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Table of contents

INTRODUCTION **12**
Chapter 1 ..... **7**
Literature review **7**
1.1 Sources of lead contamination 9
1.2 Hyperaccumulation and remediation 10
1.2.1 Hyperaccumulator plants 10
1.2.2 Remediation techniques 12
1.2.3 Examples of Pb phytoextraction 14
1.3 Metal availability and uptake 16
1.3.1 Effect of soil properties on metal bioavailability 18
1.3.2 Effect of root exudates and microbes 20
1.3.3 Effect of chelating agents on metal availability 21
1.3.4 Metal speciation 22
1.4 Metal detoxification, translocation and homeostasis 23
1.4.1 Phytochelatins (PCs) 25
1.4.2 Metallothioneins (MTs) 26
1.4.3 Metal chelators 26
1.4.4 Metal ion transporters 27
1.4.5 Oxidative stress mechanisms 29
1.5 Phytoremediation and Genetic Engineering 30
1.5.1 Genes for phytoremediation 31
1.5.2 Gene function discovery 32
1.5.3 Genetic transformation procedure 32
1.5.4 Mechanism of genetic transformation 35
1.6 Research approach and objectives 36
Chapter 2 ..... **39**
Identification of lead hyperaccumulators **39**
2.1. *A field study of lead phytoextraction by various scented Pelargonium cultivars. Chemosphere 71:2187-2192.* 41
2.2. Perspectives 48
Chapter 3 ..... **51**
Lead phytoavailability and speciation **51**
3.1 *Phytoextraction of lead by scented Pelargonium cultivars: availability and speciation. Submitted to Chemosphere.* 54
1. Introduction 56
2. Materials and methods 58
3. Results 62
4. Discussion 73
5. Conclusion and perspectives 76
3.2 Additional results 82
3.3 General discussion 83
Chapter 4 ..... **85**
Tools for functional genomics of lead hyperaccumulation **85**
4.1(A) Choice of Explants 87

4.2 (A) *High efficiency Thidiazuron-induced shoot organogenesis from leaf explants of lead hyperaccumulator scented Pelargonium capitatum cultivars. Submitted to Plant Cell, Tissue and Organ Culture*.....88
Introduction 90
Materials and methods.....92
Results and discussion.....94
Conclusion and perspectives.....103
4.4 (B) Genetic transformation of lead-hyperaccumulator scented *Pelargonium* cultivars 109
Introduction 109
Materials and methods.....113
Results and discussion.....118
Conclusions and perspectives 129
LITERATURE CITED **151**
ANNEXES **171**
Annex I: Arshad et al. 2008. 4th European Bioremediation Conference, Chania, Crete, Greece. 3-6 Sep, 2008.....173
Annex II: Arshad et al. 2008. 4th European Bioremediation Conference, Chania, Crete, Greece. 3-6 Sep, 2008.....177
Annex III: Arshad et al. 2007. Info Chimie 482 : 62-65. 181
Annex IV: Arshad et al. 2008. 4th European Bioremediation Conference, Chania, Crete, Greece. 3-6 Sep, 2008. *Poster* 185
Annex V : Arshad et al. 2007. Pollutec, Nov, 27-30, 2007. Paris - Nord Villepinte, France. *Poster*.....186

List of figures

Figure 1: Different sources of lead contamination. 10
Figure 2: Cross-section of root showing the passage of ions through apoplastic and symplastic pathways (Gobat et al, 1998) 17
Figure 3: Biogeochemical processes controlling availability of metals in soil-plant system 19
Figure 4: Schematic presentation of soil-plant interactions in the rhizosphere 23
Figure 5: Schematic diagram of *Agrobacterium*-mediated transformation of plants 33
Figure 6: *Agrobacterium*-mediated genetic transformation (Tzfira and Citovsky, 2006)..... 36
Figure 7: Schematic presentation of the research approach 37
Figure 8: Cropping device used for rhizosphere experiments. 48
Figure 9: Cd concentrations in shoots and roots of scented Pelargonium cultivars 82
Figure 10: A schematic presentation of possible strategies for improved phytoextraction... 84
Figure 11: Microscopic images of root cells of *Pelargonium capitatum* cultivar Attar..... 141
Figure 12: Schematic representation of the work focussed on understanding of soil-plant interactions 142
Figure 13: Schematic presentation of optimized regeneration and transformation protocols 143
Figure 14: Methodology outline for studying gene function for Pb hyperaccumulation by scented *Pelargonium* cultivars s 145
Figure 15: Prospects for development of improved phytoextraction technique using scented *Pelargonium* cultivars 146

List of tables

Table 1: Some examples of metal hyperaccumulating plant species. 11
Table 2: Cost comparison of some remediation techniques 12
Table 3: Various phytoremediation strategies 13
Table 4: Physiological changes in response to Pb exposure in plants 24
Table 5: Metal ion transporters/genes/molecules involved in heavy metal detoxification / homeostasis in plants 28
Table 6: Rooted plants (%) obtained from scented *Pelargonium* cuttings after 30 days culture on different media/substrates 53

List of abbreviations

AFNOR	Association Française de NORmalisation
ANOVA	Analysis Of Variance
AS	Anti sens
BAP	N^6-benzylaminopurine
BCF	BioConcentration Factor
CEC	Cation exchange capacity
DM	Dry matter
DNA	Deoxyribonucleic acid
DOC	Dissolved Organic Carbon
DW	Dry weight
ERM	Elongation and rooting medium
ESEM	Environmental Scanning Electron Microscopy
EXAFS	Extended X-Ray Absorption Fine Structure
ha	Hectare
Hyg	Hygromycin
hyp	Hygromycin phospho-transferase
IAA	Indole-3-acetic acid
ICP-OES	Induced Coupled Plasma-Optical Emission Spectrometry
Kana	Kanamycin
LMWOAs	Low Molecular Weight Organic Acids
MS	Murashige and Skoog
MTs	Metallothioneins
NAA	α-naphthaleneacetic acid
NPTII	neomycin phosphotransferase gene
OD	Optical density
OM	Organic matter
PCR	Polymerase Chain Reaction
PCs	Phytochelatins
REACH	Registration, Evaluation, Authorisation and Restriction of CHemical substances

RM	Regeneration Medium
RNA	Ribonucleic acid
ROS	Reactive oxygen species
STCM	Société de Traitement Chimique des Métaux
t	ton
T-DNA	Transferred DNA
TDZ	Thidiazuron
TF	Translocation factor
uidA (GUS)	ß-glucuronidase
X-Gluc	5-bromo-4-chloro-3-indoyl-beta-D-glucuronic acid
y	year

INTRODUCTION

Parmi les différents polluants qui induisent des risques pour la santé et l'environnement, le plomb est l'un de ceux qui se trouve le plus souvent dans les milieux contaminés (Alkorta et al. 2004). Potentiellement toxique pour les organismes vivants, même à faible concentration (Sahi et al. 2002), le plomb peut être inhalé ou ingéré par l'homme et selon Henry (2000), induire des effets délétères (impact sur le tractus gastro-intestinal, les reins et le système nerveux central, pertes de mémoire, nausées, insomnie et anorexie, effet cancérigène potentiel) en particulier sur la santé infantile.

