constitute a major sorbent phase for contaminants in soils [15]. The properties of humic substances are due to their physico-chemical characteristic with respect to molecular weight, molecular size, substructures and functionalities, depending on the nature and sources of organic matter and functional groups. The carboxylic and phenolic hydroxyl groups are the main binding sites of these compounds for metals. The great variation among different humic substances makes their functioning complex towards metal biogeochemical fate and behaviour in the system [16]. Among these humic substances, fulvic acids (FAs) are soluble at all pH, with average molecular weight less than 1,000. They are more reactive than humic acids due to higher oxygen contents, number of functional group and exchange capacity [17].

According to Shahid et al. [11], Pb-induced toxicity to plant is closely related to its applied form in the soil solution, which, after uptake, interferes with cellular metabolic processes. However, very rare data is available on Pb toxicity in relation with uptake and speciation, especially in the presence of humic substances. Therefore, this study was carried

out to investigate the effect of Pb speciation on phytoavailability and toxicity to *Vicia faba* in the presence of two different types of fulvic acids.

## 2. Materials and methods

### *2.1 Plant materials and growth conditions*

The *V. faba* (common name, broad beans; cultivar, Primabel; type, aguadulce; family, Fabaceae) seedlings were cultured according to El Hajjouji et al. [18] and Marcato-Romain et al. [7]. The *V. faba* seeds were germinated under optimal germination conditions i.e. in darkness at 22 °C temperature and 100 % of moisture. When the primary roots were about 2-3 cm in length, the seedlings were transplanted and cultured in modified Hoagland solution (Sigma) with the macro-elements: 5 mM $KNO_3$, 5 mM $Ca(NO_3)_2$, 2 mM $KH_2PO_4$ and 1.5 mM $MgSO_4$ and micro-elements: 9.11 µM $MnSO_4$, 1.53 µM $ZnSO_4$, 0.235 µM $CuSO_4$, 24.05 µM $H_3BO_3$, 0.1 µM $Na_2MoO_4$ and 268 µM Fe/EDTA. Nutrient solution was renewed on alternate days to keep the nutrient composition and pH constant. Plants were grown under controlled conditions in a phytotron with 16 h photoperiod at 70 % relative humidity and day/night temperatures of 24/22 °C. Light was supplied by 600 W Osram Nav-T Super high pressure sodium lamp providing a minimum photosynthetical photon flux density of 500 $\mu mol.m^{-2}.s^{-1}$ at the top of the plant [8].

### *2.2. Treatments*

After pre-culturing of 15 days ($5^{th}$-$6^{th}$ foliar stage), plants were exposed to 5 µM Pb in the presence or absence of two fulvic acids i.e. Suwannee River fulvic acid (SRFA) and Elliott Soil fulvic acid (ESFA) from the International Humic Substances Society (IHSS, Colorado School of Mines, Golden, CO) [19], used as model of natural dissolved organic matter. The chemical properties of these fulvic acids are given in Table 1. These substances were applied at two levels i.e. 5 and 25 $mg.L^{-1}$ to Pb treatments. The humic substances were also applied alone as control but only at higher levels (25 $mg.L^{-1}$). The concentration of $KH_2PO_4$ in the nutrient solution used for Pb treatment was reduced to 0.2 mM in order to prevent phosphate precipitation [11]. Control plants were also cultured in the appropriate uncontaminated media with reduced $PO_3^{-4}$ concentration. All the treatments were applied for 1, 12 and 24 h except for fulvic acids alone which were applied for 24 h only.

**Table-1.** Characteristic properties of two fulvic acids used (Source International Humic Substance Society).

| Parameters | SRFA | ESFA |
|---|---|---|
| Cat. No. | 2S101F | 2S102F |
| C (% w/w) | 52.34 | 50.12 |
| H (% w/w) | 4.36 | 4.28 |
| O (% w/w) | 42.98 | 42.61 |
| N (% w/w) | 0.67 | 3.75 |
| S (% w/w) | 0.46 | 0.89 |
| P (% w/w) | 0.004 | 0.12 |
| Carboxyl (meq/g C) | 11.17 | 13.24 |
| Phenolic (meq/g C) | 2.84 | 2.27 |
| Log $K_1$ | 3.76 | 3.67 |
| Log $K_2$ | 9.84 | 9.53 |

### *2.3. Calculation of Pb speciation in nutrient solution*

The speciation of Pb in nutrient solution (free $Pb^{2+}$ ions and complexed by fulvic acids, Table 2) was calculated using Windermere Humic Aqueous Model VI (WHAM VI) [20] under various applied levels of fulvic acids and experimental conditions. WHAM VI is a speciation model designed to model chemical equilibria in oxic waters, but could also be applied to soil solution where humic substances are the main complexants of metals. This model represents humic compounds by rigid spheres of homogenous size, which carry metal–humic binding sites positioned on the surface with different binding strength, bidentate and tridentate binding sites being permitted. The model considers two types of acid groups for metal binding, which are represented by COOH and OH. The WHAM VI database of metal binding constants (pKs) for the two acid groups was replaced with that obtained from IHSS (Table 1). Electrostatic effects are corrected with terms based on the Debye–Hückel and Gouy–Chapman theories.

### *2.4. Pb content Analysis*

Before all the experiments, Pb content analysis was carried out in the nutrient solution after 24 h without exposure to plants for all the treatments. The objective of this analysis was to determine the possible precipitation of Pb after 24 h. All the treatments solution (Table 2)

were prepared from mother solutions of Pb [50 µM Pb as $Pb(NO_3)_2$, Sigma] and fulvic acids (100 $mg.l^{-1}$ SRFA and ESFA) in milli-Q water. The pH of all the treatment solutions was adjusted to 5 ± 0.1 using distilled $HNO_3$ (15 M, suprapur 99.9 %, Sigma) and were kept under experimental conditions for 24 h. Inductively coupled plasma-atomic emission spectrometry (ICP-AES, Jobin Yvon) with an IRIS Intrepid II XDL/ Thermo Electron Corporation was used to determine Pb contents after filtration (0.22 µm). Both the pH and total Pb concentration remained constant in all the treatments after 24 h (Table 2).

**Table-2.** Experimental design and speciation of Pb in nutrient solution calculated using WHAM VI. Pb measured (last column) indicates the concentration of Pb (µM) calculated by ICP-OES in nutrient solution after 24 h without plants.

| Treatments | Composition | Pb-chelated (%) | Pb-free (%) | Pb measured (µM) |
|---|---|---|---|---|
| Control | Hoagland solution (HS) | – | – | 0 |
| ESFA-25 | HS + 25 $mg.l^{-1}$ ESFA | – | – | – |
| SRFA-25 | HS + 25 $mg.l^{-1}$ SRFA | – | – | – |
| Pb | HS + 5 µM Pb | 0 | 84 | 4.99 ± 0.04 |
| Pb-ESFA-5 | HS + 5 µM Pb + 5 $mg.l^{-1}$ ESFA | 12 | 74 | 5.01 ± 0.03 |
| Pb-ESFA-25 | HS + 5 µM Pb + 25 $mg.l^{-1}$ ESFA | 36 | 57 | 5.01 ± 0.02 |
| Pb-SRFA-5 | HS + 5 µM Pb + 5 $mg.l^{-1}$ SRFA | 16 | 70 | 4.98 ± 0.03 |
| Pb-SRFA-25 | HS + 5 µM Pb + 25 $mg.l^{-1}$ SRFA | 44 | 47 | 5.03 ± 0.02 |

Pb cell uptake by *V. faba* seedlings was carried out according to Pourrut et al. [8]. After harvest, the seedlings were separated into root stem and leaves. Pb bound to the rhizoderm was removed by HCl as described by Uzu et al. [10]. Plants tissues were dried at 80 °C for 48 h and were digested by a 1:1 mixture of 65 % $HNO_3$ (Sigma) and 30 % $H_2O_2$ (Sigma) at 80 °C over 6 h using DigiPrep Jr (SCP Sciences). After dilution with milli-Q water, the metal contents were analysed using ICP-AES. Virginia Tobacco leaves (CTA-VTL-2, polish certified reference material; ICHTJ) were used as a reference material for verifying the accuracy of the analytical procedure. Certified values for Pb in Tobacco leaves were given for 22.1 ± 1.2 mg $Pb.kg^{-1}$ dry weights. Measured values for the three replicates were 22.1 ± 0.9, 22.3 ± 1.0 and 22.0 ± 0.7 mg $Pb.kg^{-1}$ dry weight.

### *2.5. Determination of lipid peroxidation*

In order to evaluate the Pb-induced oxidative damages on cell membrane, thiobarbituric acid reactive substances (TBARS, Sigma) were calculated as reported by Hodges et al. [21]. Plant samples were homogenised in hydro-alcoholic solution (80/20: v/v, Sigma) under liquid nitrogen at 4 °C in darkness followed by incubation at 95 °C with thiobarbituric acid. After centrifugation, the absorbance of the supernatant was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of TBARS is calculated using the extinction coefficient (155 $mM^{-1}cm^{-1}$) and the equations reported by Hodges et al. [21].

### *2.6. Evaluation of $H_2O_2$ contents*

The measurement of $H_2O_2$ content was carried out according to Islam et al. [3]. Each sample (500 mg) was homogenized with 5 mL 0.1 % (w/v) trichloroacetic acid (Sigma) under liquid nitrogen followed by centrifugation at 12,000 g for 20 min. The mixture assay contained 0.5 mL of the supernatant added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0, Sigma) and 1 mL of 1 M KI (Sigma). Absorbance was determined at 390 nm and the content of $H_2O_2$ was evaluated using a standard curve under the same conditions.

### *2.7. Pigment content assay*

The frozen leaves samples (600 mg) of *V. faba* were ground with liquid nitrogen. Pigments were extracted by incubating leaves in 10 mL acetone 80 % (v/v, Sigma) for 24 h at 4 °C in darkness. After centrifugation at 1000 g for 10 min, absorbance of the supernatant was recorded at 663, 645 and 480 nm. Concentrations of chlorophyll a (Chl-a), chlorophyll b (Chl-b) and carotenoids were calculated according to extinction coefficients and equations reported by Lichtenthaler [22] .

### *2.8. Statistical analysis*

The variables were tested for differences between treatments using an Analysis of Variance (one-way ANOVA) followed by LSD Fisher test. This statistical analysis was performed using the software Statistica, Edition'98 (StatSoft Inc., Tulsa, OK, USA). For each bioassay, mean values with different letters represent significant difference ($p < 0.05$) as measured by LSD Fisher test.

## 3. Results and discussions

### *3.1. Effect of fulvic acids on Pb uptake by Vicia faba*

Table 3 presents Pb contents in *V. faba* roots, stem and leaves in function of time. *V. faba* roots were exposed to Pb in presence or absence of fulvic acids. In roots, in the absence of fulvic acids, data showed a rapid uptake and accumulation of 27 ± 8, 59 ± 8 and 95 ± 9 $\mu g.g^{-1}$ of Pb respectively, after 1, 12 and 24 h. In contrast to Pb uptake, its translocation towards shoot tissues was very slow and only 3.6 ± 1.1 and 3.2 ± 0.5 $\mu g.g^{-1}$ of Pb was transferred, respectively, into stem and leaves after 24 h. This limited translocation of Pb from roots to shoot tissues is very well documented in literature [2,4,23]. Indeed, in plant roots, Pb precipitates as insoluble salts or is immobilized by biological molecule such as sugar, pectins, celluloses and hemicelluloses [24]. This metal sequestration phenomenon in roots is not common to all heavy metals and its intensity is very specific to Pb. Several previous studies reported that more than 90 % of absorbed Pb is stored in the plant roots [4,10,24]. In our case, 93 % of absorbed Pb was sequestered in *V. faba* roots.

Application of both fulvic acids at low level (Pb-SRFA-5 and Pb-ESFA-5) increased non significantly Pb uptake and translocation to shoot tissues. The accumulation of Pb in roots, stem and leaves increased, respectively, by 16, 42 and 22 % for Pb-ESFA-5 and by 12, 25 and 13 % for Pb-SRFA-5 after 24 h. In contrast, for higher levels of fulvic acids (Pb-SRFA-25 and Pb-ESFA-25), a significant decrease in Pb uptake and translocation was observed except for Pb-ESFA-25 where decrease in translocation was non significant (Table 3). The decrease in Pb accumulation by Pb-ESFA-25 in roots, stem and leaves was, respectively, 22, 22 and 19 % after 24 h. In case of Pb-SRFA-25, this decrease was 35, 28 and 25 %, respectively, in roots, stem and leaves after 24 h.

Several previous studies described the interaction between Pb and humic substances (HSs). Their results are contrasted: certain authors observed a significant increased Pb uptake under HSs influence [16,25], whereas others observed a decrease [26,27]. In our experiment, the contrasting effects of fulvic acids towards Pb uptake, at low and high concentrations could be explained by FA reactivity in relation with structure [13,28,29]. When highly concentrated, FA adopts a ball structure efficient to strongly complex Pb and reduces its plant uptake. Under diluted conditions, FA adopts a more hydrophilic fibrous structure that forms

soluble and mobile complexes with Pb, which could enter the plants more easily due to small size and thus increased Pb uptake [30,31].

**Table-3.** Effect of SRFA and ESFA on Pb concentration (µg.g$^{-1}$ D.W.) and rate of uptake and translocation (µg.g$^{-1}$.h$^{-1}$ D.W.) in *V. faba* roots, stem and leaves. Values are means of two separate experiments each replicated five times. Significant differences at $P < 0.05$ are indicated with an asterisk (*) and bold value.

| | | Roots | | Stem | | Leaves | |
|---|---|---|---|---|---|---|---|
| Treatments | Time | Mean ± SD | $UR_R$ | Mean ± SD | $TR_S$ | Mean ± SD | $TR_L$ |
| Pb | 1 | 27 ± 8 | 27 | 0.14 ± 0.01 | 0.14 | 0.03 ± 0.01 | 0.03 |
| Suw2-Pb-FA-5 | | 31 ± 4 | 31 | 0.14 ± 0.05 | 0.14 | 0.03 ± 0.01 | 0.03 |
| Suw2-Pb-FA-25 | | 27 ± 2 | 27 | 0.11 ± 0.02 | 0.11 | 0.02 ± 0.01 | 0.02 |
| Ell-Pb-FA-5 | | 29 ± 8 | 29 | 0.11 ± 0.04 | 0.11 | 0.04 ± 0.03 | 0.04 |
| Ell-Pb-FA-25 | | 28 ± 2 | 28 | 0.14 ± 0.03 | 0.14 | 0.03 ± 0.01 | 0.03 |
| Pb | 12 | 59 ± 8 | 2.9 | 2.9 ± 0.7 | 0.25 | 2.2 ± 0.4 | 0.20 |
| Suw2-Pb-FA-5 | | 60 ± 13 | 2.6 | 3.1 ± 0.4 | 0.27 | 2.3 ± 0.3 | 0.21 |
| Suw2-Pb-FA-25 | | 47 ± 12 | 1.8 | 2.4 ± 0.6 | 0.21 | 2 ± 0.3 | 0.18 |
| Ell-Pb-FA-5 | | 69 ± 5 | 3.6 | 3.5 ± 0.5 | 0.31 | 2.7 ± 0.5 | 0.24 |
| Ell-Pb-FA-25 | | 50 ± 6 | 2.0 | 2.5 ± 0.3 | 0.21 | 2 ± 0.5 | 0.18 |
| Pb | 24 | 95 ± 9 | 3.0 | 3.6 ± 1.1 | 0.06 | 3.2 ± 0.5 | 0.08 |
| Suw2-Pb-FA-5 | | 106 ± 13 | 3.8 | 4.5 ± 0.8 | 0.12 | 3.6 ± 0.6 | 0.11 |
| Suw2-Pb-FA-25 | | **62 ± 19*** | 1.3 | **2.6 ± 0.6*** | 0.02 | **2.4 ± 0.2*** | 0.03 |
| Ell-Pb-FA-5 | | 110 ± 15 | 3.4 | 5.1 ± 0.3 | 0.13 | 3.9 ± 1.1 | 0.10 |
| Ell-Pb-FA-25 | | **74 ± 8*** | 2.0 | 2.8 ± 0.4 | 0.03 | 2.6 ± 0.3 | 0.05 |

$UR_R$=Uptake rate by roots, $TR_S$= Translocation rate to stem, $TR_L$= Translocation rate to leaves

The kinetic of Pb uptake by *V. faba* roots and translocation to aerial parts is shown in Table 3. The rate of Pb uptake was maximum during first hour for root, whereas translocation towards stem and leaves occur at maximum rate between 2-12 h for all the treatments. This showed that Pb uptake by *V. faba* roots and translocation to shoots tissues was not linear during the first 24 h of incubation but occurs in phases. The application of fulvic acids increased the rate of Pb uptake and translocation at low level and decreased it at high level of application. However, the trends/phases of Pb uptake and translocation remained the same for

all the treatments. There is very rare data regarding short term metal uptake kinetic. However, Nedelkoska and Doran [32] and Sgherri et al. [33] also showed triphasic kinetic profiles for cadmium, nickel and copper during 24 h. These results could have implication for the design of biotests performed in order to test the quality of soils and solutions.

### *3.2. Effect of organic ligands on lipid peroxidation and $H_2O_2$ induction by Pb*

The effect of fulvic acids alone was investigated on plant physiology. The higher concentrations of both fulvic acids (25 $mg.L^{-1}$) were applied alone without Pb for 24 h. Application of the fulvic acids alone showed, in the both cases, no significant effects on lipid peroxidation and $H_2O_2$ production compare to control (Fig. 1, 2 and 3).

The effect of fulvic acids on Pb-induced generation of $H_2O_2$ and lipid peroxidation is presented in Fig. 2 and 3. Application of Pb alone caused overproduction of $H_2O_2$ which resulted into lipid peroxidation in both roots and leaves. In roots, Pb-induced $H_2O_2$ production and lipid peroxidation started immediately after Pb exposure at 1 h and continued up to 12 h. The increase in $H_2O_2$ and TBARS contents by Pb was, respectively, 64 and 55 % at 1 h whereas 39 and 52 % at 12 h. In leaves, the Pb-induced increase in $H_2O_2$ and TBARS contents started after 12 h but the effect was significant at 24 h only (34 and 36 %, respectively, for $H_2O_2$ and TBARS compare to the control). The presence of Pb across the root-cell membrane, even in small amounts, is known to induce oxidative stress through overproduction of ROS [3,4,23,34]. This overproduction of ROS is one of the earliest responses of plant cells to heavy metal toxicity which is due to imbalance between the generation and the neutralization of ROS by antioxidant mechanisms. These free radicals react with (oxidize) various cellular components including DNA, proteins, lipids / fatty acids and cause DNA damage, mitochondrial malfunction and cell membrane damage [7,35,36]. Pb ions are known to induce lipid peroxidation, decrease the level of saturated fatty acids and increase the content of unsaturated fatty acids of membranes in several plant species [34]. Indeed, ROS remove hydrogen from unsaturated fatty acids Pbing to formation of lipid radicals and reactive aldehydes, ultimately causing distortion of lipid bilayer [36].

In roots, Pb-ESFA-5 prolonged the Pb-induced induction of $H_2O_2$ to 24 h without any effect on lipid peroxidation (Fig. 2 and 3). Application of Pb-SRFA-5 did not affect $H_2O_2$ production but delayed the initiation of lipid peroxidation from 1 to 12 h which then continued up to 24 h. At higher levels of fulvic acid application, Pb-induced $H_2O_2$ induction

and lipid peroxidation were reduced significantly at 1 h by Pb-ESFA-25 whereas at 1 an 12 h by Pb-SRFA-25. However, Pb-SRFA-25 increased slightly $H_2O_2$ contents but increased significantly TBARS values at 24 h. In leaves, addition of fulvic acids in the presence of Pb at both levels of application, have no significant effect on Pb-induced $H_2O_2$ production and lipid peroxidation, except for Pb-SRFA-25 which significantly reduced $H_2O_2$ induced by Pb at 24 h.

This is the first time that the influence of fulvic acids was studied on $H_2O_2$ induction and lipid peroxidation. Marcato-Romain et al. [37] concluded a protecting effect of soil organic matter on Pb-induced genotocity to *V. faba*. On the basis of our results (Table 2), it is assumed that Pb-induced toxicity correlates with the concentration of free $Pb^{2+}$ ions in plants. Recently, Shahid et al. [11] established a linear relationship between free $Pb^{2+}$ ions concentration in nutrient solution and genotoxicity in the presence of ethylenediaminetetraacetic acid (EDTA). Application of fulvic acids at higher levels, complexed $Pb^{2+}$ ions in the nutrient solution and reduced its uptake and consequently alleviated or delayed the toxicity. Pourrut et al. [8] previously demonstrated that Pb-induced toxicity is antagonized by the $Ca^{2+}$ ions due to the great inhibition of Pb entry into the roots, suggesting an important role for Pb level in oxidative stress induction in plants.

The other possible assumption of alleviation/delay of Pb toxicity when fulvic acids were added at high level could be the minimum threshold level of $Pb^{2+}$ ions required inside the plant to induce $H_2O_2$ and lipid peroxidation which might not be achieved in the presence of these treatments. Moreover, the plant detoxification mechanism is proposed to be more efficient at low plant Pb level than at higher levels. Mishra et al. [36] reported increased level of protein accumulation involved in the maintenance of cell redox status like ascorbate or in the sequestration of the metal like glutathione (GSH) and phytochelatins (PCs) under low Pb levels. This decreased uptake of Pb and in turn comparatively more efficient detoxification mechanism could be the consequence of reduced or delayed Pb-induced toxicity in the presence of higher levels of fulvic acids. For low level of FAs application, despite increased uptake of Pb, Pb-induced toxicity remained slightly below compare to Pb alone. Under diluted conditions, a portion of Pb could be taken up by plants as Pb-FA complex due to fiber FA structure [29]. Inside the plants, this complex either remains stable and in this way slightly reduces Pb toxicity or dissociates after some times and, thus, delays the toxicity. This kind of effect has already been demonstrated by Shahid et al. [11]. They concluded that only stable organic-metal complexes could decrease Pb genotoxicity.

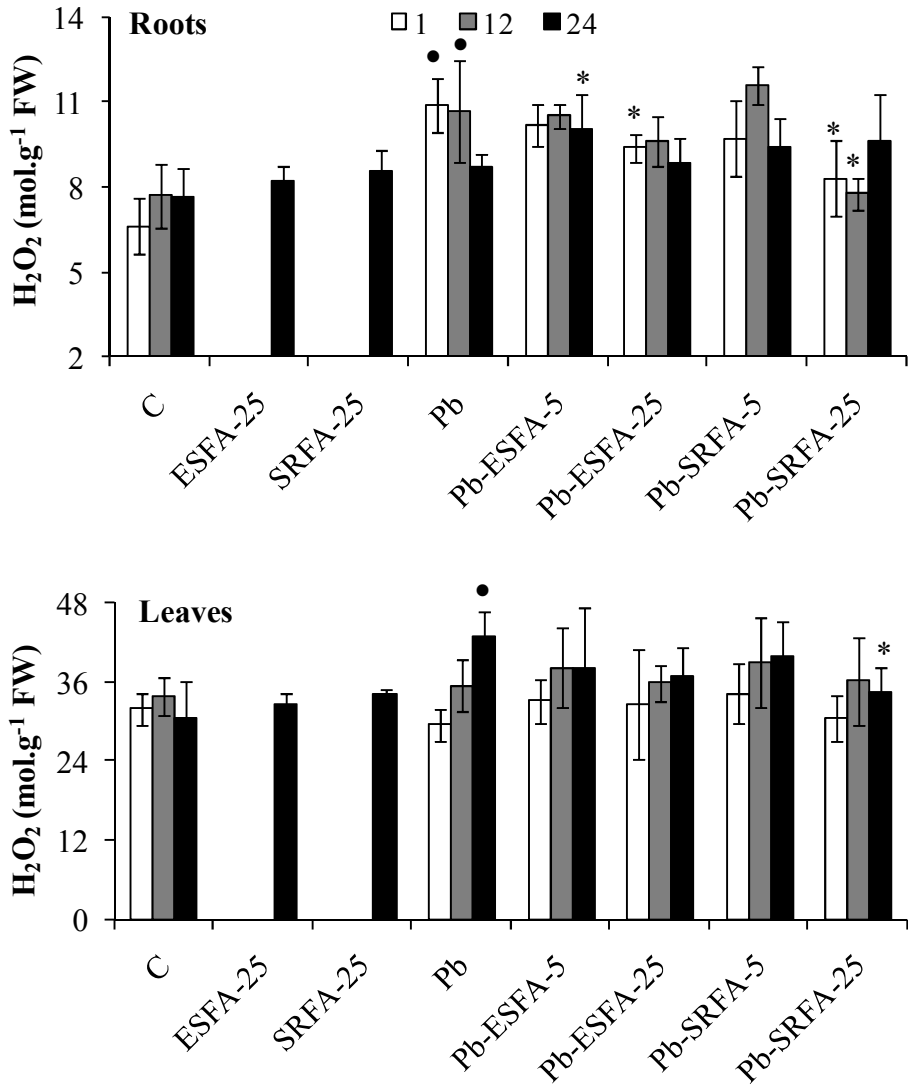


**Fig-1.** Effect of SRFA and ESFA on Pb-induced production of H2O2 (µM.g-1 FW) in *V. faba* roots and leaves. Values are means of two separate experiments each replicated six times. An asterisk (*) indicates significant differences at $P < 0.05$ for Pb-FA treatments compare to Pb alone whereas a solid dot (•) indicates significant differences at $P < 0.05$ for Pb and FAs alone compare to control.

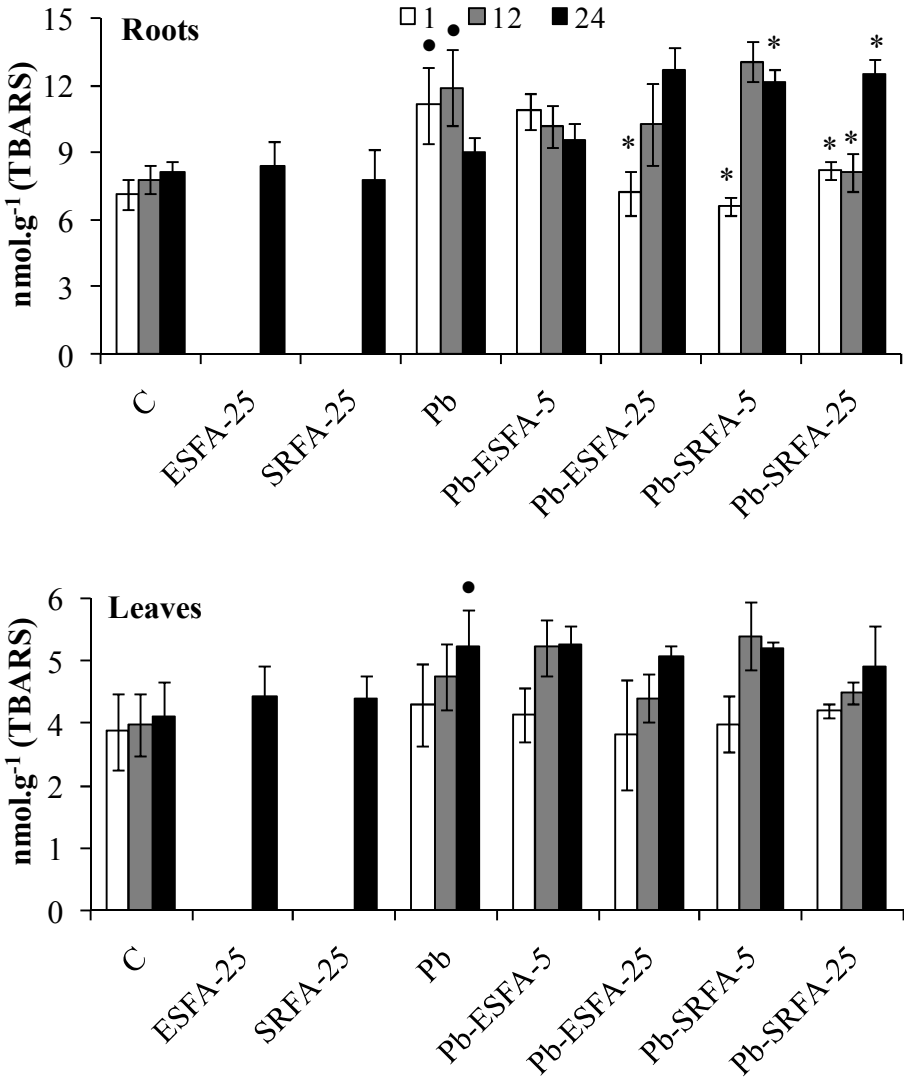


**Fig-2.** Effect of SRFA and ESFA on Pb-induced production of lipid peroxidation (TBARS in nmol.g-1 FW) in *V. faba* roots and leaves. Values are means of two separate experiments each replicated six times. An asterisk (*) indicates significant differences at $P < 0.05$ for Pb-FA treatments compare to Pb alone whereas a solid dot (•) indicates significant differences at $P < 0.05$ for Pb and FAs alone compare to control.

### *3.3. Effect of organic ligands on chlorophyll contents*

The application of fulvic acids alone has no significant effect on pigment contents compare to control except for carotenoid contents which decreased significantly by 9 % in the presence of Suwana river FA (Fig. 1, 2 and 3). Humic substances are, generally, known to increase plant tolerance against metal stress, enhance pigment contents and growth [31,38].

The Fig. 1 illustrates Pb effect on chlorophylls and carotenoids contents in the presence and absence of fulvic acids. The pigment composition remained unaffected after 1 h for all the treatments compare to control. This is due to slow Pb translocation which takes more than 1 h to reach threshold level in leaves as indicated by Pb uptake kinetic (Table 3). The application of Pb alone reduced pigment contents with time at 12 and 24 h, the effect being significant after only 24 h, except for carotenoids contents where the effect was also significant at 12 h. Pb is known to stimulate the degradation of pigments contents [2-4,23].

Pb-induced degradation of pigment has been attributed to many factors, direct or indirect, such as distortion of chloroplast ultra-structure, inhibition of the synthesis of photosynthetic pigments and enzymes of Calvin cycle [36], impaired uptake of essential elements like Mn and Fe, damage of photosynthetic apparatus or due to chlorophyll degradation by increased chlorophyllous activity [2]. Recently, Cenkci et al. [39] also reported a dose dependent negative correlation between Pb concentration and pigment contents in *Brassica rapa* exposed to 0.5-5 mM Pb for 6 days. However, in our case, the rapidity (after 12 h) of observed effect on the pigment contents compare to other studies [4,23,39] suggests the involvement of some fast mechanisms such as production of reactive oxygen species [34,35].

The low level application of both the fulvic acids (5 $mg.L^{-1}$) has no or slight effect on Pb-induced reduction of pigment contents. The effect of Pb-ESFA-5 was similar to Pb alone. In contrast, application of Pb-SRFA-5 reduced significantly Pb-induced Chl-b degradation at 24 h. The application of fulvic acids at higher levels (25 $mg.L^{-1}$) inhibited the Pb-induced toxicity to pigment contents, Pb-SRFA-25 being more effective than Pb-ESFA-25. Addition of Pb-ESFA-25 significantly reduced the degradation of Chl-b induced by Pb. In case of Chl-a and carotenoids contents, the values remained continuously higher than Pb alone. Application of Pb-SRFA-25 alleviated the Pb-induced reduction of pigment contents throughout the experiment. The values of pigments remained close to control for this treatment.

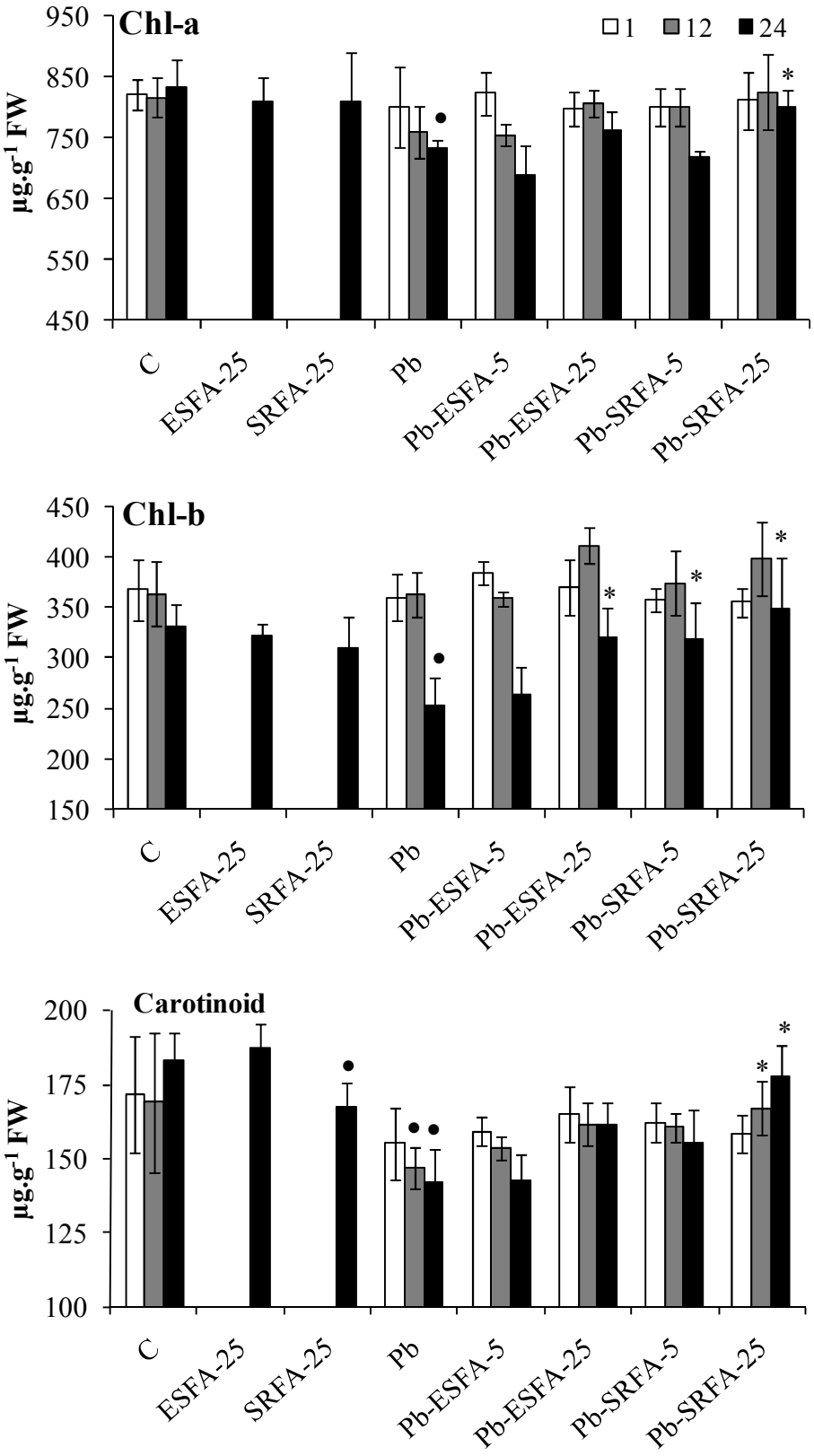


**Fig-3.** Effect of SRFA and ESFA on Pb-induced reduction of pigment contents (µg.g-1 FW) in *V. faba* leaves. Values are means of two separate experiments each replicated six times. An asterisk (*) indicates significant differences at $P < 0.05$ for Pb-FA treatments compare

to Pb alone whereas a solid dot (•) indicates significant differences at $P < 0.05$ for Pb and FAs alone compare to control.

The results show that only higher levels of fulvic acids can effectively bind and reduce Pb toxicity to chlorophyll contents. This effect was due to the reduced Pb concentration in roots and leaves in the presence of higher levels of fulvic acids. Ruley et al. [40] also reported similar effect for LMWOAs on Pb-induced degradation of chlorophyll contents in *Sesbania drummondii*. They stated that due to low binding affinity of citric acid, only high level of these acids can efficiently bind and reduce $Pb^{2+}$ toxicity. The mechanism of protection provided by humic substances against Pb toxicity could be similar to that of the clay minerals, i.e., adsorption of free $Pb^{2+}$ ions to fulvic acid and, therefore, limiting the uptake and toxicity of Pb to living organisms [41]. Kruatrachue et al. [42] reported that application of HSs inhibited the Pb-induced degradation of total chlorophyll contents and growth rate of *Lemna minor*.

## 4. Conclusions and perspectives

The present study highlights the important role of natural fulvic acids towards biogeochemical behaviour and fate of Pb in soil-plant systems. The fulvic acids are capable to alleviate Pb toxicity to plants by complexing highly toxic free $Pb^{2+}$ in solution and reducing their uptake. This protecting role of fulvic acids against Pb uptake and toxicity is function of their applied concentration: only the higher concentrations of FAs (25 $mg.L^{-1}$) could effectively bind and reduce metal toxicity. This was the reason of inefficiency of low FAs levels (5 $mg.L^{-1}$) against Pb toxicity.

Therefore, the behaviour of pollutants in terms of bioavailability, uptake and toxicity in soil systems could vary with natural levels of humic substances. Our results also proposed that Pb toxicity depends on metal binding capacity of humic substances. Due to high binding constant, the SR-FA binds more strongly Pb compare to ES-FA and was more effective against Pb toxicity. Therefore, understanding metal speciation along with nature and level of complexing agents in the natural systems are salient factors in the context of risk assessment and/or soil or water quality criteria.

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# Chapter 4:

# General Discussion

## 1. Study background

This thesis is the part of continuous projects accomplished in the "Laboratory of Functional Ecology (Ecolab)". These previous works revealed the early step of Pb-induced oxidative stress and genotoxicity on *Vicia faba* plants (Thesis Pourrut, 2008) and the importance of Pb speciation in its biogeochemical behaviour (Thesis Uzu, 2009). Based on these previous works and literature review, the thesis was conducted to evaluate the role of Pb speciation on its phytoavailability and phytotoxicity.

## 2. Metal speciation in nutrient

In this study, the Pb speciation in nutrient solution was calculated using speciation models i.e. Visual Minteq ver. 2.60 (Gustafsson, 2008) and Windermere Humic Aqueous Model VI (Tipping, 1998). This calculated $Pb^{2+}$ concentration was correlated to Pb-induced genotoxicity. A linear correlation was observed between genotoxicity and free $Pb^{2+}$ ions concentration in growth medium. This correlation proposed the possible application of speciation models in the field of risk assessment. Many attempts to test the viability or limitations of speciation models have already been made. These studies reported successful prediction of complexed and free metal ions concentrations by speciation models when compared with experimental measurements (Meylan et al. 2004; Parat et al. 2009). Christensen et al. (1999) calculated the complexation of Cu and Pb by DOC from landfill leachate-polluted groundwater and compared with those predicted by WHAM and MINTEQ2. They reported that MINTEQ2 with default database, whereas WHAM with adjusted database offers a very useful prediction of metal complexation by DOC. Guthrie et al. (2005) reported that WHAM V and WHAM VI predict the labile and free-metal-ion concentrations of Ni, Zn, and Cd reasonably well. However, most of these studies employed the speciation model to predict metal speciation or compare it with calculated values (Qu et al. 2008; Parat et al. 2009; Gandois et al. 2010; Stockdale et al. 2010; Tipping et al. 2010). Our results showed that the speciation models can also be employed with success to toxicological studies to make a link between metal speciation, transfer and toxicity in risk assessment studies. Moreover, these models are also very useful to obtain a rapid knowledge of an appropriate system.