Selon Laperche et al. (2004), les émissions totales de Pb en France étaient de 217 tonnes/an en 2002. Basol (http://basol.environnement.gouv.fr), recensait, en 2005, 3 717 sites pollués pour lesquels l'État a entrepris une action de remédiation. Les sols non contaminés contiendraient de 10 à 30 mg Pb kg^{-1} de sol sec ; des teneurs en plomb supérieures à 110 mg Pb kg^{-1} sont considérées comme des anomalies (Laperche et al. 2004). La migration du plomb dans l'environnement est essentiellement tributaire de la solubilité des espèces chimiques présentes et des interactions du plomb avec les constituants réactifs des milieux (argiles, matières organiques, oxydes…). La précipitation des complexes peu solubles, la formation de complexes organiques relativement stable, et l'adsorption sur les constituants organiques et inorganiques peuvent réduire la biodisponibilité du plomb dans le sol, les sédiments et l'eau (Hettiarachchi et Pierzynski 2004). Le comportement du plomb dans un sol dépend donc en particulier de sa spéciation chimique (Dumat et al. 2001) et de ces interactions avec les constituants du sol (Cecchi et al. 2008), qui sont la résultante des caractéristiques pédologiques et physico-chimiques du sol. Les interactions entre les différents constituants des sols modifient aussi la capacité individuelle de chacun des constituants à adsorber ou complexer les métaux (Dumat et al. 2006).

De nombreuses techniques ont été développées afin de réduire la quantité totale et/ou la fraction disponible des métaux dans les sols pollués et d'apporter des réponses adaptées aux divers contextes de pollution (He et al. 2005). Les techniques physico-chimiques sont les plus couramment utilisées (comme par exemple l'excavation du sol, « nettoyé » ensuite par des solvants), elles ont l'avantage de réduire très rapidement et efficacement les concentrations totales en métaux. Cependant ces techniques physico-chimiques sont peu respectueuses de l'environnement bio-géo-chimique du sol, qui suite à ce type de traitement devient parfois un simple matériau de remblai (Ensley 2000). Certaines plantes sont capables d'adsorber

(phénomène de surface) et d'absorber les métaux au niveau de leurs racines, puis de les transloquer vers leurs parties aériennes. L'utilisation de plantes afin de réduire la concentration ou la disponibilité des métaux d'un sol contaminé est appelée « Phytoremédiation ». Les techniques de phytoremédiation sont moins coûteuses que les techniques physico-chimiques: selon Hettiarachchi et Pierzynski (2004), la technique de décontamination « off-site » est 5.7 fois plus chère par rapport à la phytoremédiation.

Des plantes ont été utilisées pour le traitement des eaux usées, il y a environ 300 ans (Lasat 2000). L'accumulation de métaux dans différentes parties aériennes de plusieurs espèces de plantes a également été signalée au 19e siècle et au début du 20e siècle (Lasat 2000). En 1980, l'idée de l'assainissement par les plantes a été réintroduite et développée par Utsunamyia (1980) et Chaney (1983). Ils ont été rejoints par Baker et al. (1991) qui a effectué des premiers essais au champ pour la phytoextraction de Zn et Cd. Au cours des deux dernières décennies, de nombreuses recherches ont été menées pour identifier/rechercher des plantes qui ont la capacité d'extraire les métaux du sol vers les parties aériennes de plantes. Selon Prasad et Freitas (2003), plus de 400 plantes sont identifiées comme hyper-accumulatrices des métaux. Mais, malgré la disponibilité de ces 400 plantes hyper accumulatrices, l'utilisation de la phytoremédiation au champ reste encore à une échelle limitée. Cependant, la phytoextraction du nickel par *Alyssum sp.* est couramment utilisée aux Etats-Unis (Chaney et al. 2007). *Alyssum murale* et *Alyssum corsicum* peuvent accumuler plus de 20 000 mg Ni kg^{-1} dans les parties aériennes et dégager des bénéfices économiques de \$16000 ha^{-1} (dus à la vente de nickel) avec un coût de production de \$250 a \$500 ha^{-1}.

Les limitations majeures de l'utilisation de la phytoextraction du Pb à grande échelle sont : i) indisponibilité de plantes qui accumulent de fortes concentrations dans les parties aériennes et produisent en même temps des biomasses élevées ; ii) trop peu d'essais au champ ; iii) connaissance insuffisante des mécanismes d'hyperaccumulation du Pb. La plupart des plantes identifiées à ce jour ne sont pas capables de réduire rapidement (quelques mois) les concentrations totales de Pb dans le sol en raison de leur trop faible biomasse ou de leur trop faible concentration en Pb dans leurs parties aériennes. L'exploration des mécanismes d'hyperaccumulation de façon générale, a sans doute peu avancé à ce jour en raison de l'absence d'outils génomiques et moléculaires (banques génomiques, EST, transgénèse) pour les espèces autres que les espèces modèles telle *Arabidopsis thaliana*. Si cette espèce est

devenue la référence en matière de génétique moléculaire pour la compréhension de mécanismes intervenant dans le développement, dans la résistance aux stress biotiques et abiotiques, elle ne convient pas pour l'étude de l'hyperaccumulation de métaux car elle n'est pas hyperaccumulatrice. Par contre, elle est apparentée à *Arabidopsis halleri* (accumulatrice du Zn et Cd) et *Thlaspi caerulescens* (accumulatrice du Pb, Zn et Cd), qui sont devenues des modèles d'études moléculaires pour l'accumulation du cadmium et du zinc. L'homologie au niveau des séquences codantes entre ADN d'*Arabidopsis thaliana* et les deux espèces nommées ci-dessus sont respectivement de 94 et 88,5% (Becher et al. 2004; Rigola et al. 2006). Il a donc été possible d'identifier plusieurs dizaines de gènes qui sont impliqués dans la régulation de l'absorption, de la translocation, de la séquestration et la détoxication des métaux chez les hyper accumulateurs. L'attribution d'une fonction à une séquence donnée n'est faisable que si on dispose de techniques d'expression dans l'organisme ciblé. Ainsi dans le cas des variétés hyperaccumulatrices du *Pélargonium*, il serait envisageable d'aborder l'étude des mécanismes cellulaires et moléculaires régissant le phénotype hyperaccumulateur si on disposait d'une technique de transformation génétique stable.

Ce travail de thèse fait partie d'un projet, qui a été initiée en 2004 en collaboration avec la STCM (http://w3.stc-metaux.com): entreprise de recyclage de batteries au plomb, volontaire en matière de gestion environnementale. Des essais au champ ont été mis en place afin de tester la faisabilité de la phytoremédiation en conditions réelles. Des expériences sur sites industriels (Toulouse-31 et Bazoche-45) en activité, ont été réalisées afin de tester sur deux sols aux caractéristiques contrastées, les capacités d'extraction du plomb de différents cultivars de *Pélargonium.* Cette étape constitue la base des expériences effectuées par la suite en conditions contrôlées :

1) Etude des paramètres influant le transfert sol-plante du Pb
2) mise au point d'outils d'études (régénération et transformation génétique) nécessaires pour déterminer les mécanismes moléculaires mis en jeu lors de l'hyperaccumulation du plomb.

Ce manuscrit de thèse est composé de quatre parties (Chapitres) présentés sous formes de publications (acceptées, soumises ou en préparation).

Le premier chapitre présente une synthèse bibliographique des divers aspects concernant la phytoextraction du Pb.

Le deuxième chapitre traitera des résultats d'essais au champ pour la faisabilité de la phytoextraction du Pb par le *Pélargonium*.

Le troisième chapitre est consacré à l'étude en conditions contrôlées de la phyto-disponibilité du Pb et de sa spéciation dans le système sol-plante en relation avec les changements physico-chimiques au niveau de la rhizosphère.

Le quatrième chapitre comporte deux parties : le première relatera les résultats concernant la mise au point d'une technique de régénération de plantes, et la deuxième partie sera focalisée sur l'optimisation de la transformation génétique.

Les conclusions de ce travail seront finalement exposées ainsi que quelques perspectives.