### 3. Effect of organic ligands on Pb absorption and translocation

In this study, we observed that the Pb root uptake and translocation to shoot tissues varied greatly as a function of its speciation into the solution. These findings are in accordance with previous studies (Jamil et al. 2009; Kim et al. 2010), which showed that the organic ligands strongly modify Pb speciation in soil solution and in turn change its phytoaccumulation. Most of these studies correlate the metal uptake and translocation to applied level of ligands or physico-chemical properties of soil in the context of metal remediation using hyperaccumulator plants (Keller et al. 2003; Jamil et al. 2009; Sun et al. 2009; Goel et al. 2010). According to our knowledge, no study compares the effectiveness of organic ligands towards metal uptake using sensitive plants as in present study.

In this study, we compared the effectiveness of different organic ligands and observed that metal uptake or translocation is dependent on the type of organic ligand. EDTA forms relatively soluble Pb-EDTA complexes and is, therefore, proposed as the most efficient organic ligand in increasing Pb uptake (Sun et al. 2009; Goel et al. 2010; Hasegawa et al. 2010). In the case of citric acid, no effect on Pb uptake or translocation was observed. Indeed, the citric acid and other LMWOAs are only capable to enhance metal bioavailability by decreasing soil pH (Chen et al. 2003). In the case of humic substances (fulvic acids), the contrasting role at higher and lower level of application showed their complex behaviour towards metal uptake. These molecules have large size with complex and heterogeneous molecular structure (Cabaniss et al. 2000; Calace and Petronio, 2004; Shi et al. 2008). Therefore, it is very difficult to estimate or optimize their role in metal solubilization and uptake. They can increase metal uptake by forming soluble metal complexes (Antoniadis and Alloway, 2002; Evangelou et al. 2004) or can reduce uptake by adsorbing metal on exchange sites Slaveykova et al. 2003; Worms et al. 2010).

Results presented in this thesi also highlighted for the first time the role of dilution factors of humic substances against Pb uptake: increased Pb uptake at low level of application, whereas decreased at higher level. We proposed that at higher levels, HS adsorb Pb at exchange site and reduce it uptake by *V. faba* plant roots. On the other hand, at lower level, they increased Pb uptake either by forming soluble Pb-FA complexe, which enters the plant like Pb-EDTA or by exchanging the adsorbed Pb with protons of root surface charges. This shows that understanding nature and applied level of complexing agent, particularly in the case of humic substances is also important in soil-plant metal transfer studies. According

to Kleber and Johnson (2010), the polymeric nature of HSs under concentrated conditions may complex Pb very strongly and reduces its uptake.

In the presence of citric acid and HS, the translocation of Pb to shoot tissues depends on its uptake i.e. increased translocation when there was increased uptake and *vice versa*. However, a contrasting role of EDTA towards Pb translocation in *V. faba* seedlings was observed with respect to these organic ligands and most of literature (Ruley et al. 2006; Usman et al. 2009; Xu et al. 2009). The EDTA increased Pb uptake, whereas decreased its translocation in a dose dependent manner. The literature shows many fold increased translocation of Pb by EDTA (Evangelou et al. 2007; Usman et al. 2009; Saifullah et al. 2009 and 2010). This is also the most prominent features of its utilization in phytoremediation studies. The comparison of our work with literature showed that most of the studies carried out using EDTA employed hyperaccumulator plant. These plants have, generally, efficient metal detoxification mechanism. In contrast, *V. faba* is a very sensitive plant to Pb toxicity. This plant responds very quickly to metal toxicity. This difference in plant type can be the result of reduced Pb translocation in the presence of EDTA. Moreover, Pb transportation to vacuole of root cells in the form of Pb-EDTA is also possible, which decreased its translocation. Similarly, the short duration of our study (24 h *vs* more than one week) could also be the consequence of reduced Pb translocation.

**4. Effect of organic ligands on Pb uptake kinetics**

Our results showed that Pb uptake and translocation kinetic is not linear over time but occurs in phases during the first 24 h (Fig. 1 on next page). In this study, 35-40 % of total Pb uptake took place during the first hour for all the treatments. Pb uptake was maximum after the first hour, decreased continuously up to 8 h then again increased at 12 h followed by another decrease at 24 h. Concerning Pb translocation to aerial parts, Pb translocation rate increased until 8 h followed by decreased upto 24 h. Moreover, chelation of Pb by organic ligands only affects rate of Pb uptake or translocation but not the phases of uptake except for EDTA, which modifies translocation trend.

Previously, Pourrut et al. (2008) reported that Pb-induced oxidative stress to *V. faba* seedlings coincide with its entrance to plant tissues. In this work, similar results were observed for Pb alone. But this correlation between Pb entrance to plant tissues and toxicity was not valid in the presence of organic ligands. Pb toxicity was inhibited by EDTA, delayed

in time by citric acid, whereas delayed or inhibited by fulvic acids. This shows that organic ligands chelate and in turn prevent Pb effect by different mechanisms. This again confirmed the role of Pb speciation towards its uptake and toxicity. There is no bibliographic data available regarding the Pb uptake and translocation kinetics over short exposure periods in the presence of organic ligands. However, Nedelkoska and Doran (2000) and Sgherri et al. (2007) showed triphasic kinetic profiles for cadmium, nickel and copper in the absence of organic ligands.

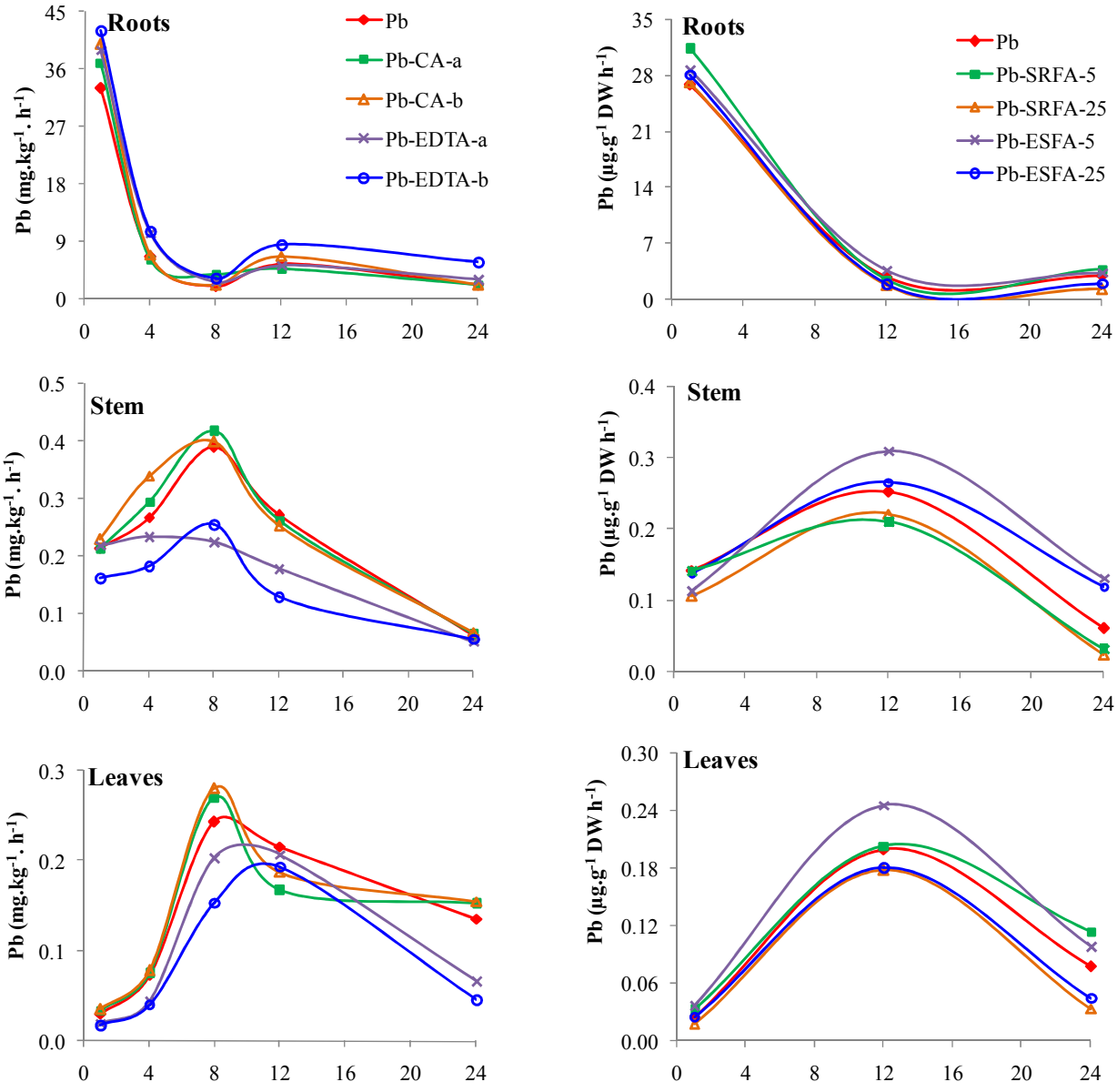


**Fig-1.** Effect of organic ligands on Pb kinetic uptake and translocation by *Vicia faba*.

**5. Effect of organic ligands on Pb-induced toxicity**

In this study, a linear curve was obtained for EDTA ($r^2 = 0.93$) when genotoxicity was plotted against the $Pb^{2+}$ concentration in solution (Fig. 2 on next page). However, this correlation between Pb-induced genotoxicity and $Pb^{2+}$ concentration is not valid in the presence of citric acid. Similarly, the correlation was very week when micronucleus (MN) frequency was plotted against Pb contents in *V. faba* roots (Fig. 2). The contrary behaviour of EDTA and citric acid toward Pb-induced genotoxicity is due to differences in their complexing constants. EDTA, having highest binding capacity for Pb forms stable and non toxic Pb-EDTA complexes. Citric acid has very low binding capacity for Pb and therefore, did not affect its toxicity but delayed the effects. We proposed that the Pb-CA complex dissociates before or after uptake. This breakdown is the result of microbial activity or low stability of Pb-CA complex. Although the behaviour of fulvic acids towards Pb toxicity varies with applied level but in both the cases Suwannee River fulvic acid was more effective than Elliott Soil fulvic acid alleviating its toxicity to *Vicia faba* due to strong binding affinity for Pb. Although, in literature the studies using different organic ligands do not describe this comparison but the results reported have similar pattern (Ruley et al. 2004 and 2006). This effect of binding capacity could be better traced in literature with respect to metal uptake than toxicity due to scarcity of data in the case of toxicity to plants.

Our results proposed that the Pb-induced toxicity is directly associated with the uptake of free $Pb^{2+}$ ions. In a previous work Pourrut et al. (2008) demonstrated that Pb-induced oxidative stress was antagonized by the calcium entry blocker $LaCl_3$ or high concentrations of $Ca^{2+}$, suggesting an important role for the free ion $Pb^{2+}$. Sauvé et al. (1998) also found similar results for $Pb^{2+}$ upon a variety of crop plants. The role of organic ligands in this study was also in accordance with this hypothesis. EDTA complex this toxic form, reduce its exposure to plants and ultimately alleviated toxicity. In case of CA, we suggest that Pb-CA complex was taken up by *V. faba* seedlings just like Pb-EDTA. But inside the plant, Pb-EDTA remained stable, whereas Pb-CA dissociate and causes increased and delayed production of $Pb^{2+}$ ions inside the plant. This delayed production of $Pb^{2+}$ ions was the possible result of delayed Pb toxicity. Fulvic acids also reduced Pb toxicity by decreasing $Pb^{2+}$ uptake by plants at higher levels of application, whereas delayed slightly at lower level of application.

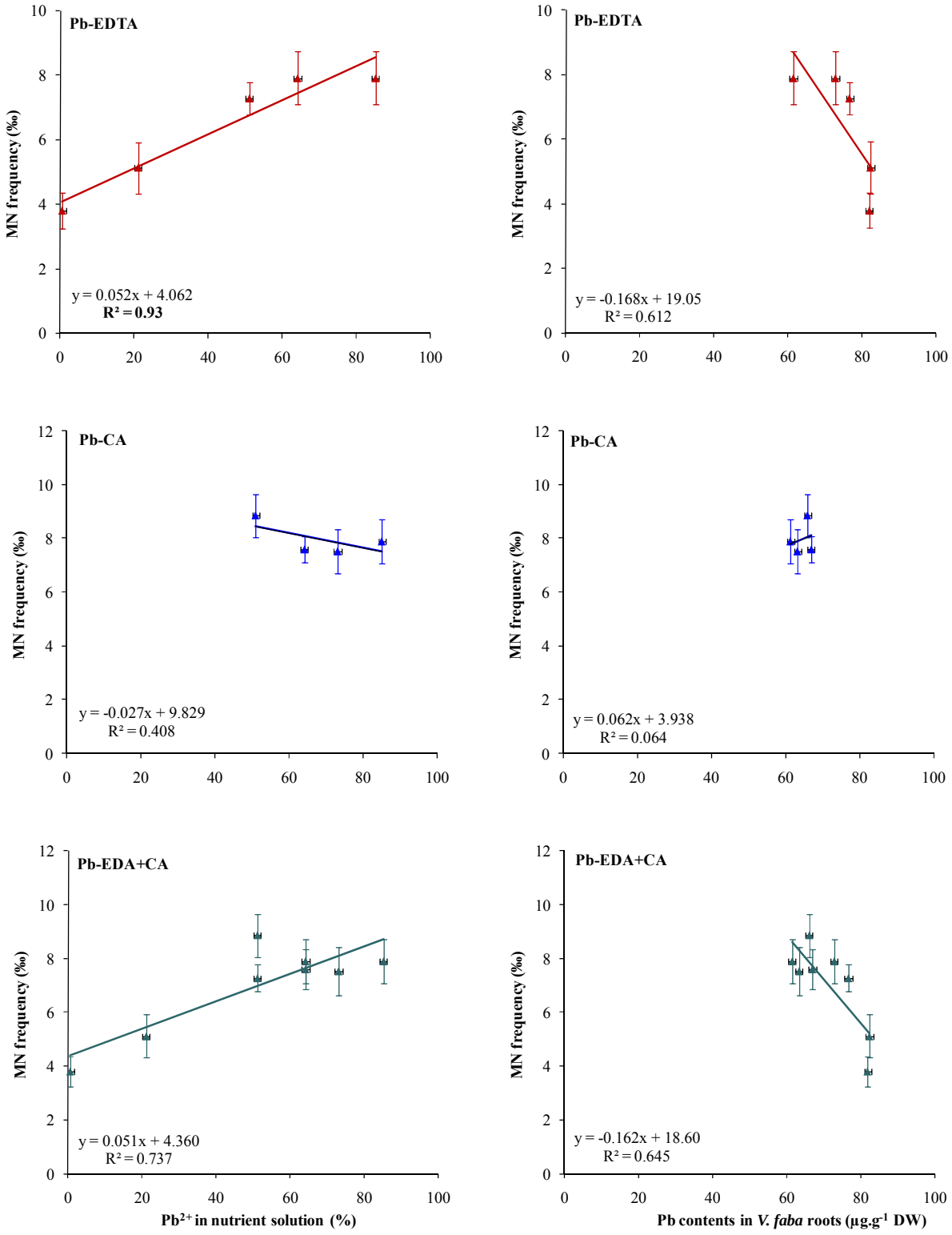


**Fig-2.** Micronucleus frequency plotted against free Pb ion concentration in solution (left side) and Pb contents in *V. faba* roots under EDTA and citric acid.

## 6. Effect of organic ligands on Pb-induced oxidative stress

The oxidative stress, during which a large quantity of reactive oxygen species are generated, is one of the earliest responses of plant cells to Pb toxicity (Mishra et al. 2006; Pourrut et al. 2008; Singh et al. 2010). This is basically the result of imbalance between the generation and the neutralization of ROS by antioxidant mechanisms (Mishra et al. 2006, Fig. 3). These free radicals are unstable molecules that have an unpaired electron in their outer shell. They react with (oxidize) various cellular components including DNA, proteins and lipids / fatty acids (Fig. 3). These reactions between cellular components and free radicals causes DNA damage, mitochondrial malfunction, cell membrane damages and eventually cell death (Reddy et al. 2005; Clemens 2006; Hu et al. 2007; Wang et al. 2007; Pourrut et al. 2008; Yadav 2010). The same results are also observed in this study where application of Pb induced ROS production and lipid peroxidation. Moreover, Pb-induced genotoxicity in term of micronuclei is also proposed to be the result of ROS production (Shahid et al. 2011). Our data clearly illustrates the effect of Pb speciation on ROS production and DNA damages.

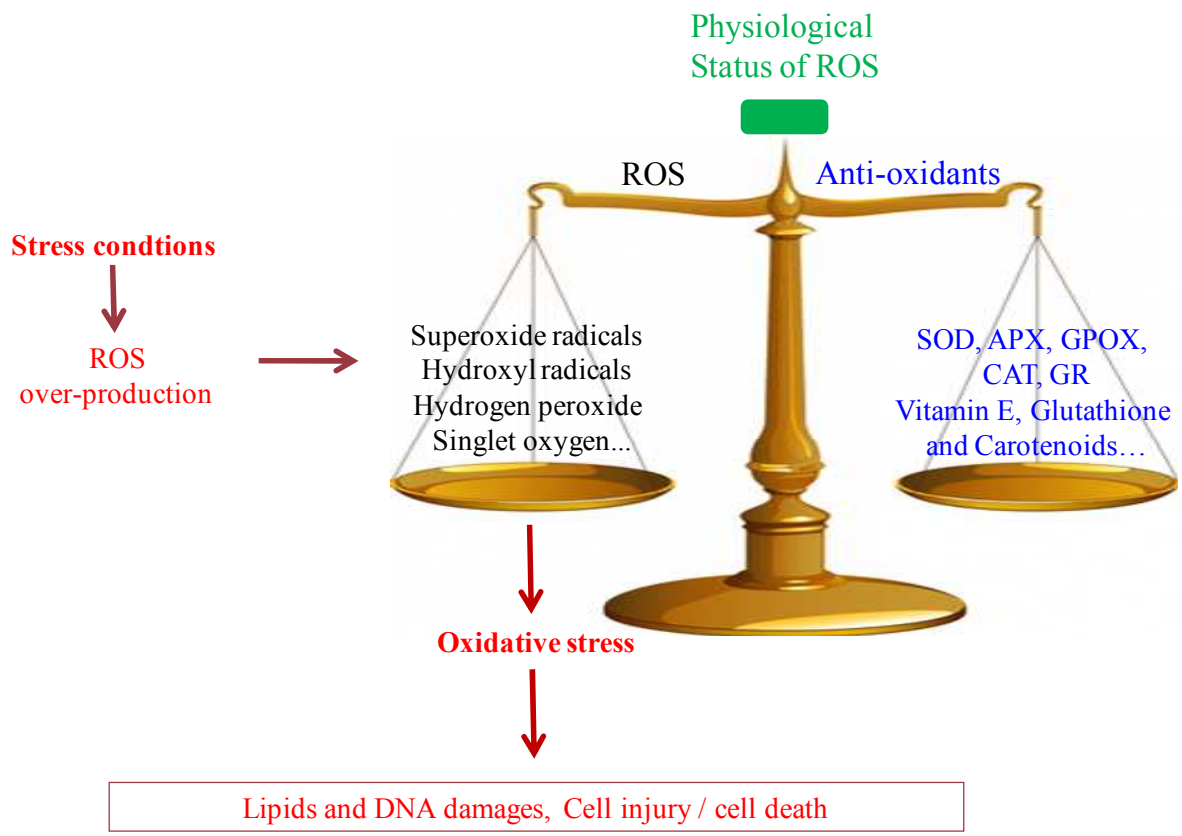


**Fig-3.** Mechanism of Pb-induced oxidative stress in plants.

The time course experiment showed that Pb-induced LPO occurred after Pb concentration reached a threshold value in roots (after 1 h) and leaves (after 8 h). This suggests that Pb-induced production of $H_2O_2$ and lipid peroxidation (LPO) is a very rapid phenomenon both in roots and leaves of *V. faba* seedlings (Fig. 4). These results highlighted the importance of time dependent short-duration oxidative studies. Moreover, oxidative stress appears to persist in the leaves compare to roots under very low concentration of Pb (almost 35 times lower compared to roots) showing high sensitivity of *V. faba* leaves to Pb. This persistence of oxidative stress was also valid in the presence of organic ligands except in presence of high concentrations of EDTA.

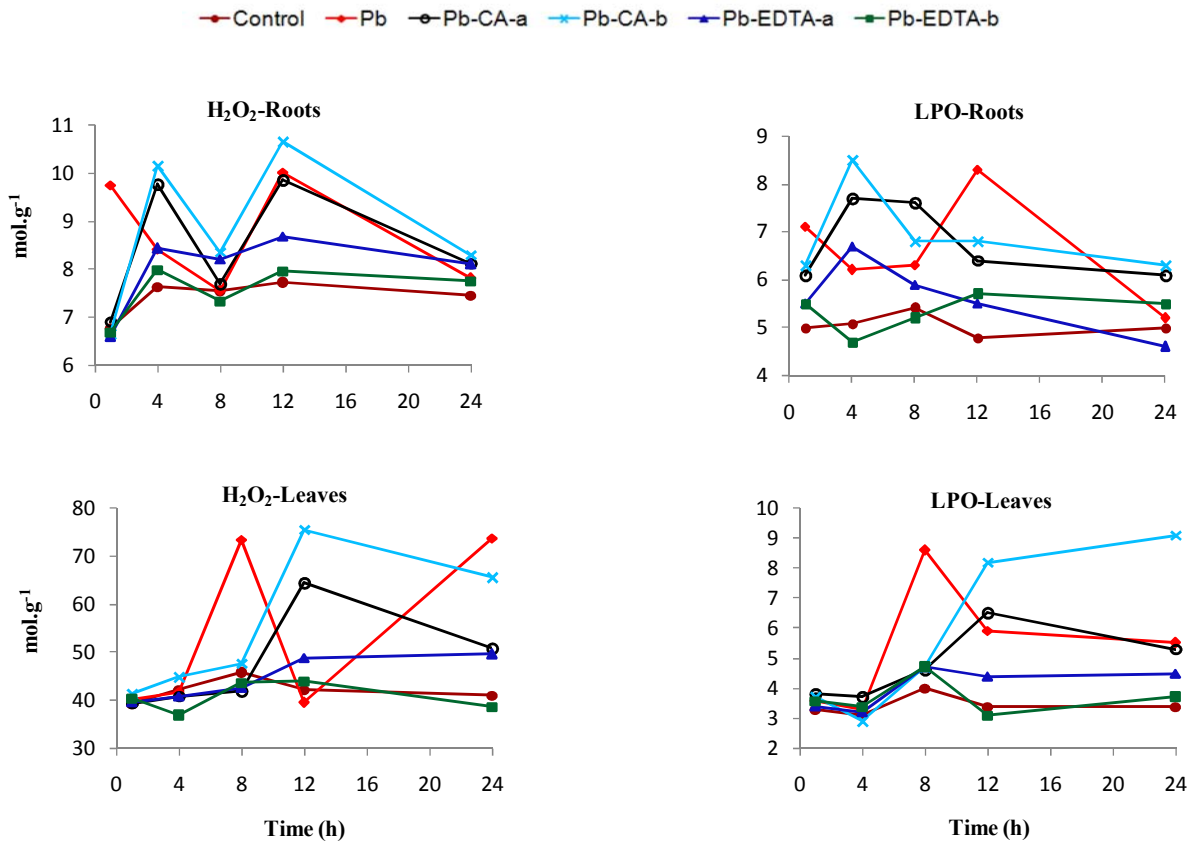


**Fig-4.** Relationship between Pb-induced ROS production and oxidative stress.

## 7. Effect of organic ligands on pigment contents

In addition to oxidative stress induction, the presence of Pb into the leaves also adversely affects the photosynthetic pigment content. This could be associated to Pb effect on decline in photosynthetic rate due to distorted chloroplast ultrastructure, restrained synthesis of chlorophyll, plastoquinone and carotenoids, obstructed electron transport, inhibited activities of Calvin cycle enzymes, impaired uptake of essential elements such as Mg and Fe

as well as deficiency of $CO_2$ as a result of stomatal closure (Fig. 5) (Mishra et al. 2006; Liu et al. 2008; Gupta et al. 2009, Cenkci et al. 2010; Sing et al. 2010). However, these effects of Pb were observed for long time exposure of plant to this metal. The rapidity of the alteration observed in our experiments can not be explained by these different pathways. It seems that the Pb acts on pigments by other mechanisms, directly or indirectly associated to the presence of this metal. This rapid reduction of pigment contents strongly suggests the involvement of ROS in altering pigment observed (Fig. 5). Very few studies have assessed the impact of metal contamination on photosynthetic pigments at short times. On an aquatic plant, Ralph and Burchett (1998) showed a significant decrease in chlorophyll content after five hours of exposure to Pb, cadmium, copper or zinc. These authors also reported a sharp decrease in the carotenoid content in plants exposed to Pb. They suggest a common mechanism of interaction between metals and the chlorophylls, in the first hours of the exposition. ROS production could be involved in this mechanism.

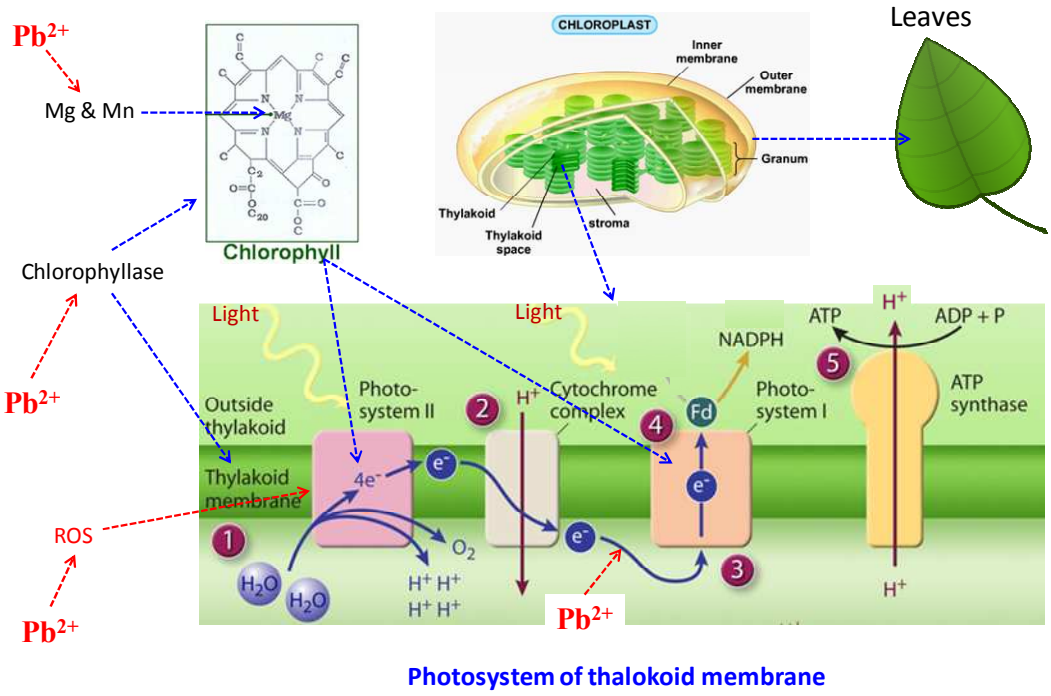


**Fig-5.** Pb-induced reduction of pigment contents.

## 8. Effect of organic ligands on plant detoxification mechanisms

To combat oxidative damage, plants contain a wide range of protective mechanisms. Fig. 6 shows the activites of antioxidant enzymes in *V.faba* roots and leaves.

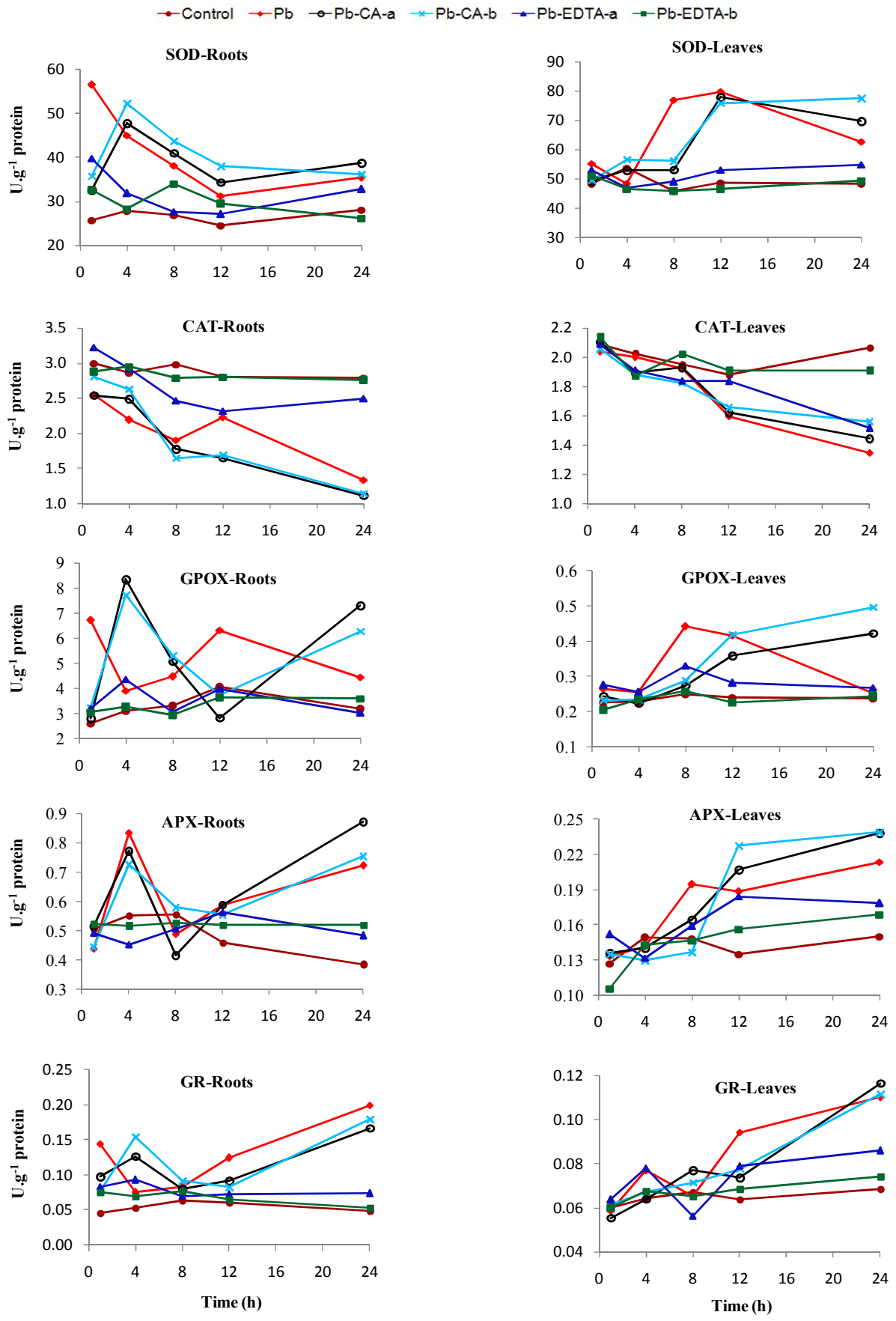


**Fig-6.** Comparison of antioxidant enzymes activities in *V. faba* roots and leaves.

First is either the avoidance of metal entry into the cell via exclusion or binding metal to cell wall and other ligands *viz*., organic acids, amino acids, GSH or PCs to render them harmless at primary level of metal entry to the cell. For Pb, binding to cell wall is one of the major mechanisms of detoxification (Islam et al. 2007; Kopittke et al. 2007; Arias et al. 2010). In this study, the increased Pb sequestration in root cell wall or sequestration to vacuole in the presence of EDTA could be the proposed detoxification mechanism. However, this mechanism was not found to be active in the presence of citric or fulvic acid.

Secondary defense system constitutes various antioxidants to combat increased production of ROS caused by metal (Fig. 6). These enzymes scavenge different types of ROS, thereby prohibiting cell injury and tissue dysfunction (Singh et al. 2010). Most antioxidants are electron donors and react with the free radicals to form innocuous end products such as water. In this study, the activation of these enzymes indicated the activation of this detoxification mechanism.

Enzymes of ascorbate–glutathione cycle, APX and GR, are localized mainly in chloroplasts and also in other cellular organelles and cytoplasm where they play important role in combating oxidative stress (Fig. 7) (Singh et al. 2010). APX utilizes ascorbate to reduce $H_2O_2$ to water and oxygen (Fig. 7). In the process ascorbate is oxidized to monodehydroascorbate, which may then either be directly reduced back to ascorbate by monodehydroascorbate reductase (MDHAR) or may get first converted to dehydroascorbate and then reduced by dehydroascorbate reductase (DHAR). This reduction consumes GSH as reductant, which is oxidized to GSSG. Induced activity of GR maintains high GSH/GSSG ratio and thus plays a bridging role between primary and secondary responses of plant against metal toxicity.

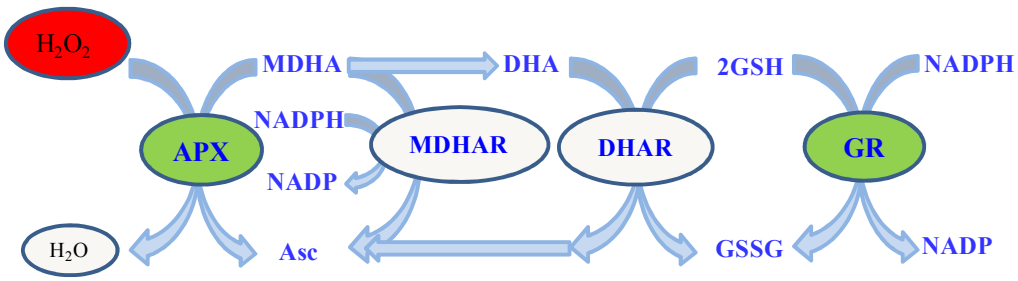


**Fig-7.** Function of APX and GR in Halliwell-Asada pathway for ROD detoxification.

The comparison of different toxicity tests using PCA in our study highlighted the important role of GR in roots and APX and GR in leaves. We suggested that these enzymes play a key role in accordance with Pb speciation and signal the major earlier defense against Pb toxicity. Moreover, GSH and PC play an important role in protecting membranes from free radical damage by trapping oxygen radicals in the aqueous phase (Sharma and Dubey, 2005). The Pb-induced PCs are documented by Pourrut (Thesis 2008) under same experimental conditions in *V. faba*.

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# Conclusions and Perspectives

This section summarize the conclusion (in English and French) of all the different studies presend above. Moreover, some future perspective are also discuised at the end.

## 9. Conclusions

La génotoxicité du plomb induite sur *Vicia faba* est fonction de sa concentration en ions libres dans la solution nutritive. Sous forme ionique, le plomb est capable d'intervenir directement ou indirectement sur le métabolisme des plantes. La phytotoxicité du plomb réduit la teneur en pigments chlorophylliens et induit la peroxydation lipidique et l'activation des enzymes en raison de la surproduction d'espèces réactives de l'oxygène. Cette surproduction d'espèces réactives de l'oxygène par le Pb est un mécanisme prépondérant de sa phytotoxicité : les acides nucléiques, les protéines et lipides peuvent ainsi être oxydés. Le Pb peut également causer des altérations de l'ADN à l'origine de l'induction des micronoyaux, soit directement par liaison directe avec l'ADN, soit indirectement par l'intermédiaire de la production d'espèces réactives de l'oxygène. Les résultats obtenus au cours de cette thèse posent plusieurs questions scientifiques en lien avec : (I) la compréhension du comportement biogéochimique des polluants dans les écosystèmes et (II) les études d'évaluation des risques environnementaux.

Nous avons révélé que le Pb pénétrait rapidement dans le système racinaire et induisait une forte activation du stress oxydatif. Cette induction diminuait après 4 heures, puis re-augmentait jusqu'à 12 ou 24. Cette production de ROS induite par le plomb ainsi que l'induction d'enzymes antioxydantes est un phénomène très rapide et coïncide bien avec l'entrée de plomb dans les tissus végétaux. Ce travail a révélé l'importance des études menées en cinétique et sur de courtes durées concernant à la fois la peroxydation lipidique, l'activation des enzymes et la production de ROS, afin de mieux comprendre les étapes précoces de la toxicité des métaux.

Après absorption, le plomb s'accumule principalement (95 %) dans les racines de *Vicia faba*. Selon de nombreuses études, la séquestration du Pb dans les racines ou sa translocation vers les parties aériennes dépend, en particulier, des protéines transporteurs spécifiques. Selon nos observations, ces protéines transporteurs spécifiques du Pb seraient absentes chez *Vicia faba*.

L'application d'EDTA augmente l'absorption du Pb, mais diminue sa translocation vers les parties aériennes. Ces données suggèrent deux voies possibles dans le transfert de Pb: (I) une forme ionique par les canaux ioniques et interaction avec les composants cellulaires (II) une forme non-ionique (forme chélaté), qui peut entrer rapidement, s'accumuler dans les racines, et être stockée, par exemple, dans les vacuoles ou les parois des cellules. La présence

d'EDTA réduit la toxicité induite par le Pb en formant avec les ions $Pb^{2+}$ des complexes bio-disponibles pour la plante, mais stables (fortes constantes de complexation) et faiblement toxiques. Cependant, contrairement à d'autres plantes, la translocation du Pb vers les partie aériennes de *Vicia faba* a diminué en présence d'EDTA. Cela est dû aux courts temps d'exposition, à la nature de la plante *Vicia faba* (extrêmement sensible au Pb) et à l'absence de transporteurs du Pb.

L'acide citrique n'a eu aucun effet significatif sur l'absorption du plomb, son transfert ou sa toxicité. Ceci serait du à la faible stabilité du complexe avec le Pb, qui est susceptible de se dissocier juste avant ou après l'absorption par les plantes.

Les substances humiques ont montré un comportement non linéaire à des niveaux différents d'exposition pour l'absorption et la toxicité du plomb en raison de leur structure complexe. Le mécanisme de protection proposé pour les substances humiques contre la toxicité du plomb est similaire à celle des minéraux argileux, à savoir, l'adsorption des ions $Pb^{2+}$ libre qui limite l'absorption et la toxicité du plomb pour les organismes vivants.