Chapter 1

Literature review

Bio-accumulative nature of potentially toxic metals and metalloids through entrance into the food chain represents an important ecological and health hazard due to their toxic effects. The European Environmental Agency (2007) has reported the occurrence of 250,000 polluted sites, mostly with heavy metals in 32-member states of the European Union. The identification process is going on and the number may increase up to three million sites (Jensen et al. 2009). Highly toxic nature of Pb, As, Cd and Hg makes them the most important among other pollutants (Chojnacka et al. 2005). Lead is comparatively less studied element with reference to Cd, Ni and Zn, due to the difficulties for remediation i.e. low mobility in soil, lack of hyperaccumulators with high biomass under field conditions, safe disposal of the biomass produced etc. Search for hyperaccumulators with high biomass and understanding accumulation, particularly of Pb, are the areas of particular interest, to develop environmental friendly techniques i.e. phytoremediation, for soil cleanup.

1.1 Sources of lead contamination

Anthropogenic and geogenic processes are the sources of heavy metals in the soil. Lead remains one of the most common metal (Alkorta et al. 2004) in the environment due to its persistence and numerous past or present uses (Fig 1). Half-life, the time taken to reach half concentration naturally, for Pb has been reported to be about 740–5900 years, depending upon soil physical and chemical properties (Alloway and Ayres 1993). Although some sources of Pb contamination have been reduced worldwide (e.g. from Pb alkyls in gasoline), Pb emission to the environment is still increasing in many countries (Adriano 2001). Nowadays, battery manufacturing is the principal current use of lead and the batteries represent 70% of the raw material for lead recycling industries reaching 160,000 t per year in France (Cecchi et al. 2008; Uzu et al. 2009). To limit the emission of Pb into the environment, it was recently classified as a substance of very high concern in the European REACH law (European Parliament Regulation EC 1907/ 2006 and the Council for Registration, Evaluation, Authorization and Restriction of Chemicals, 18 December 2006). However, its uses stay justified by industries in terms of cost-benefits analysis.

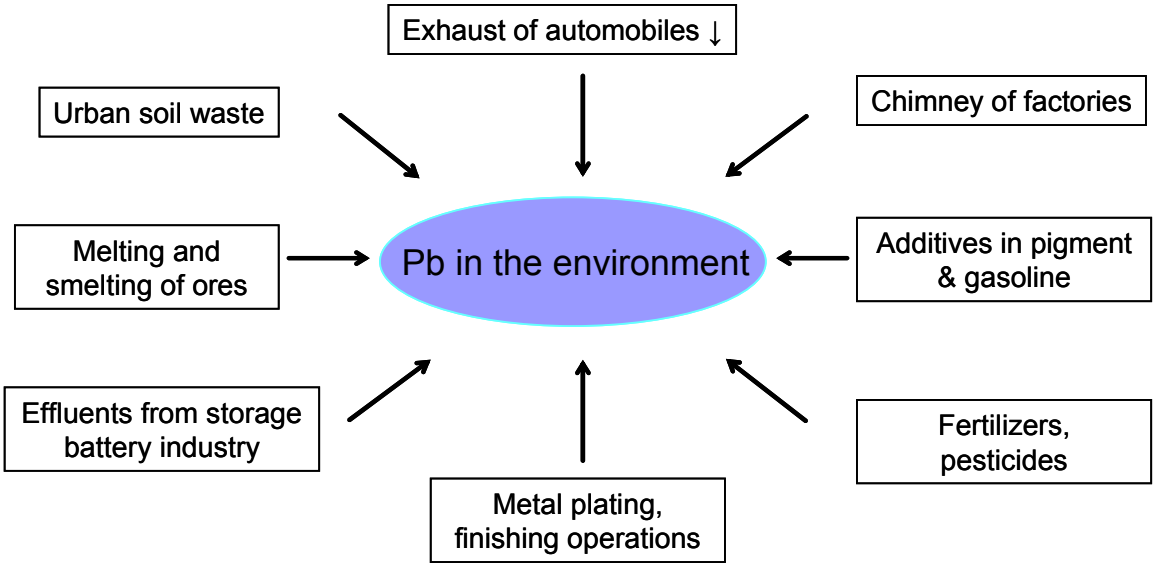

Figure 1: Different sources of lead contamination. Adapted from Sharma and Dubey 2005.

1.2 Hyperaccumulation and remediation

All the land plants have natural ability to take up essential and non-essential elements from the soil. Although most plant species are affected by the presence of metal ions in the environment, a few higher plants have evolved populations with ability to tolerate and thrive in metal-rich soils. These plants, known as metal accumulators, can sequester excessive amounts of metal ions in their biomass—the phenomenon is termed as hyperaccumulation—without incurring damage to basic metabolic functions—termed as tolerance (Cunningham et al. 1997; Reeves and Baker 2000).

1.2.1 Hyperaccumulator plants

The plants which can accumulate a certain amount of the target metal in shoots (Table 1), e.g. for Pb more than 0.1% of DW (Cunningham et al. 1997; Reeves and Baker 2000) are called “hyperaccumulators”. The hyperaccumulator plants could tolerate to heavy metal ions through various detoxification mechanisms, which may include selective metal uptake, excretion, complexing by specific ligands, and compartmentation of metal–ligand complexes (Rauser 1999; Cobbet 2000; Clemens 2001). Currently more than 400 plant species have been characterized as hyperaccumulators (Prasad and Freitas 2003). But, most of them are

characterized by a low biomass production or a low translocation rate, slow growth habit (Gleba et al. 1999). Some examples of hyperaccumulator species are presented in Table 1.

Table 1: Some examples of metal hyperaccumulating plant species.

Metal	CC DW (%)	Plant species	Concentration (mg kg^{-1} DW)	Exp. Conditions	Biomass (t ha^{-1} y^{-1})	Reference
Cd	≥ 0.01	*Thlaspi caerulescens*	3000	Field	4	Reeves et al. 1995
Co	≥ 0.1	*Haumaniastrum robertii*	10200	Field	4	Brooks et al. 1977
Cu	≥ 0.1	*Haumaniastrum* katangese	8356	Field	5	Brooks et al. 1977
Mn	≥ 1.0	*Macadamia neurophylla*	55000	Field	30	Jaffre, 1980
Ni	≥ 0.1	*Phyllanthus serpentinus*	38100	Field	-	Kersten et al. 1979
Zn	≥ 1.0	*Thlaspi caerulescens*	39600	Field	4	Reeves and Brooks 1983
Pb	≥ 0.1	*Brassica juncea (L.) Czern*	10300	Sand/perlite mixture	-	Kumar et al. 1995
		Pelargonium sp. Frensham	1700	Sand/perlite mixture	-	KrishnaRaj et al. 2000
		Avena sativa L.	5324	Hydroponics	-	Hernandez-Allica et al. 2008
		Helianthus annuus L.	4600	Hydroponics	-	"
		Triticum aestivum L.	4193	Hydroponics	-	"
		Triticale L.	4094	Hydroponics	-	"
		Thlaspi rotundifolium subsp.	8200	Field	4	Reeves and Brooks 1983
		Stellaria vestita Kurz.	3141	Field	-	Yanqun et al. 2005

CC = Concentration Criterion to be classified as hyperaccumulator; DW = shoot dry weight

By looking on the table 1, it can easily be figured out that the most of the hyperaccumulators reported for Pb have been only tested in hydroponics or on salt/perlite mixture. These conditions are very far from reality. Moreover, there is no information about their biomass which is of prime importance for phytoextraction purpose. The only field tested case is of *Thlaspi* which is capable of accumulating 33 kg Pb ha^{-1} y^{-1}. With this effectiveness, it would take too long to decontaminate even moderately contaminated soil. So it is of great interest to find new plants which are able to accumulate high concentrations of Pb as well as elevated biomass, resulting into increased extracted quantity of Pb and fasten the process of phytoextraction.