L'ensemble de nos résultats soulignent la sensibilité et l'efficacité des essais biologiques est fonction des conditions environnementales, en plus du type d'organisme ciblé ou des formes chimique du plomb. Dans cette étude, la comparaison des différents essais d'évaluation des risques à l'aide de l'APC a montré que GR et APX sont les principales enzymes qui réagissent contre la toxicité du Pb en relation avec son absorption et spéciation dans les racines ; tandis que seule GR réagit dans les feuilles. Ces données suggèrent que l'activité GR est un bon indicateur du stress induit par le plomb à la fois dans les racines et les feuilles et doit donc être mesuré en priorité pour une évaluation de la phytotoxicité du plomb.

**Perspectives**

Les résultats obtenus au cours de cette thèse posent plusieurs questions scientifiques en lien avec : (I) la compréhension du comportement biogéochimique des polluants dans les écosystèmes et (II) les études d'évaluation des risques environnementaux.

Le plomb est en effet connu pour induire une toxicité directement ou indirecte sur les plantes par la production de ROS qui interfèrent avec le métabolisme des plantes. Cependant les mécanismes d'action des effets délétères du plomb sont encore mal connus. La connaissance de ces mécanismes d'action pourrait permettre d'améliorer les outils d'évaluation des risques. De plus, le séquençage génétique de *Vicia faba* n'a pas encore été réalisé, pourtant ces données génétiques pourraient mettre en lumière certains mécanismes de la toxicité du Pb chez les plantes sensibles utilisées comme biotests.

Nos résultats montrent que les réponses des plantes à la toxicité induite par le plomb (production ROS, activités des enzymes, etc.) ne sont pas corrélées de façon linéaire avec la durée d'exposition. En effet ces réponses pourraient coïncider avec l'entrée du Pb dans les différents compartiments de *Vicia-Faba*. Il serait donc nécessaire de mieux connaitre les cinétiques de transfert du plomb dans la plante et de réaction de la plante au stress pour améliorer l'utilisation de la plante comme biotest. Des mesures effectuées avec des pas de temps et des durées totales d'exposition plus ou moins grands pourraient renseigner ces cinétiques. Cette réflexion serait extrapolable à priori pour d'autres organismes vivants que *Vicia-Faba* (choisie comme plante modèle de la thèse) et d'autres polluants comme les pesticides.

De nombreuses études ont conclu que l'EDTA pouvait accroître la translocation du Pb dans le cas de plantes accumulatrices pour des expériences généralement de plusieurs semaines. Cependant, dans le cas de *V. faba*, plante sensible au stress métallique, exposée au plomb sur une durée maximale de 24 h (intérêt du biotest de courte durée), la translocation a été réduite en présence d'EDTA. Cela pourrait être dû à un effet espèce : *V. faba*, plante sensible n'a pas de transporteurs de métaux actifs identifiés ; et/ou à un effet durée d'exposition. Ces deux hypothèses pourraient être évaluées en effectuant une expérience à plus long terme avec *V. faba* et également en utilisant d'autres plantes sensibles au Pb comme *Allium cepa*.

Nos résultats ont démontré que la toxicité du Pb sur *V. faba* est fonction de la concentration en ions métalliques libre dans la solution nutritive. Une hypothèse avancée repose sur la stabilité des complexes organo-métalliques formés. Cependant, des expériences avec d'autres ligands permettraient d'affiner la compréhension des mécanismes. Par exemple : d'autres ligands organiques forts mais avec des caractéristiques différents d'EDTA (encombrement stérique, polarité). De même, différents ligands organiques pourraient être utilisés pour tester leur effet sur la phytotoxicté d'autres polluants que le plomb.

Le modèle de génotoxicité induite par le plomb développé dans cette thèse pourrait être appliqué à des solutions de divers sols pollués (à partir du moment où les paramètres d'entrée du modèle sont disponibles). Il serait en particulier possible d'évaluer à priori l'influence de composés organiques parfois ajoutés lors de la décontamination des sols pollués. De plus l'application et le développement de ce type de modèle qui permet de lier des paramètres du sol (ou de sa solution) avec l'écotoxicité des substances sont des outils utiles dans le contexte des recherches menées à Ecolab sur les charges critiques.

La comparaison des différentes techniques d'évaluation du stress oxydatif à l'aide de l'APC a montré que dans un contexte donné (une plante, un métal, etc.), certaines mesures étaient bien plus sensibles et pertinentes que d'autres. Cette conclusion peut être généralisée : quelque soit l'organisme et le polluant étudiés, il semble nécessaire de mettre en place des batteries de tests écotoxicologiques et de les comparer lors d'un test statistique afin de déterminer le ou les plus pertinents. Ces optimisations (en fonction des conditions environnementales, du polluant appliqué, de l'organisme cible, etc.) permettraient une évaluation plus rapide et efficace des risques environnementaux induits par les polluants.

## 11. Conclusions

The results of present study concluded that Pb toxicity and genotoxicity to *Vicia faba* are the function of its free ion concentration in nutrient solution rather than total contents in plants tissues or nutrient solution. In ionic form, Pb is capable to interfere directly or indirectly with plant physiological metabolism and genetic material and, in turn, could cause a series of negative effects. Pb phytotoxicity reduced pigment contents and caused lipid peroxidation and enzymes activation due to over-production of reactive oxygen species in *Vicia faba* seedlings. This over-production of reactive oxygen species is the most prominent feature of Pb phytotoxicity, which could rapidly attack all types of biomolecules through oxidation, such as nucleic acids, proteins and lipids. The Pb caused DNA alterations and particularly DNA breakdowns at the origin of micronucleus either directly by binding with DNA or indirectly through reactive oxygen species production.

We revealed that Pb quickly penetrated into the root system and caused significant oxidative stress after only a few hours before being scavenged by enzymes. This production of ROS by Pb and scavenging by antioxidant enzymes is a very rapid phenomenon and coincide well with Pb entrance in the plant tissues. This revealed the importance of continuous and short duration studies regarding lipid peroxidation, enzymes activations and ROS production to understand better the early steps of metal toxicity.

After uptake Pb accumulate mainly (95 %) in *Vicia faba* roots. The sequestration of Pb in roots or translocation to aerial parts depends, in particular, by metal transporter proteins, which are specific to metal and plant species types. This showed the absence of these specific Pb transporter proteins in *Vicia faba* seedlings.

The results proved that the efficiency of organic ligands towards Pb solubilisation and mobility in soil, uptake by plants, translocation to shoot tissues, and phytotoxicity varies greatly by their metal binding capacity. This binding capacity in turn depends on their molecular structure and amount and type of functional groups.

EDTA, having highest binding capacity for Pb, forms relatively high soluble and stable Pb-EDTA complexes and is, therefore, the most efficient organic ligand in increasing Pb solubilisation and phytouptake. However, the translocation of Pb to tissues parts is the function of treatments exposure time, nature of plant and the absence/effectiveness of Pb

transporter proteins. The protection induced by EDTA against Pb toxicity is due to the binding of toxic free Pb ions, reducing their exposure to plants and carrying them to vacuoles.

Citric acid is a very week Pb complexent and had no or very little effect on Pb uptake, translocation or toxicity due to weak Pb-CA complexation. CA affects metal behaviour only in nutrient solution mainly by changing pH. Buffering solution pH as in our case makes them inefficient with respect to metal uptake and toxicity to plants.

The humic substances showed non linear behaviour at different levels of exposure towards metal uptake and toxicity due to complex structure. They can effectively bind and reduce metal uptake and toxicity only at higher concentrations. The mechanism of protection provided by humic substances against Pb toxicity is similar to that of the clay minerals, i.e., adsorption of free $Pb^{2+}$ ions and, therefore, limiting the uptake and toxicity of Pb to living organisms.

The sensitivity and efficiency of short term and long term bioassays is the function of environmental conditions in addition to pollutant and target organism type. In this study, the comparison of different risk assessment assays using PCA showed that the enzymes of ascorbate–glutathione cycle, APX and GR, play important role in combating Pb-induced oxidative stress in accordance with Pb uptake and speciation. These data suggest that GR activity in both roots and leaves is a good indicator of Pb-induced stress in Pb-sensitive plants and should be measured in priority for phyto-toxicity assessment.

## 12. Perspectives

The results of present study arosed several scientific questions, which need attention in order to improve the understanding regarding the bio-geochemical behaviour of pollutants in ecosystem and risk assessment studies.

Pb is known to induce toxicity to plants directly or indirectly by producing ROS, which interfere with plant metabolism. However the mechanisms of actions behind these noxious effects of Pb in living organisms are still unknown. These mechanisms could further improve the tools in the context of risk assessment and quality criterian. Moreover, the genetic makeup (sequencing) of *Vicia faba* plants is still unknown. The research in this field can disclose many unknown mechanisms of Pb toxicity in sensitive plants.

Our results showed that Pb-induced production of ROS and resulting oxidative stress and activation or suppression of enzymes are very rapid and non linear mechanisms with respect to exposure time. They coincided with the entrance of Pb in different plant tissues. It is, therefore, necessary to know the kinetics of pollutant transfer in plants in addition to plant response to stress to improve the use of plants as bioassay. Therefore, further studies using different organisms and pollutants such as pesticides or heavy metals could be performed over short and continuous exposure time to assess their early step of toxicity induction in the target organisms.

Literature showed that EDTA increase Pb translocation to aerial parts by many fold. However, in our case the translocation was decreased by EDTA. We hypothised that this might be due to plant nature (sensitive *V. faba* to Pb), absence of Pb transporters or short exposure time. This hypothesis could be evaluated by performing a long term experiment using *V. faba* or using other Pb sensitive plants like *Allium cepa*.

Our results proved that Pb toxicity to *V. faba* seedlings is the function of free metal ions concentration in nutrient solution. However, this relation persists only in the presence of strong chelating agents like EDTA in our case. This relationship can be assessed using other strong organic ligands like EDDS. Similarly, the organic ligands used in this study could be used for other pollutants to evaluate their effect with respect to metal type.

The model of Pb-induced genotoxicity *vs* free ion concentration in nutrient solution developed by us in this study could be applied to soil solution of polluted soil or where

organic ligands are used for polluted soil remediation. The application and optimization of this model in soil solution conditions will further help the researchers to quickly analyse the toxicity behaviour of metal in soil solution.

The comparison of different oxidative stress assessment techniques using PCA showed that GR and APX are the main enzymes reacting against Pb toxicity in relation with Pb uptake and speciation in *V. faba*. Similarly other toxicity assessment assays could be compared using different statistical comparison tools to optimize the most sensitive and efficient assays with respect to environmental conditions like applied metal and organic ligand form, physic-chemical parameters of medium and metal and plant type. These optimizations will Pb to a fast and efficace assessment of pollutants risk and environmental quality.

# Annexes

In this section, I have presented a short CV of all the publications, communications and posters presented in different journals and conferences (published, submitted and under preparation). Some of these articles, posters and scientific communications also include my M2R research work, which I conducted here in INP-ENSAT under the supervision of C. Dumat.

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## List of publications accepted & under review

### Publications accepted

- **M. Shahid,** C. Dumat, B. Pourrut, P. Winterton and E. Pinelli. 2011. Mechanisms of Pb uptake, toxicity and detoxification in plants. (Accepted in Reviews of Environmental Contamination and Toxicology & will be published in vol 213, 2011).

- **Shahid, M.,** Pinelli, E., Pourrut, B., Silvestre, J., Dumat, C., 2011. Pb-induced genotoxicity to *Vicia faba* L. roots in relation with metal cell uptake and initial speciation. Ecotoxicology and Environmental Safety. 74, 78–84.

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### Publications under review

- **M. Shahid,** C. Dumat, C. Laplanche, B. Pourrut, J. Silvestre, E. Pinelli. Comparison of *Vicia faba* metabolic responses in relation with Pb speciation and uptake. (Submitted to Ecotoxicology, ECTX1562).

- **M. Shahid**, C. Dumat, E.Pinelli. Effect of fulvic acids on Pb-induced oxidative stress to metal sensitive *Vicia faba* L. plant (Submitted to "Journal of Hazardous Materials" HAZMAT-D-10-05879).

- **M. Shahid,** E. Pinelli, P. Winterton and C. Dumat. Review of Pb availability and toxicity to plants in relation with metal speciation; role of synthetic and natural organic ligands (To submit in "Journal of Hazardous Materials").

## Papers & poster in Conferences Proceedings

- **M. Shahid,** E. Pinelli, J. Silvestre and C. Dumat. Effect of fulvic acids on Pb-induced oxidative stress to sensitive *V. faba* plant used as biotest. Accepted in 11th International Conference on the Biogeochemistry of Trace Elements (ICOBTE), 3-7 July 2011, Florence-Italy.

- **M. Shahid**, C. Dumat, B. Pourrut, J. Silvestre & E. Pinelli. Role of speciation in uptake and toxicity of Pb to *Vicia faba* L. SETAC Europe 20th Annual Meeting, Science and Technology for Environmental Protection, 23-27 May 2010, Palacio de Congresos y Exposiciones - FIBES Seville-Spain.

- **M. Shahid**, E. Pinelli, B. Pourrut, J. Silvestre & C. Dumat. Effects of organic ligand (EDTA) on speciation, transfer, and toxicity of Pb to *Vicia faba.* 2ème rencontres nationales de la Recherche sur les sites et sols pollués : pollutions locales et diffuses 20 et 21 octobre 2009, Paris-France.

- M. Arshad, **M. Shahid**, P. Pradere & C.J. Dumat, Gestion floristique de sols pollue par metaux : étude long terme de la phytoextraction du plomb. 2ème rencontres nationales de la Recherche sur les sites et sols pollués : pollutions locales et diffuses 20 et 21 octobre 2009, Paris-France.

- **M. Shahid**, L'adaptation des plantes dans des conditions de stress des métaux lourds. Fête de la Science (Festival of Science), 16-22 November 2009 at University of Toulouse, Toulouse-France.

- **M. Shahid**, L'origine et évolution de la vie sur Terre: La Sagesse de la Nature. Fête de la science (Festival of Science), 16-22 November 2009, University of Toulouse, Toulouse-France.

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## Scientific Formations attended

- Attended three days workshop of CHESS (Chemical Equilibrium with Species and Surfaces) at Ecole de Mine-Paris. 19-21 Nov 2008.

- Attended 15 days formations of CIES (Initiation Center for Higher Education) to improve teaching skills (How to prepare and deliver lecture, Methods of Communications, Social Psychology etc….)

- Participated in organizing Fête de la Sciences (Festival of Science) at University of Paul Sabatier Toulouse-France 16-22 November 2009.

*11th International Conference on the Biogeochemistry of Trace Elements. 3-8 July 2011. Florence, Italy*

# Effect of fulvic acids on lead-induced oxidative stress to sensitive *V. faba* plant used as biotest

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***Key Words:*** lead, fulvic acids, speciation, *Vicia faba*, oxidative stress.

**Abstract**
Lead (Pb) is a known toxic and persistent pollutant with no biological role. Only few studies concern Pb uptake and phytotoxicity in relation to its speciation. Therefore, Pb toxicity was studied for sensitive *V. faba* plants in relation with Pb uptake and speciation by calculating lipid peroxidation (LPO), hydrogen peroxide ($H_2O_2$) induction and photosynthetic pigments contents. Three weeks old *V. faba* seedlings were exposed to Pb alone, or complexed at two levels by two types of fulvic acids (FAs) for 1, 12 and 24 h in controlled hydroponic conditions. For both FAs, Pb uptake by *V. faba* roots and translocation to aerial parts increased at low level (5 $mg.L^{-1}$), whereas decreased at higher level of application (25 $mg.L^{-1}$). FAs did not influence significantly Pb-induced toxicity at low level of application. However, at higher concentrations, FAs reduced Pb toxicity by reducing its uptake, highlighted the role of dilution factor for FAs reactivity in relation with structure.

## Introduction

Lead is widely used in many industrial processes, which led to its toxic levels in different environmental compartments (Uzu et al. 2010). This metal interferes with morphological, physiological and biochemical functioning of exposed plants and could induce a broad range of noxious effects (Shahid et al. 2011a). However, the toxicity of Pb is the function of its speciation; not only in predicting metal mobility and bioavailability, but also in assessment of risk to living organisms (Shahid et al. 2011b). Therefore, predicting the role of metal speciation is of utmost importance in understanding of metal toxicity mechanisms.

Humic substances influence speciation, bioavailability and acute toxicity of metals in soils and natural waters. These substances are a complex mixture of partially "decomposed" and otherwise transformed organic materials and are major sorbent phase for contaminants in soils. Carboxylic and phenolic hydroxyl groups are main binding sites of these substances.

However, very rare data is available on Pb toxicity in relation with uptake and speciation, especially in the presence of humic substances. Therefore, this study was carried out to investigate the effect of Pb speciation on phytoavailability and toxicity to *V. faba* in relation with speciation.

## Materials and Methods

The dry *V. faba* seeds were germinated for 6 days in germination chamber before transplanting to hydroponic conditions with the macro-elements: 5 mM $KNO_3$, 5 mM $Ca(NO_3)_2$, 2mM $KH_2PO_4$ and 1.5 mM $MgSO_4$ and micro-elements: 9.11 µM $MnSO_4$, 1.53 µM $ZnSO_4$, 0.235 µM $CuSO_4$, 24.05 µM $H_3BO_3$, 0.1 µM $Na_2MoO_4$ and 268.6 µM Fe (Uzu et al. 2009). After a culture period of three weeks, the *V. faba* seedlings were exposed to different treatments (Table 1) for 1, 12 & 24 h. The Suwannee River fulvic acid (SRFA) and Elliott Soil fulvic acid (ESFA) were obtained from the International Humic Substances Society (IHSS, Colorado School of Mines, Golden, CO) and used as model of natural dissolved organic matter. The speciation of Pb in nutrient solution was calculated using Windermere Humic Aqueous Model VI (WHAM VI). Calculations were used to design experimental setup. After harvest, seedlings were analyzed for Pb contents by ICP-OES and LPO, $H_2O_2$ and photosynthetic pigment by spectrophotometry.

*Table 1* Treatments and Pb speciation (WHAM).

| Treatments | WHAM VI input | WHAM VI outpu (%) | |
|---|---|---|---|
| Notations | Composition | Pb-chelated | $Pb^{2+}$ |
| Control | Hoagland solution | - | - |
| ESFA-25 | 25 $mg.l^{-1}$ ESFA | - | - |
| SRFA-25 | 25 $mg.l^{-1}$ SRFA | - | - |
| Pb | 5 µM $Pb(NO_3)_2$ | 0 | 84 |
| Pb-ESFA-5 | Pb + 5 $mg.l^{-1}$ ESFA | 12 | 74 |
| Pb-ESFA-25 | Pb + 25 $mg.l^{-1}$ ESFA | 36 | 57 |
| Pb-SRFA-5 | Pb + 5 $mg.l^{-1}$ SRFA | 16 | 70 |
| Pb-SRFA-25 | Pb + 25 $mg.l^{-1}$ SRFA | 44 | 47 |

## Results

*Pb uptake:* Application of both FAs at low level (Pb-SRFA-5 and Pb-ESFA-5) increased non significantly Pb uptake and translocation to shoot tissues (Table 2). In contrast, for higher

levels of FAs (Pb-SRFA-25 and Pb-ESFA-25), a significant decrease in Pb uptake and translocation was observed after 24 h except for Pb-ESFA-25 where decrease in translocation was non significant. In all cases more than 95 % of Pb accumulated in roots with limited translocation to aerial tissues.

*Table 2* Effect of SRFA and ESFA on Pb concentration (µg.g-1 D.W.) in *V. faba* roots and leaves. Significant differences at $P < 0.05$ are indicated by bold value.

| | Roots | | | Leaves | | |
|---|---|---|---|---|---|---|
| Treatments | Mean | ± | SD | Mean | ± | SD |
| Pb | 95 | ± | 9 | 3.2 | ± | 0.5 |
| Pb-ESFA-5 | 110 | ± | 15 | 3.9 | ± | 1.1 |
| Pb-ESFA-25 | **74** | ± | **8** | 2.6 | ± | 0.3 |
| Pb-SRFA-5 | 106 | ± | 13 | 3.6 | ± | 0.6 |
| Pb-SRFA-25 | **62** | ± | **19** | **2.4** | ± | **0.2** |

*Pigment contents* Application of Pb alone reduced pigment contents with time at 12 and 24 h, the effect being significant after only 24 h (Table 3). Addition of both FAs low level has no effect on Pb-induced toxicity to pigment contents whereas higher levels inhibited the Pb-induced toxicity to pigment contents, SRFA-25 being more effective.

*Table 3* Effect of SRFA and ESFA on Pb-induced reduction of pigment contents (µg.g-1 FW) in *V. faba* leaves. The pigments contents remained unaffected before 12 h. therefore, the results are shown at 24 h only. Significant differences are indicated by bold value. Pb alone is compared with control whereas other Pb treatments are compared with Pb alone.

| | Chl-a | | | Chl-b | | | Carotenoid | | |
|---|---|---|---|---|---|---|---|---|---|
| Treatments | Mean | ± | SD | Mean | ± | SD | Mean | ± | SD |
| Control | 832 | ± | 46 | 331 | ± | 22 | 183 | ± | 9 |
| Pb | **733** | ± | **15** | **254** | ± | **28** | **142** | ± | **11** |
| Pb-ESFA-5 | 688 | ± | 50 | 264 | ± | 27 | 142 | ± | 9 |
| Pb-ESFA-25 | 762 | ± | 30 | **320** | ± | **30** | 161 | ± | 8 |
| Pb-SRFA-5 | 719 | ± | 11 | **318** | ± | **37** | 155 | ± | 11 |
| Pb-SRFA-25 | **802** | ± | **26** | **348** | ± | **52** | **177** | ± | **11** |

*Oxidative stress*: Application of Pb alone caused overproduction of $H_2O_2$ which resulted into LPO in both roots and leaves (Table 4). In roots, Pb-induced $H_2O_2$ production and LPO started immediately after Pb exposure at 1 h whereas in leaves at 24 h. Application of both the FAs at low level has no effect on Pb induced $H_2O_2$ production or LPO except for Pb-SRFA-5 which delayed the initiation of LPO from 1 to 12 h in roots. At higher levels of FAs application, Pb-induced $H_2O_2$ induction and LPO in roots were reduced significantly, SRFA-25 being more effective. In leaves, application of both FAs has no effect on $H_2O_2$ production or LPO.

*Table 4* Effect of SRFA and ESFA on Pb-induced production of $H_2O_2$ (µM.g-1 FW) and LPO (TBARS in nmol.g-1 FW) in *V. faba* roots and leaves. Significant differences at $P < 0.05$ are indicated by bold value.

| | | Roots | | | Leaves | | |
|---|---|---|---|---|---|---|---|
| Treatments | | 1 h | 12 h | 24 h | 1 h | 12 h | 24 h |
| Control | $H_2O_2$ | 6.6 | 7.7 | 7.6 | 32 | 34 | 30 |
| Pb | | **10.9** | **10.7** | 8.7 | 29 | 35 | **43** |
| Pb-ESFA-5 | | 10.2 | 10.5 | **10.1** | 33 | 38 | 38 |
| Pb-ESFA-25 | | **9.4** | 9.6 | 8.9 | 33 | 36 | 37 |
| Pb-SRFA-5 | | 9.7 | 11.6 | 9.4 | 34 | 39 | 40 |
| Pb-SRFA-25 | | **8.3** | **7.8** | 9.7 | 30 | 36 | **35** |
| Control | LPO | 7.2 | 7.8 | 8.1 | 3.5 | 3.6 | 3.8 |
| Pb | | **11.1** | **11.9** | 9.0 | 4.0 | 4.5 | **5.1** |
| Pb-ESFA-5 | | 10.9 | 10.2 | 9.6 | 3.8 | 5.1 | 5.1 |
| Pb-ESFA-25 | | **7.2** | 10.3 | 12.6 | 3.4 | 4.1 | 4.9 |
| Pb-SRFA-5 | | **6.6** | 13.1 | **12.1** | 3.6 | 5.3 | 5.0 |
| Pb-SRFA-25 | | **8.2** | 8.1 | **12.5** | 3.9 | 4.2 | 4.7 |

## Discussion

The contrasting effects of FAs towards Pb uptake & translocation, at low and high concentrations could be explained by FA reactivity in relation with structure. When highly concentrated, FA adopts a ball structure efficient to strongly complex Pb and reduces its plant uptake. This was also the result of reduced Pb toxicity in the presence of higher levels of FAs. Under diluted conditions, FAs adopts a more hydrophilic fibrous structure that forms soluble and mobile complexes with Pb, which could enter the plants more easily due to small size and thus increased Pb uptake and in turn the toxicity. These results have implications on ecotoxicity studies performed to characterize quality soils and waters.

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# Role of speciation in uptake and toxicity of Pb to *Vicia faba* L.

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## Introduction

The fate of heavy metals in the environment, as well as their toxicity and other associated ecological risks, are still topical. Recent literature indicates that the toxicity and/or bioavailability of trace metals depend on their speciation [1]. Therefore, in addition to knowing the total metal concentration, predicting the role of free ionic species of metals is of utmost importance in improving our understanding of the mechanisms of metal toxicity. The present study is carried out to evaluate the Pb-induced toxicity to *V. faba*, a metal sensitive plant used as biomarker of toxicity, in relation with cellular uptake and speciation.

## Materials and methods

*V. faba* seedlings were grown in modified Hoagland's nutrient solution [1], with the macro-elements: 5 mM $KNO_3$, 5 mM $Ca(NO_3)_2$, 2mM $KH_2PO_4$ and 1.5 mM $MgSO_4$ and micro-elements: 9.11 mM $MnSO_4$, 1.53 mM $ZnSO_4$, 0.235 mM $CuSO_4$, 24.05 mM $H_3BO_3$, 0.1mM $Na_2MoO_4$ and 268.6 mM Fe/EDTA under controlled conditions in phytotron. After three weeks of culture period, seedlings were exposed to different treatments (Table 1) at pH 5 for 1, 4, 6, 8, 12 and 24 h.

| Treatments | Composition |
|---|---|
| Negative control (NC) | Hoagland solution |
| Positive control (PC) | Maleic hydrazeide (40 µM) |
| EDTA-b | EDTA (10 µM) |
| CA-b | citric acid (1000 µM) |
| Pb | Pb [5 µM $Pb(NO_3)_2$] |
| Pb-EDTA-a | Pb + EDTA (2.25 µM) |
| Pb-EDTA-b | Pb + EDTA (10 µM) |
| Pb-CA-a | Pb + citric acid (550 µM) |
| Pb-CA-b | Pb + citric acid (1000 µM) |

*Table 1: Experimental layout of treatments.*

For Pb contents analysis, different plant parts were separated, oven-dried at 80 °C for 48 h, milled in a micronizing mill, and mineralized in a 1:1 mixture of 65 % $HNO_3$ and 30 % $H_2O_2$ at 80 °C over 6 h using DigiPrep Jr. After dilution, Pb content analysis was carried out with an IRIS Intrepid II XDL ICP-OES. The *V. faba* micronucleus (MN) test was carried out according to El Hajjouji et al. [2]. All treatments were exposed for 30 h (6 h for the treated groups followed by a 24-h recovery period). After treatment, root tips were fixed in Conroy's solution (glacial acetic acid/ethanol 1:3 v/v), hydrolyzed with 1 M HCl and stained with 1 % aceto-orcein. For each root tip, 5000 cells were observed under 1000X magnification.

The frozen samples (600mg) were ground under liquid nitrogen and incubated with 80 % acetone. After centrifugation, the absorbance of supernatant was recorded at 663, 645 and 480 nm and chlorophylls (a+b) contents were calculated according to extinction coefficients and equations reported by Lichtenthaler [3].

**Results and discussion**

**Cellular uptake of Pb by *V. faba* seedlings in the presence of organic ligands**

Results showed that the addition of ethylenediaminetetraacetic acid (EDTA) dose dependently increased the uptake of Pb by *Vicia faba* (*V. faba*) roots (Figure 1). The result is in agreement with

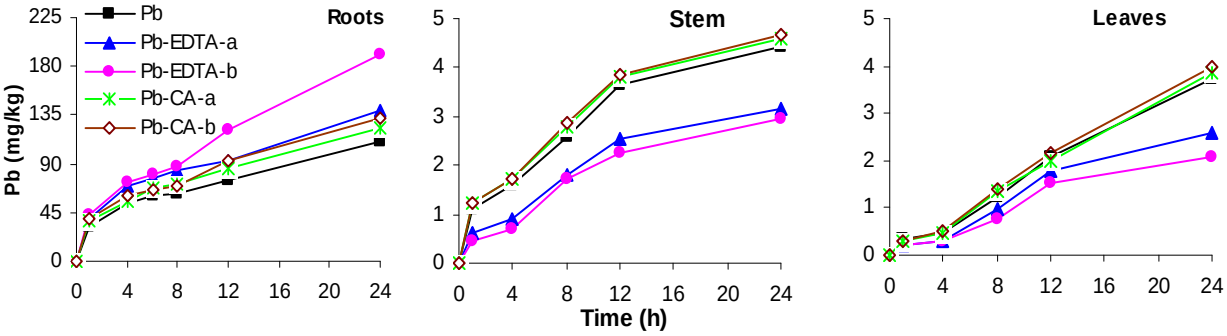


*Figure 1: Effect of EDTA and CA on Pb uptake and translocation by V. faba.*

previous studies [4] which reported many fold increase in Pb uptake by various plants in the presence of EDTA. In contrast to literature, the translocation of Pb to aerial parts (stem and leaves) was reduced in the presence of EDTA (Figure 1). In our experimental conditions, decrease in Pb translocation in the presence of EDTA might be due to short exposure time, nature of *V. faba* plant (sensitive to Pb) or presence or absence of some proteins involved in Pb translocation. In the case of citric acid (CA), only a slight, but not significant increase in the uptake and translocation of Pb to aerial parts was observed. The effect of CA on metal uptake is plant dependent and is, generally, attributed to decrease in the pH of culture media. That acidification did not happen in our experimental condition, because the pH value was always adjusted at 5.

**Effect of organic ligands on Pb induced genotoxicity to *V. faba* L.**

When EDTA or CA alone as controls (grey bars Figure 2) was added to the nutrient solution, no significant effect was observed on MN frequency compared to the negative control. A four-fold increase in MN frequency compared to the negative control was observed for Pb alone treatment. In the presence of EDTA, Pb-induced genotoxicity decreased significantly and dose dependently. Results are in line with those of Ruley et al., [5] where the

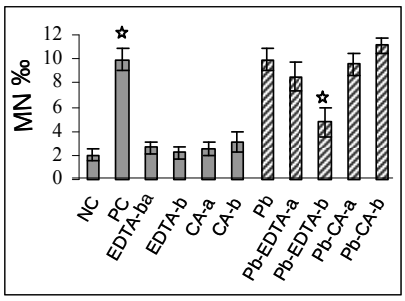


*Figure 2: MN frequency in V. faba root tips.*

** indicates significant differences at P < 0.05.*

presence of chelators (EDYA) increased Pb uptake while reduced Pb oxicity. Addition of CA, however, had no significant effect on Pb genotoxicity. Results suggest that Pb-CA complex can dissociate due to lower stability constant (log $K$ = 5.67), thus, increasing slightly $Pb^{2+}$ levels in *V. faba* roots and ultimately genotoxicity.

**Chlorophyll contents**

Results showed that pigment composition was not affected until 8 h for all treatments compare to control (data not shown). After 8 h, chlorophylls contents remained close to control for EDTA and CA when used without Pb. However, Pb alone strongly modified chlorophyll contents after 12 and 24 h of incubation (Figure 3). Addition of EDTA dose dependently inhibited Pb induced reduction in Pb contents. In the presence of EDTA, chlorophyll contents were not significantly different from those of control. Ruley et al., [5] also reported that addition of EDTA alleviated Pb-induced toxicity to chlorophyll contents. Like cellular uptake, no effect of CA was observed on Pb induced reduction of chlorophyll contents and the values remained close to that of Pb alone. The results obtained for lipid peroxidation, $H_2O_2$ induction and antioxidant activities (not presented) also showed similar affects of EDTA and CA on Pb induced toxicity.

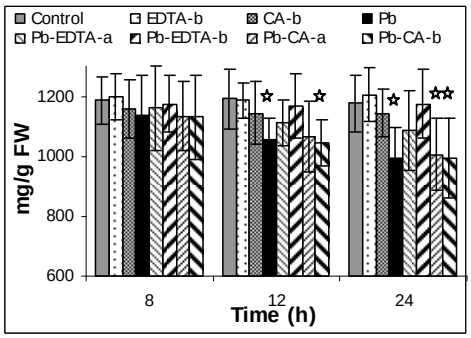


*Figure 3: Chlorophyll (a+b) contents of V. faba leaves.*

**Conclusions**

Metal speciation plays an important role in cellular uptake and toxicity of Pb. EDTA with strong complexation constant (log $K$ = 17.88) increase Pb uptake and reduce toxicity by converting toxic form of Pb into non toxic form. In contrast, CA inducing labile organo-metallic complexes did not demonstrate any significant change in genotoxicity or uptake of Pb.

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*2eme rencontres nationales de la recherche sur les sites et sols pollués. 20-21 octobre 2009 à Paris.*

# Les effets d'un ligand organique, l'EDTA sur la spéciation, le transfert, et la toxicité de plomb sur *Vicia faba*.

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## *Contexte & Objectifs*

Malgré les progrès considérables accomplis au cours des dernières années, la spéciation des métaux traces, leur transfert sol-plante ainsi que les risques écologiques associés demeurent des sujets d'investigations importants. L'accumulation excessive de plomb (Pb) dans les milieux abiotiques et biotiques (Cecchi *et al.*, 2008), induit en particulier des aberrations chromosomiques dans les tissus végétaux, l'induction de micronoyaux, l'inhibition de la biosynthèse de la chlorophylle ainsi que la génotoxicité et la cancérogénicité (Chantachon *et al.*, 2004). Le Plomb a la particularité de se fixer aux membranes biologiques ainsi qu'aux parois cellulaires, ce qui expliquerait la perturbation des ultrastructures et les problèmes de croissance. Cependant, la présence de ligands organiques dans les milieux naturels modifie la spéciation et la réactivité des métaux (Ferrand et al., 2006).

Pour étudier le transfert de Pb dans le système sol-plante, il est nécessaire de faire le lien entre la spéciation chimique, la mobilité, la solubilité, la phyto-toxicité et l'absorption. Selon Blaylock *et al.*, (1997) et (Huang *et al.*, 1997), l'EDTA améliore considérablement l'absorption et la translocation du plomb dans les plantes. Nous avons choisi d'étudier dans le cadre d'une pollution « réaliste » au plomb, comment la chélation du Pb par l'EDTA pouvait modifier son absorption, l'activité photosynthétique, l'activation des enzymes antioxydants et l'induction de micro-noyaux.

### *Matériel & Méthodes*

Des plantes de *Vicia faba* âgées de 3 semaines sont exposés au plomb (5 µM) avec ou sans EDTA (20 µM) pour 1, 4, 8, 12 et 24 heures. L'analyse de la teneur en plomb a été réalisée comme décrit par Pourrut *et al.*, (2008) en utilisant 1:1 mélange de 65% $HNO_3$ et 30% de $H_2O_2$ à 80 °C pour 6 h. Après filtration, les concentrations de plomb ont été déterminées avec un IRIS Intrépide II XDL ICP-AES. Pour l'analyse des activités enzymatiques antioxydants, la teneur en protéines a été déterminée en fonction de Bradford (1976), en utilisant l'albumine de bovin sérum (BSA, Sigma) comme standard. APX activité a été évaluée par la diminution de l'absorbance à 290 nm à cause de l'ascorbate de l'oxydation (Nakano et Asada, 1981) et l'activité de catalase (CAT) a été mesurée en suivant la consommation de $H_2O_2$ à 240 nm (Aebi, 1984). La fréquence de micronoyaux a été quantifiés par *V.faba* test du micronoyau selon Ma *et al* (1995).

### *Résultats et Perspectives*

● L'Accumulation de Pb dans les racines de *Vicia faba* augmente avec le temps d'exposition et lorsque le plomb est apporté en présence d'EDTA. Sans EDTA, la concentration en Pb dans les racines passe de 5ppm pour une heure d'exposition à 20 ppm pour 24h d'exposition. En présence d'EDTA, à 1h la concentration est de 15 ppm et de 75 ppm pour 24h d'exposition. Concernant la concentration dans les feuilles les tendances observées sont très différentes. Sans EDTA, la concentration en Pb est d'environ 1 ppm et ne varie pas de façon significative entre 1h et 12h d'exposition et selon que de l'EDTA est ajouté ou non. Puis pour 24h d'exposition la concentration augmente jusqu'à 3,3 ppm si le Pb est seul alors qu'en présence d'EDTA la translocation est bloquée.

● Dans les feuilles, les activités enzymatiques de CAT et APX sont influencées par la présence de plomb et ces activités sont modifiées par la présence d'EDTA et également par le

temps d'exposition (après 1, 4, 8, 12 et 24 h). Pour une exposition de *Vicia faba* au plomb seul, aucun changement significatif de l'activité enzymatique (CAT et APX) n'est observé avant 8 h (valeur d'environ 1,1 U et 0, 6 U pour CAT et APX respectivement). À 24 h, l'activité de CAT a diminué (0,7 U) et celle d'APX a augmenté (0,8) de manière significative par rapport au contrôle (valeur de 1,1 U et 0,6 U pour les contrôles de CAT et APX respectivement). Cette augmentation de l'activité d'APX par la présence du Pb est réduite lorsque le plomb est apporté en présence d'EDTA (valeur de 0,3 à 24h). Ceci est aussi observé pour l'activité de CAT.

● Dans les racines, l'activité de l'APX augmente rapidement après 1 h par exposition au plomb seul (6 U par rapport au contrôle de 3,3 U). Puis à 8 h, une diminution est observée (4,5 U) et cette activité d'APX augmente jusqu'à atteindre une valeur maximale de 8 après 24 h d'exposition. L'ajout d'EDTA augmente l'activité d'APX jusqu'à 8 h (6,6 U) et ensuite diminue l'activité pour atteindre une valeur de 4 U à 24 h. Concernant l'activité enzymatique de la CAT, une réduction est observée à partir de 4h (0,7 U) en comparaison avec le contrôle non exposé au plomb (1,1 U). La présence d'EDTA augmente de façon significative l'activité de la CAT : la valeur du contrôle est retrouvée.

● Le test de micronoyau a montré que la présence d'EDTA modifie la fréquence de micronoyau induite par l'exposition des racines au Pb et modifie également l'index mitotique de *Vicia faba* dans l'apex racinaire. Une modification de la spéciation chimique du plomb pourrait expliquer ce phénomène.

● Des expériences complémentaires sont en cours pour relier les résultats de transferts aux effets sur la plante. D'autres ligands sont également étudiés : LWOAs et substances humiques.

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# Gestion floristique de sols pollue par métaux : etude long terme de la phytoextraction du plomb

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## Contexte & Objectifs

Le plomb est l'un des principaux contaminants persistants des sites industriels ou de leurs abords et il peut parfois être transporté sur de très longues distances (Elless et Blaylock, 2000 ; Cecchi et al., 2008). Dudka et Miller (1999) ont conclu qu'une concentration du sol de 300 mg $kg^{-1}$ de Pb pouvait induire des problèmes de santé humaine et en particulier chez les enfants: anémie, impact sur le système nerveux central... Selon Laperche et al. (2004), les émissions totales de Pb en France étaient 217 tonnes/an en 2002 et la base de données Basol (http://basol.environnement.gouv.fr), recensait, en 2005, 3 717 sites pollués en plomb pour lesquels l'État a entrepris une action de remédiation.