1.2.2 Remediation techniques

Different retrieval techniques are being employed to reduce the total and/or available metal concentration in polluted soils (He et al. 2005). Current technologies exploit soil excavation and, either land filling or soil washing followed by physical or chemical separation of the contaminants. Unfortunately, these techniques are labour-intensive, costly, and moreover affect the soil properties together with its agricultural potential (Ensley 2000). Whereas, plant based techniques offer economic (Table 2) and environmental advantages (Tanhan et al. 2007; Alkorta et al. 2004) and are considered as promising techniques due to their multi-fold advantages: large scale application, aesthetic value to the landscape, increased aeration of the soil in favour of a healthy ecosystem and, stabilization of the top soil that reduces erosion and health risks (Deng et al. 2006; Saxena et al. 1999; Ruby et al. 1996).

Table 2: Cost comparison of some remediation techniques

Decontamination Technique	Net present cost (ha^{-1})
	(USD)
Off-site	1 600 000
Soil washing	790 000
Phytoextraction	279 000

(Hettiarachchi and Pierzynski, 2004)

Plant based techniques are further named depending upon their mode of action e.g. phytoextraction, phytostabilization, rhizofiltration …etc. These remediation strategies are grouped into a collective name "Phytoremediation". These strategies and their mode of action have been listed in Table 3.

Table 3: Various phytoremediation strategies

Technique	Action mechanism	Medium treated
Phytoextraction	Direct accumulation of contaminants into plant shoots with subsequent removal of the plant shoots.	Soil
Rhizofiltration (phytofiltration)	Absorb and adsorb pollutants in plant roots.	Surface water and water pumped through roots
Phytostabilization	Root exudates cause metals to precipitate and becomes less bioavailable.	Groundwater, soil, mine tailings
Phytovolatilization	Evaporation of certain metal ions and volatile organics from plant parts.	Soil, groundwater
Phytodegradation	Plant-assisted bioremediation; microbial degradation in the rhizosphere region.	Rhizosphere
Phytotransformation	Uptake of organic contaminants & degradation.	Surface and groundwater
Removal of aerial contaminants	Uptake of various volatile organics by leaves.	Air

(Adapted from Yang et al. 2005)

Of these plant based techniques, phytoextraction offers a better solution, particularly for non-degradable contaminants or metals. The phytoextraction involves the removal of the contaminants from the soil and stockage in aerial parts which are subsequently removed away. The application of phytoextraction in field is hampered by the time required to decontaminate completely (Alkorta and Garbisu 2001). The time required is dependent on the type and extent of metal contamination, the length of the growing season, and the efficiency of metal removal by plants. Phytoextraction is applicable only to sites that contain low to moderate levels of metal pollution, because plant growth is not sustained in heavily polluted soils and/or it may take centuries to decontaminate.

1.2.3 Examples of Pb phytoextraction

Currently, two kinds of plants are being tested for phytoextraction; i) metal hyperaccumulators, ii) high biomass producing which accumulate low to average metal concentrations, but which compensate for this by their high biomass, such as *Brassica juncea*, (Lasat 2002), tobacco (Keller et al. 2003). The hyperaccumulating plants take up one or two specific metals and have a low biomass that is compensated by very high metal concentrations in the shoots (Reeves and Baker 2000; Chiang et al. 2006).

Pelargonium sp. "Frensham", scented geranium was identified as one of the most efficient metal hyperaccumulator plants (Saxena et al. 1999). In a greenhouse study, young cuttings of scented geranium grown in artificial soil and fed with different metal solutions, were capable of taking up large amounts of three metal contaminants i.e. Pb, Cd and Ni in a 14-day experiment. These plants were capable of extracting from the feeding solution and stocking in their roots, amounts of lead, cadmium and nickel equivalent to 9%, 2.7% and 1.9% of their dry weight material, respectively. With an average root mass of 0.5-1.0 g in dry weight, scented geranium cuttings could extract 90 mg of Pb, 27 mg of Cd and 19 mg of Ni from the feeding solution in 14 days. These values easily satisfy the conditions for being hyperaccumulator and, are even multi-folds to the desired amounts. If these rates of uptake could be maintained under field conditions, scented geranium should be able to cleanup heavily contaminated sites in less than 10 years. However, growth and uptake in nutrient solution can be extremely different to that in soil (Prasad and Freitas 2003), and scientific studies indicate the hydroponics culture is not indicative of a real-world situation, due to ion competition, root impedance, and the fact that plants do not grow root hairs when they are grown in solution (Prasad and Freitas 2003).

KrishnaRaj et al. (2000) reported the ability of scented geranium plants (*Pelargonium sp.* 'Frensham') to tolerate and maintain normal metabolic processes, when exposed to various levels of lead under greenhouse conditions. According to Dan et al. (2002), *Pelargonium* genera (*Geraniaceae* family) offers several hyperaccumulators species with high biomass levels and translocation rates. Moreover the crop could be exploited through the production of essential oils, reducing the cost of the remediation treatment. Recently, Hassan et al. (2008) tested *Pelargonium zonale*, for lead extraction from artificially contaminated soil. The plants accumulated 54, 478 and 672 mg kg^{-1} during 3 weak pot culture on soil containing 2000,

5000, 7000 mg kg^{-1} Pb, respectively. Utilization of EDTA increased Pb accumulation and respective values of Pb concentrations in shoots were 257, 727 and 2291 mg kg^{-1}. However, they have not mentioned the biomass produced that could have given an idea for the time required for soil cleanup.

Apart from *Pelargonium spp.,* some other species have also been reported as lead Hyperaccumulators. Wang et al. (2007) has described *Bidens maximowicziana* as a Pb hyperaccumulator offering remarkable tolerance and accumulation of Pb, simultaneously. Lead concentration in roots was 1509 mg kg^{-1} and 2164 mg kg^{-1} in over-ground tissues. EDTA application promoted translocation of Pb and its concentrations in over-ground parts was increased from 24–680 mg kg^{-1} to 29–1905 mg kg^{-1}. *Typha orientalis Presl* is another Pb hyperaccumulator reported by Li et al. (2008). The average lead concentrations in the leaves and roots were 619 and 1233 mg kg^{-1}, respectively, in plants collected from mine tailings. In hydroponics, Pb concentrations in the leaves and roots increased with increasing of Pb level in the modified Hoagland's nutrient solution resulting into 16190 and 64405 mg kg^{-1} in the leaves and roots, respectively. The observations of Li et al. (2008) also confirm the differences in accumulation in hydroponics as compared to field conditions.

As a plant-based technology, the success of phytoextraction is inherently dependent upon several plant characteristics. The combination of high metal accumulation and elevated biomass would result in the best output of metal removal. Other desirable plant characteristics include the ability to tolerate difficult soil conditions i.e. soil pH, salinity, soil structure and water content, the production of a dense root system, ease of care and establishment, and few disease and insect problems. Although some plants show promise for phytoextraction, there is no plant which possesses all of these desirable traits. Finding the perfect plant continues to be the focus of many plant-breeding and genetic-engineering research efforts (Prasad and Freitas, 2003). Despite the efforts in developing phytoextraction, the understanding of plant mechanisms involved in metal extraction is still emerging. In addition to this, relevant applied aspects, such as the optimisation of agronomic practices on metal removal by plants are still largely unknown, probably due to lack of field research studies and relevant data. Most of the experimentation has been carried out in hydroponics and greenhouse conditions. All the

plants reported to-date for Pb hyperaccumulation are required to be tested in field conditions to have the real efficiency and feasibility.

1.3 Metal availability and uptake

Soil-root interface comprising of few millimeters for soil surrounding the plant roots and directly affected by root activities is considered as rhizosphere. Plant roots represent highly dynamic systems that explore the rooting medium, typically soil, to stabilize the plant mechanically and to take up water and mineral nutrients, depleting both in the rhizosphere relative to the bulk soil. In response to this, plant roots provide structural elements as the rhizoplane and act as a continuous source of energy and materials creating specific conditions in the rhizosphere soil.

The movement of mineral elements to the root surface depends on different factors including;

i) Diffusion of elements along the concentration gradient formed due to uptake and subsequent decrease of the concentration in the root vicinity.

ii) Root interception, displacement of soil volume by root volume due to root growth.

iii) Mass flow, transport from bulk soil solution along the water potential gradient driven by transpiration (Marschner 1995).

Different metals have different localities for being taken up by plants. Some metals in plants are taken up primarily at the apical region and others may be taken up over the entire root surface. All the above stated mechanisms may apply singly, or in combination depending on conditions in root zone and plant capacities for uptake of concerned element (Fig 2).

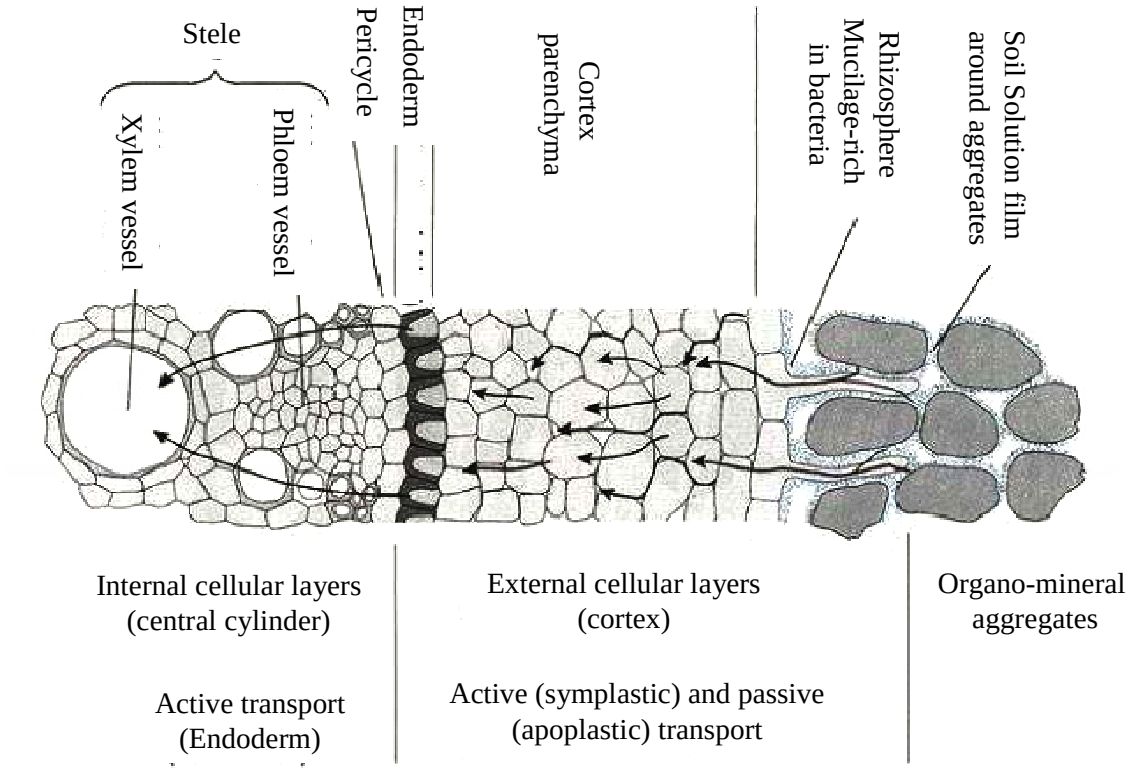

Figure 2: Cross-section of root showing the passage of ions through apoplastic and symplastic pathways (Gobat et al, 1998).

During uptake, metals first enter into apoplast of the roots. Then, apart of total amount of metal is transported further into the cells symplastically (Clemens 2006; Verbruggen et al. 2009); some in the apoplast and some may be bound to cell wall substances (Greger and Johansson 2004). Distribution of the total metal taken up between these three depends on the type of metal, plant genotype as well as many other external factors. For example, a major portion of Pb is bound to the cell walls (Wierbzicka 1998) and deposited in apoplast region (Laperche et al. 1997). According to Marschner (1995), heavy metals are transported apoplastically in plant tissue. For translocation, metals have to reach the xylem vessels by crossing endodermis and subrinized cell wall called 'Casparian strips'. Ultimately, most of the metal uptake is performed by the younger parts of the root where the Casparian strips are not yet fully developed. All the mechanisms involved in metal uptake are interdependent on amount of particular element present in the soil and its availability to the plant system. Metal

availability may be influenced by multiple factors including soil characteristics, plant's ability to mobilize the element in the rhizosphere through root exudates or external addition of similar products i.e. chelators.

1.3.1 Effect of soil properties on metal bioavailability

Metal mobility and bioavailability in the soil are also determinant factors for field application of phytoextraction of heavy metals. Plants can only take up available fraction of a metal or must have a mechanism to make the metals available. Different biogeochemical processes controlling availability of elements in soil-plant system have been schematized in fig 3. The availability of heavy metals is controlled by soil chemical properties (pH, Eh, CEC, metal speciation), physical properties (texture, clay content, organic matter percentage), biological factors (plant action, bacteria, fungi), their interactions (Ernst et al. 1992) and soil mineralogy (Navarro et al. 2006). The chemical forms of heavy metals in soil are affected by soil pH modifications. An increase in pH results in increased adsorption of Cd, Zn and Cu to soil particles and reduces the uptake of Cd, Zn and Pb by plants (Kuo et al. 1985). In contrast to this, acidification increases the metal absorption by plants through a reduction of metal adsorption to soil particles (Brown et al., 1994). In acidic soils, metal desorption from soil binding sites into solution is stimulated due to H^+ competition for binding sites. Soil pH affects not only metal bioavailability but also the process of metal uptake by roots (Brown et al. 1995). Li et al. (2007) has reported that external Pb loading decreased soil pH and increased Pb bioavailability in soil, thus resulting in increased Pb uptake and accumulation in the edible parts of rice. This indicate that plant availability of externally loaded Pb is related to Pb transformation and fractionation in soils, which are affected by basic soil properties such as clay, oxide content and composition, pH, and organic matter.

The redox potential (Eh) of the soil is a measure of the tendency of the soil solution to accept or donate electrons. As the redox potential decreases, heavy metal ions are converted from insoluble to soluble forms, thus increasing bioavailability (Pendias- Kabata and Pendias 1984). The cation exchange capacity increases with increasing clay content in the soil while the availability of the metal ions decreases (Pendias- Kabata and Pendias 1984). Thus, the higher the cation exchange capacity of the soil, the greater the sorption and immobilization of the metals.