Parmi les nombreuses techniques de décontamination des sols pollués en métaux (He et al., 2005), la phytoremédiation offre des avantages économiques et environnementaux (Tanhan et al., 2007). Récemment, Arshad et al. (2008) ont démontré que le *Pélargonium* odorants cultivars Attar, Clorinda et Atomic étaient des hyperaccumulateurs de plomb. Les principaux objectifs du présent travail étaient: (i) étudier la cinétique de phytoextraction de plomb pendant 3 ans par le *Pélargonium* cultivar Attar sur un sol acide fortement contaminés (ii) étudier les effets de la plante sur la rhizosphere pour modifier la biodisponibilité de Pb. En effet la spéciation chimique des métaux influence leur mobilité (Dumat et al. 2001 et 2000) et disponibilité (Ferrand et al., 2006). Donc étudier le sol rhizosphèrique augmente les connaissances des mécanismes impliqués dans le sol-plante transfert.

## Matériel & Méthodes

Les plantes bouturées du *Pélargonium* cultivars Attar of Roses (appelé Attar ici) ont été cultivées en serre, et irriguées régulièrement avec la solution nutritive. Le sol utilisé pour les cultures en conditions contrôlées provient des environs d'une usine de recyclage de batteries au Pb (la STCM). Ce même sol a également été utilisé pour les expériences au champ. Le contexte de pollution, la caractérisation des sources sont décrits en détail par Uzu et al. (2008) et Cecchi et al. (2008). Ce sol acide contient 39250 mg Pb $kg^{-1}$, et rentre dans la gamme considérée comme "niveau d'intervention" en France. Les caractéristiques physico-chimiques de sol sont : $pH_{eau}$ = 6, la matière organique (MO) = 8,5% et l'argile = 45%, As = 1060 mg $kg^{-1}$, Cu = 2084 mg $kg^{-1}$, Zn = 3995 mg $kg^{-1}$ et Cd = 706 mg $kg^{-1}$. Les essais de phytoextraction au champ ont été réalisés de Mai 2004 à Juillet 2006. De Mai à Septembre 2004, une première étape d'extraction de plomb a été réalisée. Après 150 jours de culture, les parties aériennes ont été récoltées. Les racines ont été laissées en vue d'effectuer une deuxième étape de culture avec les mêmes plantes. La deuxième étape d'extraction a été réalisée d'avril à Juillet 2005 (120 jours). Enfin, une culture a été réalisée au cours de 2005-2006. Pour mesurer le Pb adsorbé à la surface, les feuilles fraîches ont été traitées selon le protocole de Ferrand et al. (2006). Puis les parties aériennes (5 répétitions) ont été séchés (48 heures à 80 ° C). Matériel végétal séché a été broyé et le poudre (1g) a été digérée avec un mélange d'$HNO_3$ et $H_2O_2$ (1:1) à 80 °C pendant 4H en réacteur fermé (DigiPrep) pour la minéralisation. La concentration de Pb a été mesurée en solution par l'ICP-OES.

Pour les études rhizosphèriques, des dispositifs de microculture ont été utilisés (Guivarch et al., 1999). Ces dispositifs nous permettent de séparer physiquement les racines et le sol physiquement mais en assurant un espace privilégiant un transfert entre les éléments de la rhizosphère et la racine. Le sol obtenu après cultures est considéré comme rhizosphérique. Les plantes enracinées sont cultivées pendant 6 semaines pour avoir un tapis de racines sur les dispositifs adaptés pour les études rhizosphériques. Ces plantes ont été mises en contact avec sol contaminé (10 g de sol de Bazoche sur chaque dispositif) pendant deux semaines. Sol de Bazoche est un sol calcaire, $pH_{eau}$ = 8 et teneur en Pb = 1830 mg $kg^{-1}$ Pb. Le sol sur les dispositifs sans plantes a été mis comme témoins. Le changement du pH a été suivi sur les témoins sans plante au cours de l'expérimentation. A la récolte, les parties aériennes et racinaires été séparées. La biomasse fraîche et sèche, les concentrations de Pb ont été mesurées. La solution de sol a été récupérée par centrifugation pour le dosage de Pb des métaux et la

mesure du pH (AFNOR 1994). Le pH du surnageant a été mesuré et la solution d'extraction $CaCl_2$ correspond à la fraction disponible du plomb.

**Résultats et Perspectives**

Le tableau ci-dessous présente les résultats de biomasse sèche (g), la concentration de plomb total dans les parties aériennes (mg Pb/kg PS) et la quantité de plomb extrait (mg Pb/plante) pour les trois périodes de culture (2004, 2005 et 2006) sur le sol acide fortement contaminé. Les biomasses, les concentrations et les quantités de Pb ont été augmentées significativement en 2006 par rapport en 2004. Ces Résultats démontrent que l'augmentation de durée, induit une forte augmentation de l'efficacité d'extraction. Comme les racines sont restées dans le sol pendant trois ans, l'hypothèse avancée est une saturation des sites racinaires en Pb favoriserait le transfert de Pb vers les parties aériennes.

| Année | Biomasse (PS) (g) | [Pb] (mg Pb/kg PS) | Q de Pb extrait (mg/plante) |
|---|---|---|---|
| 2004 | 43±11 | 3914±486 | 172±63 |
| 2005 | 68±10 | 3463±118 | 183±33 |
| 2006 | 97±14 | 8644±357 | 838±39 |

Pour les microcultures, deux semaines de contact sol-plante, le pH-$CaCl_2$ du sol a été diminué de 7.50 à 6.8 par l'activité du cultivar Attar. En solution de sol, le changement de pH était de 7.8 à 7.4. Nos expérimentations ont montré que la concentration du Pb dans la solution de sol a été diminuée sur les sols en contact avec les plantes par rapport aux témoins sans plantes (témoin 70.65 mg Pb $kg^{-1}$, Attar 15.04 mg Pb $kg^{-1}$). Ceci montre que le plomb a été effectivement transféré de la solution du sol vers les plantes. Les dosages du plomb dans la fraction disponible est de 3.86% par rapport à la concentration du départ. Ces concentrations dans la solution de sol sont élevées. Selon Huang et al. (1997), les concentrations de Pb en solution de sol sont généralement moins de 0.1% de Pb total dans la phase solide.

Ces études ont démontré la possibilité de décontaminer les sols plombés par le *Pélargonium* cultivar Attar et surtout d'utiliser cette solution pour la gestion de sols qui sont en attente d'une décision de traitement de remédiation lourd. En outre, les *Pélargonium* odorants testés en phyto-remédiation ont plusieurs avantages significatifs en comparaison avec les techniques physico-chimiques classiques de remédiation: (i) une réduction de la fraction labile des métaux, (ii) une stabilisation des particules de sol contaminées, (iii) une amélioration de l'impact visuel des sites industriels, facteur non négligeable pour les riverains du site et (iv) un coût bien moindre, (v) le respect de la biodiversité et du fonctionnement biogéochimique du sol. Des expériences complémentaires sont nécessaires afin de vérifier la qualité de ces produits et d'étudier le comportement de Pb (spéciation et transferts) dans la rhizosphère, les racines et la sève de *Pélargonium*.

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**Field Demonstration of Age Dependent Increase in Pb Extraction and Uptake Efficiency of Attar of Roses *Pelargonium* Cultivar in Acidic Contaminated Soil**

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Society of Chemical Treatment of Metals, STCM

Unnecessary for living organisms, Pb (Pb) is one of the major widespread toxic metals found in the environment with potential danger to human health and to ecosystems. A field study was carried out in the vicinity of Pb recycling plant near Toulouse-France, and contaminated by atmospheric fallouts to evaluate Pb extraction and uptake efficiency of hyperaccumulater Attar of Roses *Pelargonium* cultivar with aging between 2005 and 2007. It was found that Attar of Roses has ability to accumulate (8644 mgPb/kg DW plant) and survive on highly contaminated acidic soil (39250 mg $kg^{-1}$ of total Pb) without any morpho-phytotoxicity symptoms. Moreover Attar showed increased extraction of Pb from bulk soil to rhizosphere through Pb mobilization and ultimately increased uptake by roots and translocation to shoots. The studied contaminated soil could be cleaned up in few years by planting hyperaccumulater Attar of Rose for longer time period. Under optimum fertlization, irrigation and use of natural or synthetic chelates (EDTA, LMOWA, humic substances etc.) along with old Attar of rose plants, time requires for complete remediation of contaminated site can be reduced to practically applicable time period. Moreover, the use of Pelargonium for remediation has several additional practical, esthetical and economic advantages. The extraction of value-added essential oils from harvested biomass could offset the cost of deploying phytoremediation and renders it as a viable approach for remediating highly contaminated soils, on large scale.

*International Research Conference, University of South Asia, Pakistan, 6-8 october 2008*

# Kinetic of Pb phytoextraction by Attar of Roses *Pelargonium* cultivar cultivated on industrially contaminated soil: a field study

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## Abstract

According to several laboratory studies, phytoremediation seems a promising technique for trace metal clean up, but its successful application in the field is still limited. Our field experiments on highly contaminated acidic soil (40000 ppm of total Pb), demonstrated the ability of Attar of Roses *Pelargonium* cultivar to hyper-accumulate Pb (a relatively low available metal in soils) producing high biomass without morpho-phytotoxicity symptoms (Arshad et al., 2008).

Moreover an increase of Pb phyto-extraction was observed with the age of the plant (between 2005 and 2007) which the activity increase the mobilization of Pb in rhizosphere. The phytoextraction of the total Pb quantities from the studied surface soil could be realised in few decades using that hyper-accumulator *Pelargonium* plant. Optimisation of NPK and irrigation conditions and/or the use of chelates, i.e EDTA, humic substances and low weight organic acids (LMWOAs) could reasonably reduce the time of treatment at few months. Moreover, the use of Pelargonium for remediation has several additional practical, esthetical and economic advantages. The extraction of value-added essential oils from harvested biomass could offset the cost of deploying phytoremediation and renders it as a viable approach for remediating highly contaminated soils, on large scale.

**Keywords:** Pb uptake; *Pelargonium*; Phytoremediation; Cultivar; Soil-plant transfer; kinetic.

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Short Communication

# A field study of lead phytoextraction by various scented *Pelargonium* cultivars

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**Abstract**

Phytoremediation appears to be a promising technique for metal soil clean up, although its successful application on a large scale still remains a challenge. Field experiments for six scented *Pelargonium* cultivars, conducted on two Pb-contaminated calcareous and acidic soils, revealed vigorous plant growth, with no symptoms of morpho-phytotoxicity in spite of high Pb accumulation levels. Lead contents in the harvestable parts of all plants grown on the acidic and more contaminated soil were significantly higher than those grown on the calcareous soil. Three cultivars (Attar of Roses, Clorinda and Atomic Snowflake) are Pb-hyperaccumulator plants: they accumulated more than 1000 mg Pb $kg^{-1}$ DW, with high biomass produced.


*Keywords:* Phytoremediation; Pelargonium; Cultivar; Pb; Soil–plant transfer

## 1. Introduction

Owing to its high persistence and several past and present uses (Chen et al., 2006), Pb is one of the most frequently encountered inorganic pollutants in soils (Alkorta et al., 2004). It is potentially toxic even at low concentrations, and above 400–500 mg Pb $kg^{-1}$ soil is considered as a risk for human health by the US-EPA (2001). Clean up of contaminated soils, is a major challenge in environmental engineering.

*Ex situ* decontamination using physico-chemical techniques is labour intensive, expensive, and affects the soil's biological properties. The use of plants to decontaminate soils, known as phytoremediation could therefore offer an environment-friendly solution to soil remediation (Tanhan et al., 2007). The possibility of generating additional value from by-products of the biomass would improve the economic balance of this *in situ* decontamination technique. The most limiting factor is the time required to successfully clean contaminated soil to reach the goals set by legislation (Keller et al., 2005). Its successful application is inherently dependent upon the choice of an appropriate plant which should produce high biomass through rapid growth and accumulate abundant quantities of metals in easily harvestable parts of the plant (Prasad and Freitas, 2003). However, the combination of these two characteristics is rarely observed, as most of the high biomass producing plants, such as *Brassica juncea*, concentrate only moderate amounts of metals (Lasat, 2002) and hyper accumulating plants often produce low biomass (Reeves and Baker, 2000). Current research on the development of phytoremediation is thus targeted towards the identification of new plant species (Prasad and Freitas, 2003).

In a greenhouse study, cuttings of *Pelargonium sp.* "Frensham" grown on artificial soil and fed with different

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# UPTAKE AND TOXICITY OF LEAD TO *VICIA FABA* L. IN RELATION WITH SPECIATION

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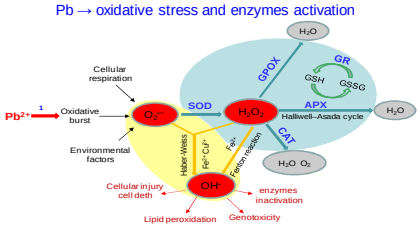
## Introduction

- Lead is a toxic, even at low level (1µM), and commonly observed pollutant in the environment.
- Pb phytoavailability and toxicity depend on speciation / organic ligands & free $Pb^{2+}$.

Pb → oxidative stress and enzymes activation




## Objective

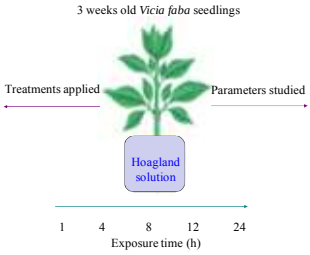
(1) Assessing the effect of speciation on lead-induced oxidative stress to *V. faba* plants

(2) Comparison of various techniques used to asses lead-induced oxidative stress in order to progress in risk assessment.

## Materials & methods

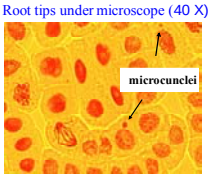
| Notations | Medium composition | Pb-chelated | $Pb^{2+}$ |
|---|---|---|---|
| *NC*[1] | Hoagland solution | – | – |
| *PC*[2] | MH[3] (40 µM) | – | – |
| *EDTA-b* | EDTA (10 µM) | – | – |
| *CA-b* | Citric acid (1000 µM) | – | – |
| *Pb* | Pb [5 µM $Pb(NO_3)_2$] | 0 | 85 |
| *Pb-EDTA-a* | Pb + EDTA (2.25 µM) | 40 | 51 |
| *Pb-EDTA-b* | Pb + EDTA (10 µM) | 99 | 1 |
| *Pb-CA-a* | Pb + citric acid (550 µM) | 25 | 64 |
| *Pb-CA-b* | Pb + citric acid (1000 µM) | 40 | 51 |

[1] *Negative control,* [2] *Positive control,* [3] *Maleic hydrazeide*
*Pb-chelated & $Pb^{2+}$ in %*



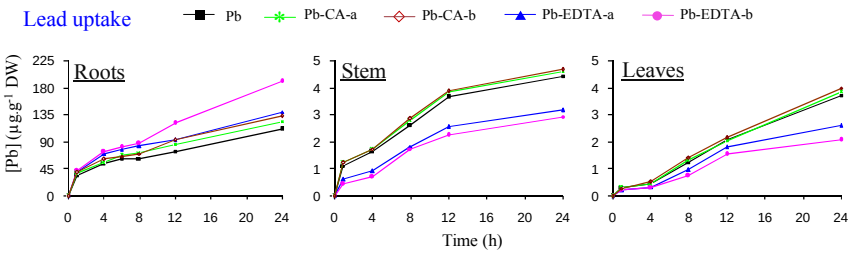

- Visual Minteq software version 2.60 → Pb speciation
- Lead uptake by roots & translocation to aerial parts (ICP-OES)
- Pigment content analysis (Chl-a, Chl-b, Carotenoids)
- Lead-induced genotoxicity (micronucleus test)
- Lead-induced oxidative stress (Lipid peroxidation & antioxidant enzymes: SOD, APX, GPOX, CAT, GR)

Root tips under microscope (40 X)



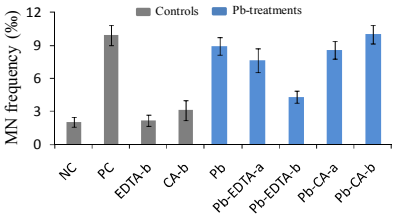

## Results & Discussions

### Lead uptake



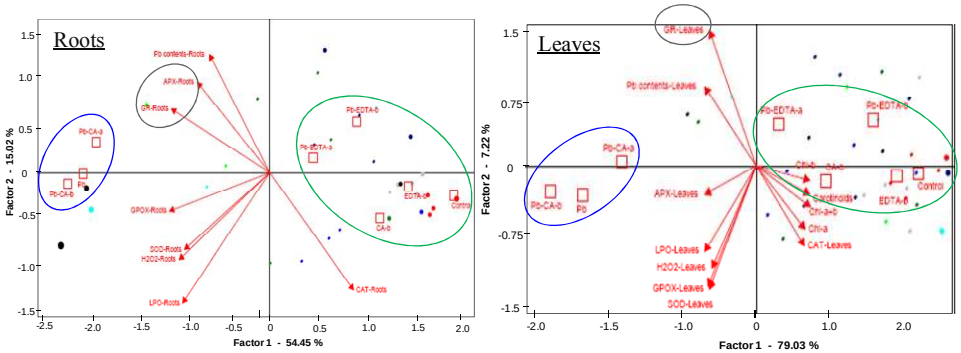

- Pb uptake started immediately after exposure, ↑ over time & reached a maximum value of 111 µg.g$^{-1}$ DW after 24h.
- EDTA dose dependently ↑ lead uptake & accumulation in roots but ↓ translocation to aerial parts.
- CA has no effect on lead uptake by roots or translocation to aerial parts at both levels of application.

### Lead-induced genotoxicity




- Application of Pb ↑ MN frequency 4 times / control.
- EDTA ↓ lead-induced genotoxicity dose dependently but CA has no effect (labile complex).

### Comparison of treatments & techniques of oxidative stress - Principal component analysis




Pb, Pb-CA-a and Pb-CA-b alone: **Non toxic group of treatments**

Control, EDTA-b, CA-b, Pb-EDTA-a and Pb-EDTA-b: **Toxic treatments**.

GR indirectly & APX directly detoxify lead-induced ROS in roots while GR alone in leaves.

Pigment contents and CAT activities grouped together showed reduction whereas SOD, GPOX, LPO & $H_2O_2$ showed induction under Pb toxicity.

## Conclusions

- **EDTA ↑ Pb uptake while ↓ its toxicity by forming stable, non-toxic and bio- available Pb-EDTA complexes.**
- **CA forms labile and week Pb-CA complexes → no effect on Pb toxicity to *V. faba*.**
- **GR is involved indirectly in detoxifying Pb induced ROS both in roots & leaves and hence better indicator of Pb toxicity whereas APX only in roots.**

**Abbreviations**: SOD; Superoxide dismutase, APX; Ascorbate peroxidise, GPOX; Guaiacol peroxidase, CAT; Catalase, GR; Glutathione reductase, EDTA; Ethylenediaminetetraacetic acid, ICP-OES; Inductively Coupled Plasma Optical Emission Spectrometers, LPO; Lipid peroxidation, Chl; Chlorophyll, MN; Micronucleus, ROS; Reactive oxygen species, CA; Citric acid, NC; Negative control, PC; Positivr control

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# Les effets d'un ligand organique, l'EDTA sur la spéciation, le transfert et la toxicité du plomb sur *Vicia faba*

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## Introduction

**Pb**, métal toxique même à très faible concentration.

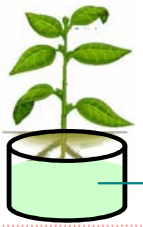
***Vicia faba (VF)***, plante très sensible, utilisée comme bio-marker de toxicité des substances.