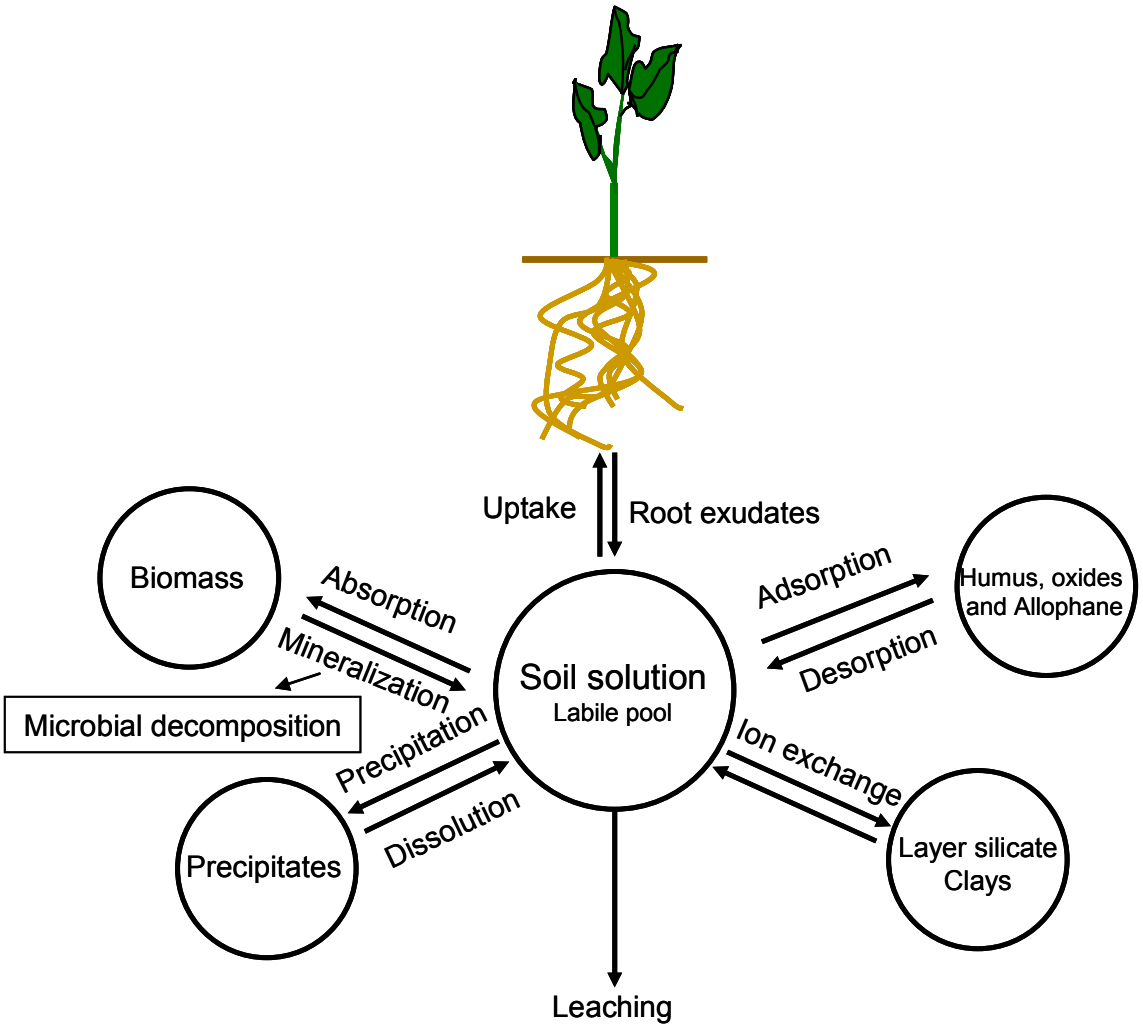

Figure 3: Biogeochemical processes controlling availability of metals in soil-plant system. The above figure shows various mechanisms involved in the equilibrium of labile pool. The availability of any element in the soil-plant system is the function of all the factors capable of disturbing equilibrium. However, the extent of one process may differ from the other in a given set of climatic, biological and soil conditions.

The soil texture also plays an important role for the bioavailability of heavy metals. Generally, metal ion availability is the lowest in clay soils, followed by clay loam and finally loam and sand (Webber and Singh, 1995). The binding of heavy metals with organic matter, humic acid in particular, has been well documented (Chen et al. 2006; Dumat et al. 2006; Quenea et al. 2009). High organic matter content enhances the retention of the metals, drastically reducing the metal availability.

1.3.2 Effect of root exudates and microbes

An essential component of the bioavailability process is the exudation of metal chelating compounds by plant roots (ex. phytosiderophores). These chelators can mobilize heavy metals such as copper, lead and cadmium by formation of stable complexes. Chelators are usually low molecular weight compounds such as sugars, organic acids, amino acids and phenolics that can change the metal speciation and thus, metal bioavailability (Salt et al. 1995). Organic compounds released into the rhizosphere provide the substrate and the energy source for microbial populations. Both plant roots and microbes are responsible for secretion of inorganic and organic compounds e.g. protons, HCO_3^-, and various functional groups capable of acidification, chelation and/or reduction. These compounds can affect a range of chemical reactions and biological transformations. Due to continued accelerated input and output of energy, materials and chemical reactivity, the rhizosphere soil far from equilibrium, thus quickly differentiating from non-rhizosphere. Due to root activity, even relatively static soil properties, such as mineralogy (Hinsinger et al. 1992), may be affected within a relatively short time.

The organic compounds released from roots (rhizodeposits or exudates) stimulate the growth of the rhizosphere microbial community. They may be responsible for the differences in the structure of the microbial communities commonly observed between the rhizosphere and the bulk soil. Rhizodeposits consists of a broad range of compounds including root mucilage. Depending upon plant species, low molecular weight organic acids (LMWOAs) can play a significant role in the bioavailability and transport (Lagier et al. 2000). These acids compete for free metal ions to form soluble complexes and reduce metal adsorption onto soil surfaces (Antoniadis and Alloway 2002). The organo-metallic complexes of LMWOAs and heavy metals could then be taken up by the plant roots (Evangelou et al. 2004). The interactions of LMWOAs are different in their extents and ways of affecting the mobility, bioavailability, degradation and phyto-toxicity of different metals (Lagier et al. 2000, Clapp et al. 2001).

Soil microbes in the rhizosphere including plant growth promoting rhizo-bacteria, P-solubilizing bacteria, mycorrhizal-helping bacteria and arbuscular mycorrhizal fungi play a significant role in nutrient dynamics including trace elements. For example arbuscular mycorrhizal fungi produce an insoluble glycoprotein, glomalin, which sequester trace

elements and could help in remediation of contaminated soils. The inoculation with appropriate heavy metal adapted rhizobial microflora could lead to enhanced phytoextraction by plants (Khan 2005).

1.3.3 Effect of chelating agents on metal availability

Use of chelators in the environment has received considerable attention for more than 50 years now. Ethylene-Diamine-Tetra-acetic Acid (EDTA) occurs at higher concentrations in European surface waters than any other identified anthropogenic organic compound (Reemtsma et al. 2006). Chelating agents potentially perturb the natural speciation of metals (Nowack, 2002) and, consequently influence metal bioavailability. However many chelating agents biodegrade slowly and are persistent in the environment (Bucheli-Witschel and Egli 2001). In the recent past, extensive research on EDTA have given rise to its environmental concerns (Nowack and VanBriesen 2005), and its low biodegradability has shaped the discussion about the problems of chelating agents (Williams 1998), including leaching of heavy metals to underground water due to increased dissolution. Dramatic increase in the research on chelating agents in the environment in the last decade was mainly due to the proposed use of chelating agents for soil remediation, both for extraction of metals and for chelant-enhanced phytoremediation (Nowack et al. 2006). A variety of chelating agents are in use with recent additions of growth hormones which promote phytoextraction multifolds (Israr and Sahi 2008). Lead accumulation in *Sesbania drummondii* shoots was enhanced by 654 and 415% in the presence of 100 mM IAA and 100 mM NAA, respectively, compared to control plants exposed to Pb alone. Application of IAA or NAA along with EDTA, Pb accumulation was further increased in shoots by 1349% and 1252%, respectively. The photosynthetic efficiency and strength of the treated plants were not affected in the presence of IAA or NAA and EDTA.

Due to reports of potential problem from the use of chelating agents, the research has also been focused towards selection of suitable chelator. Epelde et al. (2008) compared EDTA and Ethylene Diamine Di-Succinate (EDDS) in a greenhouse experiment for chelate-induced Pb phytoextraction with *Cynara cardunculus*, as well as to investigate the toxicity of these two chelates to both cardoon plants and soil microorganisms. Shoot concentration was 1332 mg Pb kg^{-1} DW with the addition of 1 mg kg^{-1} EDTA in a soil polluted with 5000 mg Pb kg^{-1} soil,

whereas, shoot Pb accumulation was 310 mg Pb kg^{-1} DW with EDDS application. The biomass was also lower in case of EDDS as compared to those plants treated with EDTA. On the other hand, EDDS degraded rapidly and was less toxic to the soil microbial community in control non-polluted soils. Basal and substrate-induced respiration values were significantly higher in Pb-polluted EDDS-treated soils than those treated with EDTA.

Careful application of chelators can potentially help phytoremediation but to-date; guidelines about their use are inconclusive. Creating new problems for the environment for the sake of already existing or replacing one with another would not be the real solution. Judicial use of these agents will rely upon the availability of comprehensive data about the effects and interactions of these compounds with soil type, soil structure and texture, kind and extent of metal pollution, genotype and, climatic conditions.

1.3.4 Metal speciation

Metals have different affinities for different elements, thus influencing complex formation and binding to different macromolecules. For example, Hg and Pb can form organic metal complexes. Mobility of different metals also varies. Cd and Zn are mobile while Pb is relatively immobile and easily forms complexes with fulvic acids (Sposito, 1989). In acidic soil, lead is either as Pb^{2+} or $PbSO_4$ while in alkaline soils, $PbCO_3$, $Pb(CO_3)_2^{2-}$ or $PbOH^+$ less available species are present (Sposito, 1989). Once the metal is taken up from the rhizosphere, it can be either in free ionic form or may bind to organic substances within plant. In xylem sap of *A. halleri*, the dominant form of Cd was free Cd^{2+} ions (Ueno et al., 2008). According to Salt et al. (1999), the major proportion of Zn in the xylem sap of *T. caerulescens* was the free hydrated Zn^{2+} ions whereas Straczek et al. (2008) have observed the Zn bonding to organic acids. Nickel was transported in a complex with histidine in hyperaccumulators (Kramer et al., 1996).

The complex nature of interactions present between soil and root in the rhizosphere are summarized in Fig 4. Good knowledge of the factors influencing the availability could help to successful application of the plant based techniques for remediation purposes. Increased bioavailability of the metal element is directly proportional to the uptake of the element. So the knowledge of the factors capable of affecting the solubility and uptake of Pb are of crucial importance. How the plant reacts to Pb stress; modifications in soil pH,

exudation of organic acids, changes in DOC contents, difference in cultivars and cultivar specific capacity to enhance availability of Pb, possible binding of Pb within plant tissues, etc? These are the questions needed to be addressed for development of Pb phytoextraction technique.

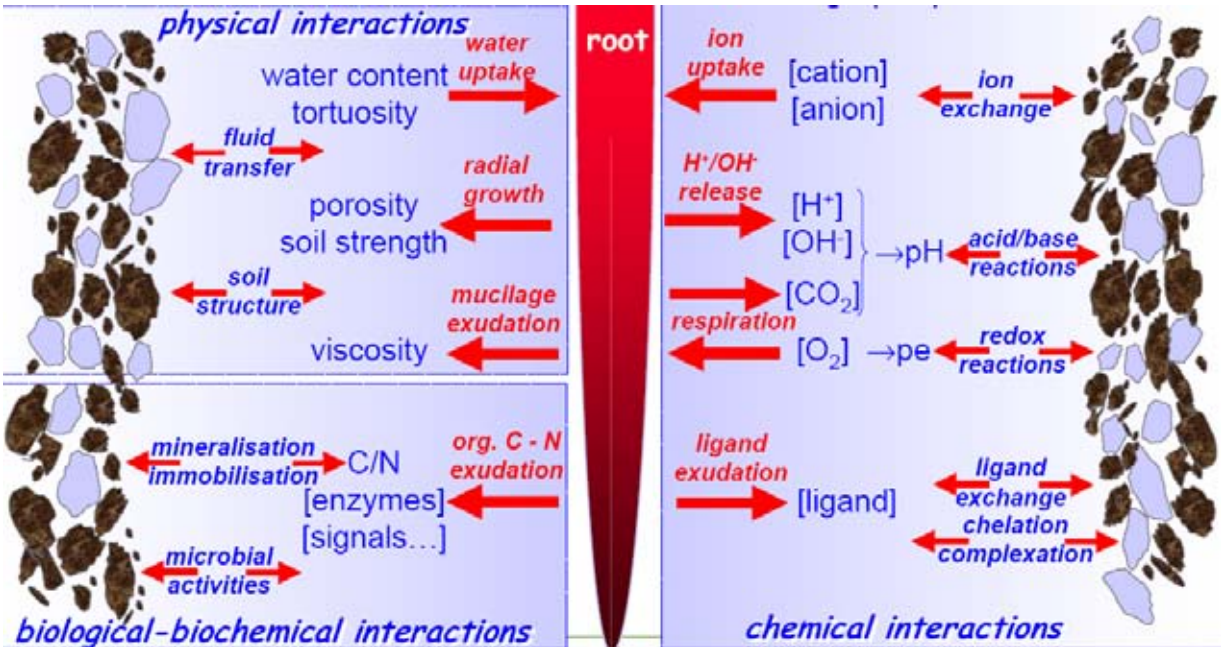

Figure 4: Schematic presentation of soil-plant interactions in the rhizosphere (Hinsinger, 2004)

1.4 Metal detoxification, translocation and homeostasis

Plants respond to heavy metal toxicity in a variety of different ways. Lead can cause deleterious effects in metal sensitive plants. Lead accumulation could reduce the concentration of chlorophyll, iron, sulphur, Hill reaction activity and catalase activity whereas increased the concentration of phosphorus, sulphur and activity of peroxidase, acid phosphatase and ribonuclease in leaves of radish (Gopal and Rizvi 2008). A summary of physiological changes in response to Pb exposure are presented in Table 4.

Organ and cell functioning
➢Nutrient uptake Alterations in uptake of cations (K^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{3+}) and anions (NO_3^-). ➢Water regimes Decrease in compounds maintaining cell turgor and cell wall, guard cell size, stomata opening, level of abscisic acid and leaf area.
Pb^{2+}
Subcellular functioning
➢Chloroplast-Photosynthesis Alteration in lipid composition of thylakoid membrane, Decrease in synthesis of chlorophyll, plastoquinone, carotenoids, activity of NADP oxyreductase, electron transport and activities of Calvin cycle enzymes. ➢Nucleus-Mitotic irregularities Increase in irregular shapes, decomposed nuclear material, chromosome stickiness, anaphase bridges, c-mitosis and formation of micronuclei. ➢Mitochondria-Respiration Decrease in electron transport, proton transport and activities of enzymes of Kreb's cycle.

Table 4: Physiological changes in response to Pb exposure in plants. Modified from Sharma and Dubey, 2005.