## Objectifs

Évaluation de l'influence de la spéciation du Pb (effet des ligands organiques) sur sa phyto-disponibilité et phyto-toxicité.

## Matériel et Méthodes

*Vicia faba*, 3 semaines



***Conditions de germination***

*14h de jour / 10h de nuit.*
*T: 24 °C le jour / 20 °C la nuit.*
*Hygrométrie: 70 % d'humidité le jour et la nuit.*

Solution Hoagland
(pH 5)

### Traitements

**Témoin** = Solution Hoagland (SH)
**Pb** = SH + $Pb(NO_3)_2$ (5 µM)
**Pb-EDTA-a, 50%** = SH + EDTA (2.25 µM) + $Pb(NO_3)_2$ (5 µM)
**Pb-EDTA-b, 1%** = SH + EDTA (10 µM) + $Pb(NO_3)_2$ (5 µM)

**Les % de $Pb^{2+}$ libre en solution ont été calculés par le logiciel Visual Mineteq.**

### Paramètres étudiés

- [Pb] racines, tiges et feuille / ICP-OES.
- [pigments photosynthétiques] et [enzymes anti-oxydantes] / Spectrophotométrie

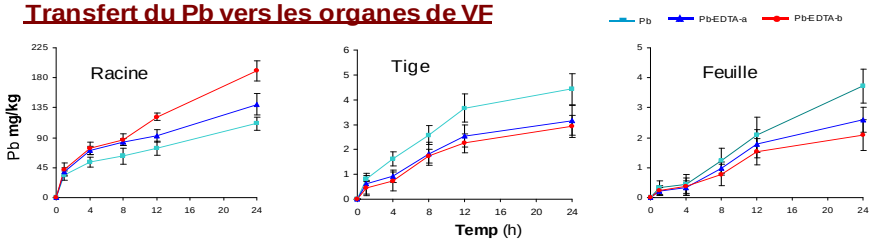
## CONCLUSIONS

- La complexation du plomb par l'EDTA modifie sa spéciation en solution, son transfert et sa phytotoxicité:

l'activité photosynthétique est « protégée » et la production des enzymes APX, CAT et GPOX est régulée.

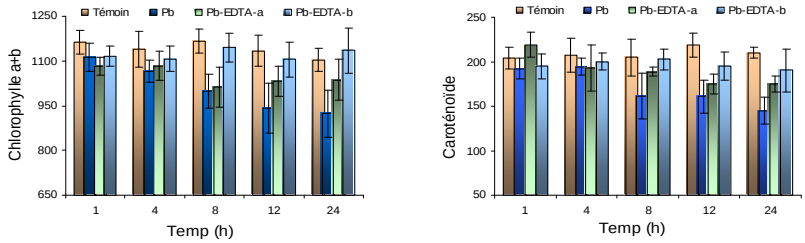
## Résultats

### Transfert du Pb vers les organes de VF




**L'EDTA ↑ l'absorption racinaire de Pb mais ↓ sa translocation (tige et feuilles).**

### Pigments photosynthétiques

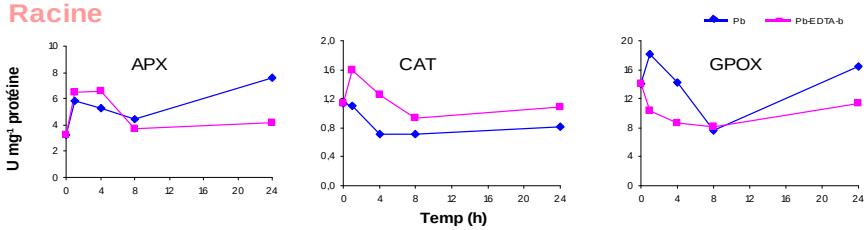



Le plomb seul ↓ [pigments photosynthétiques] après 8 h.

L'application d'EDTA ↓ significative cet effet et donc réduit la phyto-toxicité du plomb

### Enzymes anti-oxydantes

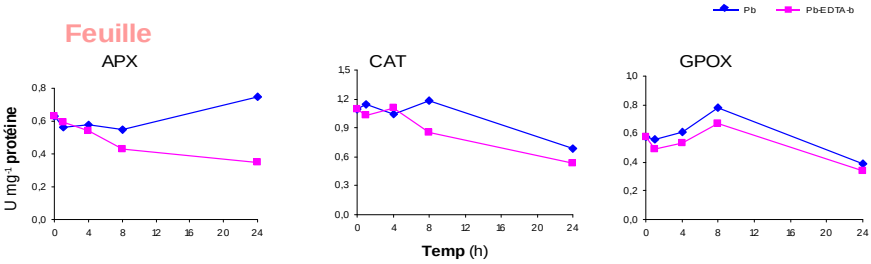
#### Racine




Pour le Pb seul, dans les racines, les activités d'APX et GPOX ↑ rapidement après 1 h puis ↓ à 8 h, et finalement ↑ après 24 h d'exposition. Concernant l'activité de la CAT, une réduction est observée à partir de 4h d'exposition au plomb.

Par ajout d'EDTA on observe : ↑ l'activité d'APX à 1 et 4 h puis une diminution à 8 et 24 h ; ↓ activité de GPOX sauf à 8 h; ↑ l'activité de la CAT jusqu'à une valeur proche du contrôle.

#### Feuille




Quelque soit le traitement, aucun changement significatif de l'activité enzymatique (CAT, APX et GPOX) n'est observé avant 8 h en raison de la faible vitesse de translocation du Pb. Cependant, à 24h, en présence de Pb seul, l'activité de CAT a diminué et celle d'APX et GPOX ont augmenté de manière significative par rapport au contrôle. Un ajout d'EDTA réduit alors significativement l'activité d'APX à 8 et 24 h dans les feuilles.

# GESTION FLORISTIQUE DE SOLS POLLUE PAR METAUX : ETUDE LONG TERME DE LA PHYTOEXTRACTION DU PLOMB

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## Contexte Scientifique et Objectifs

−Les métaux: problèmes majeurs en terme de qualité du sol, eaux superficielles et souterraines, végétaux cultivés.
−Le plomb: un des principaux contaminants persistants des sites industriels.
−[Pb] ≥ 300 mg $kg^{-1}$ de sol pouvait induire des problèmes de santé humaine et en particulier chez les enfants: anémie, impact sur le système nerveux central (Dudka et Miller, 1999).
−Plusieurs techniques pour réduire la quantité totale et/ou la fraction disponible des métaux: (a) méthodes physico-chimiques, rapides et efficaces mais coûteuses et peu respectueuses de l'environnement bio-géo-chimique du sol;
(b) **Phyto-remédiation**, utilisation des plantes pour décontaminer les sols, relativement lente mais efficace et respectueuse de l'environnement.

**Objectifs:**
➢ étudier la cinétique de phytoextraction de plomb pendant 3 ans par le *Pélargonium* cultivar Attar sur un sol acide fortement contaminés.
➢ les effets de la plante sur la rhizosphere pour modifier la biodisponibilité de Pb.

## Méthodologie

❖Le sol : des environs d'une usine de recyclage de batteries au Pb de la région de Toulouse (la STCM).
❖[Pb] = 39250 mg Pb $kg^{-1}$ de sol.
❖Les caractéristiques physico-chimiques : $pH_{eau}$ = 6, matière organique (MO) = 8,5% et argile = 45%.
❖Les essais de phytoextraction au champ ont été réalisés de Mai 2004 à Juillet 2006.
❖Un dispositif (Guivarch et al., 1999) a été utilise pour les essaies rhizospheriques, 2 semaines de contact sol-plante.

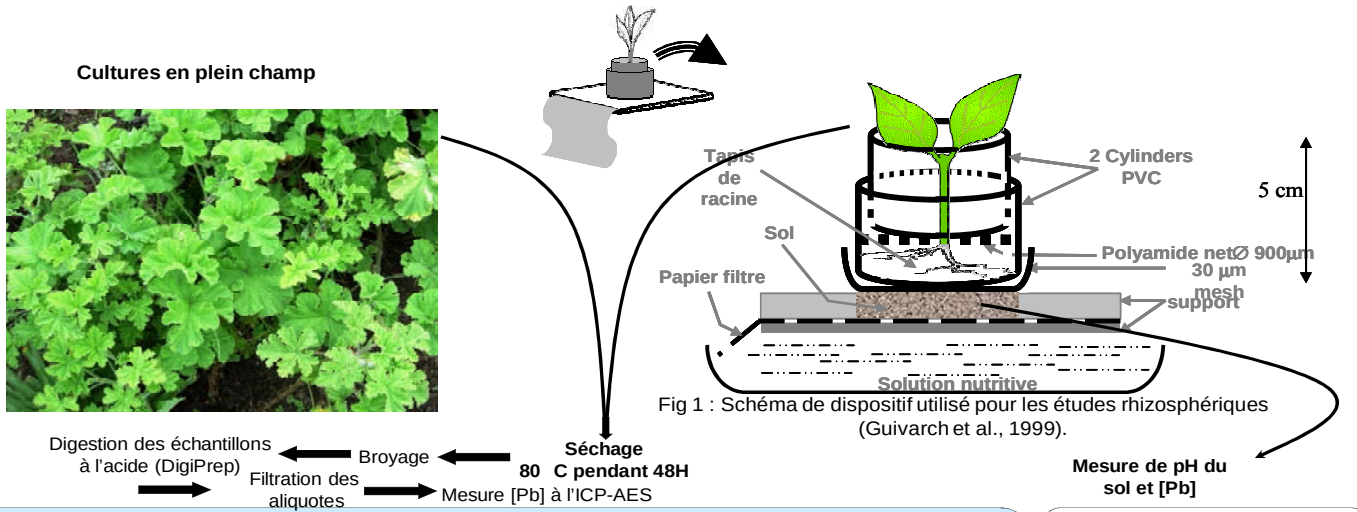


Fig 1 : Schéma de dispositif utilisé pour les études rhizosphériques (Guivarch et al., 1999).

## Résultats

❖ Fig 2 (a, b et c) présente les résultats de biomasse sèche (g), la concentration de plomb total dans les parties aériennes (mg Pb/kg PS) et la quantité de plomb extrait (mg Pb/plante) pour les trois périodes de culture (2004, 2005 et 2006) sur le sol acide fortement contaminé. Les biomasses, les concentrations et les quantités de Pb ont été augmentées significativement en 2006 par rapport en 2004. Ces Résultats démontrent que l'augmentation de durée, induit une forte augmentation de l'efficacité d'extraction. Comme les racines sont restées dans le sol pendant trois ans, l'hypothèse avancée est une saturation des sites racinaires en Pb favoriserait le transfert de Pb vers les parties aériennes. Les résultats de essaies microcultures (Tableau 1), montrent les variation de pH du sol. Le Pb a été effectivement transféré de la solution du sol vers les plantes. Les dosages du plomb dans la fraction disponible est de 3.86% par rapport à la concentration du départ.

| | $pH_{CaCl2}$ | $Pb_{CaCl2}$ (mg $kg^{-1}$) | $pH_{Solution\ de\ sol}$ | $Pb_{Solution\ de\ sol}$ (mg $kg^{-1}$) |
|---|---|---|---|---|
| Témoin | 7.50 ± 0.12 | 39.7 ± 15 | 7.8 ± 0.1 | 70.6 ± 14.7 |
| Attar | 6.79 ± 0.02 | 9.4 ± 2 | 7.35 ± 0.1 | 15 ± 0.7 |

Tableau 1 : Effets de la plante (Attar) sur le pH et la [Pb] dans la rhizosphere, pendant deux semaines de contact sol-plante.

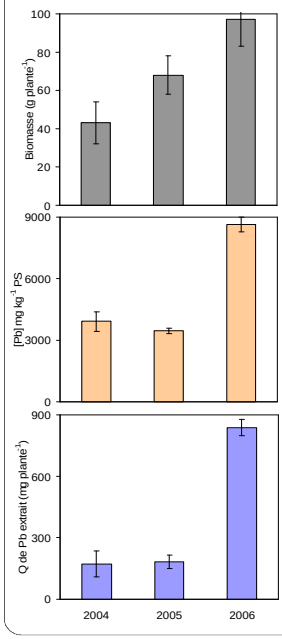


Fig 2 : Comparaison de biomasse et l'accumulation par le *Pélargonium* cultivar Attar sur trois ans de culture.

## Conclusions et perspectives

**➔Ces études ont démontré la possibilité de décontaminer les sols plombés par le Pélargonium cultivar Attar et surtout d'utiliser cette solution pour la gestion de sols qui sont en attente d'une décision de traitement de remédiation physico-chimique.**

➔En outre, les *Pélargonium* odorants testés en phyto-remédiation ont plusieurs avantages significatifs en comparaison avec les techniques physico-chimiques classiques de remédiation:

- une réduction de la fraction labile des métaux, (ii) une stabilisation des particules de sol contaminées, (iii) une amélioration de l'impact visuel des sites industriels, (iv) un coût bien moindre, (v) le respect de la biodiversité et du fonctionnement biogéochimique du sol, (vi) une possible commercialisation des huiles essentielles, acides organiques et alcools, qui pourraient dégager des bénéfices (Zheljazkov et al., 2006).

**➔Des expériences complémentaires sont nécessaires afin de vérifier la qualité de ces produits et d'étudier le comportement de Pb (spéciation et transferts) dans la rhizosphère, les racines et la sève de *Pélargonium*.**

Dudka, S., Miller, D.P. 1999. Water Air Soil Pollut. 113, 127-132; Guivarch, A., Hinsinger, P., Staunton, S. 1999. Plant Soil. 211, 131–138; Zheljazkov, V.D., Craker, L.E., Xing, B., 2006. Environ. Exp. Bot. 58, 9-16.

# Des géraniums odorants pour la phyto-remédiation des sols contaminés par les métaux

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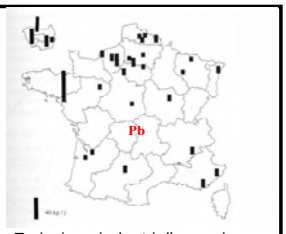
## *Contexte Scientifique et Objectifs*

−Les métaux: problèmes majeur en terme de qualité de sol, eaux superficielles et souterraines, végétaux cultivés.
−Un risque sanitaire potentiel
−Plusieurs technique pour réduire la quantité totale et/ou la fraction disponible des métaux: (a) les méthodes physico-chimiques, rapides et efficaces mais coûteuses et peu respectueuses de l'environnement bio-géo-chimique du sol; (b) **Phytorémédiation** = utilisation des plantes pour décontaminer les sols, relativement lente mais efficace et respectueuse de l'environnement.

Objectifs du projet:

➢Tester les capacités d'extraction du plomb de 6 cultivars de géraniums odorants (*Pélargonium*), sur deux sols aux caractéristiques contrastées.

➢Déterminer les mécanismes en jeu dans le transfert sol-plante et l'hyper-accumulation des métaux.




*Emissions industrielles majeures de Pb en France (1998).*

**Devenir des métaux dans le système sol-plante**

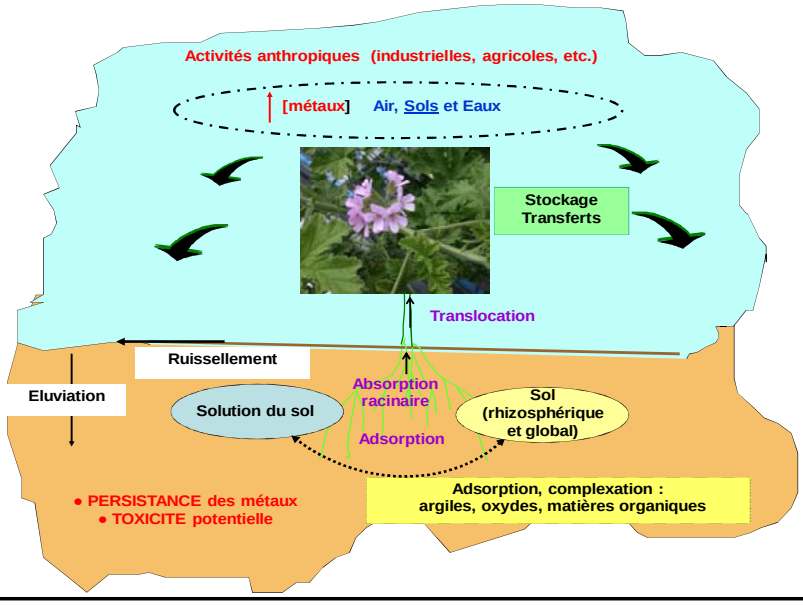
Le transfert des métaux présents dans un sol contaminé, vers les plantes utilisées en phyto-remédiation, va dépendre en particulier des caractéristiques :

(i) des sources de contamination (concentrations totales en métaux, spéciation chimique, taille..)

(ii) de la plante (espèce, cultivar, stade de maturité…)

(iii) du sol (pH, nature et teneurs des argiles, oxydes et matières organiques).

Nombreuses interactions dans le système sol-plante, entre les constituants des sols et les métaux et entre la plante et le sol (sol rhizosphérique).

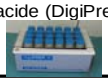



## *Méthodologie*

➢**Plantes bouturées de 6 cultivars de Pélargonium**: Attar of Roses, Clorinda, Atomic Snowflake, Gravelons, Sweet et Conclor.

➢Deux sols contaminés; sol calcaire moyennement contaminé (1830 mg Pb $kg^{-1}$ de sol sec) et sol acide fortement contaminé (39250 mg Pb $kg^{-1}$ de sol sec).

→ Séchage 80 C pendant 48H → Broyage → Digestion des échantillons à l'acide (DigiPrep)



→ Filtration des aliquotes → Mesure [Pb] à l'ICP-AES

## *Résultats et applications*

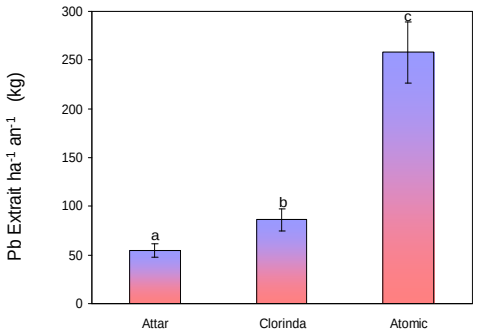



Fig: $Q_{Pb}$ extraites par les cultivars Attar, Clorinda et Atomic, sur sol acide fortement contaminé.

❖3 cultivars ont démontré un intérêt certain pour la phyto-remédiation des sols contaminés.
❖Forte concentration en plomb dans leurs parties aériennes (Atomic-T : 6904 mg Pb $kg^{-1}$ MS).
❖Biomasse élevée (Atomic-T : 45 tonnes/hectare/an) en comparaison avec d'autres plantes *(Schnoor, 1997)* et aucun signe de toxicité.

Les *Pélargoniums* odorants testés en phyto-remédiation ont plusieurs avantages significatifs en comparaison avec les techniques physico-chimiques classiques de remédiation:

(i) ↓ fraction labile des métaux en quelques semaines,
(ii) Stabilisation des particules de sol contaminées,
(iii) Amélioration de l'impact visuel des sites industriels,
(iv) Coût moindre,
(v) Respect de la biodiversité et du fonctionnement biogéochimique du sol
(vi) Fractionnement intégré des *Pelargonium* à l'étude qui pourrait dégager des bénéfices (Zheljazkov et al., 2006).

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