The type and extent of the reaction to environmental stress may be different in hyperaccumulators as compared to metal sensitive plants. Hyperaccumulators could immobilize, exclude, chelate and compartmentalize the metal ions, and the expression of more general stress response mechanisms such as ethylene and stress proteins could result. Multiple genes may be involved in hyperaccumulation. According to Kramer (2005), approximately ten key metal homeostasis genes are expressed at very high levels during Zn hyperaccumulation in a natural hyperaccumulator plant. One of the major defense mechanisms involves the production of proteinaceous compounds e.g. phytochelatins and metallothioneins (Zenk 1996). In the last decade, phytochelatins, metallothioneins, metal chelators and transporters were the major focus of the research on metal hyperaccumulation, translocation and detoxification.

Their role has been well established in metal homeostasis (Reviewed by Cobbett and Goldsbrough 2002; Yang et al. 2005; Clemens 2006; Milner and Kochian 2008; Memon and Schroeder 2009). The production of reactive oxygen species (ROS) has also been demonstrated in response to heavy metal exposure (Pourrut et al. 2008). A summary about their function in metal homeostasis is presented in the followings.

1.4.1 Phytochelatins (PCs)

Heavy metals activate PCs (Cobbett and Goldsbrough, 2002) production and they play major roles in metal detoxification in plants and fungi. The general formula of PCs is $(\gamma\text{-Glu-Cys})_nX$ where n is a variable number from 2 to 11 depending on the organism, although most common forms have 2-4 peptides, X represents an amino acid such as Gly, β-Ala, Ser, Glu or Gln (Cobbett and Goldsbrough 2002). Heavy metals including Cd, Hg, Ag, Cu, Ni, Au, Pb, As and Zn induce biosynthesis of PCs. However, Cd is by far strongest inducer (Grill et al. 1989). In the presence of the thiol groups of Cys, PCs chelate Cd, forming complexes protecting the cytosol from free Cd ions (Cobbett 2000). The metal binds to the constitutively expressed enzyme γ-glutamylcysteinyl dipeptidyl transpeptidase (PC Synthase), thereby activating it to catalyse the conversion of glutathione to phytochelatin (Zenk 1996).

PCs are considered very important in cellular homeostasis and translocation of essential nutrients such as Cu and Zn (Thumann et al. 1991) due to their metal ion affinity. PCs are required for detoxification of toxic metals, particularly to Cd, as confirmed in both *Arabidopsis* and *Schizosaccharomyces pombe*, by the Cd sensitive phenotype of *cad1* mutants defective in PCs activity (Ha et al. 1999). In some cases, the production of PCs in excessive amount may not ensure hyper-tolerance. Enhanced PCs synthesis seems to increase heavy metals accumulation in transgenic plants (Pomponi et al. 2006), whereas excessive expression of *AtPCS* induced hypersensitivity to Cd stress (Lee et al. 2003). PCs could help in the transport of heavy metal ions by forming complexes, into the vacuole (Clemens 2006). Raab et al. (2005) have studied As-PC complexes in extracts of the As-tolerant grass *Holcus lanatus* and the As-hyperaccumulator *Pteris cretica*. The dominant form was non-bound inorganic As, with 13% being present in PC complexes for *H. lanatus* and 1% in *P. cretica*.

1.4.2 Metallothioneins (MTs)

Detoxification of metals by the formation of complexes is documented in most of the eukaryotes (Kramer et al. 2007). MTs are other cysteine-rich peptides with a low molecular weight, able to bind metal ions by means of mercaptide bonds. MTs are the products of mRNA translation, induced in response to heavy metal stress (Cobbett and Goldsbrough 2002). MTs are divided into three different classes depending upon their cysteine content and structure. The Cys-Cys, Cys-X-Cys and Cys-X-X-Cys motifs (in which X denotes any amino acid) are characteristic and invariant for MTs (Yang et al. 2005). The pea MT (PsMTa) can bind Cd, Zn and Cu when expressed in *Escherichia coli* (Tommey et al. 1991). *Arabidopsis* MTs are able to restore tolerance to copper in MT-deficient yeast strains (Zhou and Goldsbrough 1995). In a study, over-expression of mouse MT in tobacco plants increased Cd tolerance *in vitro* (Pan et al. 1994), whereas *Brassica juncea* MT2, ectopically expressed in *A. thaliana*, confers enhanced tolerance to Cd and Cu (Zhigang et al. 2006). In terms of transcript amount, many plant MT genes are expressed at very high levels in all tissues. Salt el al. (1995) has reported that *Arabidopsis* MT1a and MT2a seemed to accumulate in trichomes rendering sequestration of heavy metal ions. Since *Arabidopsis* MT expression has been detected in phloem elements, MTS are potentially involved in metal ion transport (Garcia-Hernandez et al. 1998).

The biosynthesis of MTs may also be triggered by several factors other than metals, including hormones and cytogenetic agents. MT genes are expressed during various stages of plant development and in response to different environmental conditions (Rauser 1999). Abiotic stresses, such as high temperature and deficiency of nutrients can also result in production of MTs (Cobbett and Goldsbrough 2002). Despite the concept that MTs might play a key role in metal metabolism, their precise function in plants remains to be determined owing to a lack of information (Hall 2002; Yang et al. 2005; DalCorso 2008).

1.4.3 Metal chelators

Extracellular chelation by organic acids, such as citrate and malate, is important in mechanisms of aluminum tolerance. For example, malate efflux from root apices is stimulated by exposure to aluminum and is correlated with aluminum tolerance in wheat (Delhaize and Ryan 1995). Some aluminum-resistant mutants of *Arabidopsis* also have increased organic

acid efflux from roots (Larsen et al. 1998). Organic acids and some amino acids, particularly *His*, also have roles in the chelation of metal ions both within cells and in xylem sap (Kramer et al. 1996; Rauser 1999). Recently Olko et al. (2008) have reported that the accumulation of metals can only be explained on the basis of organic acid changes in *Armeria maritima* plants. The content of organic acids, especially malate, decreased in the roots and increased in the leaves. These changes may suggest their role in metal translocation from roots to shoots.

1.4.4 Metal ion transporters

Terrestrial plants have native effective ions uptake system that enables them the acquisition of nutrients as well as metal ions from soil through roots. Therefore, metal cation transport and homeostasis is essential for plant nutrition and heavy metal tolerance. Several classes of metal transporters/genes identified in plants are presented in (Table 5). They are situated in the tonoplast or plasma membrane, playing an important role in metal homeostasis within physiological limits. These include heavy metal (or CPx-type) ATPases that are involved in the overall metal ion homeostasis and tolerance in plants, the natural resistance-associated macrophage protein (Nramp) family of proteins, cation diffusion facilitator (CDF) family proteins (Williams et al. 2000), and the zinc-iron permease (ZIP) family (Guerinot 2000). Despite these identifications, many plant metal transporters remain to be explored at the molecular level (Yang et al. 2005).

Table 5: Metal ion transporters/genes/molecules involved in heavy metal detoxification / homeostasis in plants

Gene/molecules	Proposed function	Reference
AtDX1	Detoxifying Cd efflux carrier?	Li et al. 2002
AtHMA1-6 (*P-type ATPase*)	Resemble bacterial heavy metal pumps	Axelsen & Palmgren 2001; Clemens et al. 2002; Cobbett et al. 2003
AtMHX1 (*Antiporters*)	Vacuolar membrane exchanger of protons with Mg and Zn	Shaul et al. 1999
AtMRPs (*ATP-binding cassette*)	Cd, Pb transport across the tonoplast?	Tommasini et al 1998; Bovet et al. 2003
AtNramp 1,3,4 (*Nramp family*)	Iron homeostasis, Cd uptake?	Curie et al. 2000; Thomine et al. 2000
CAX1-2 (*Divalent cation/proton*)	Cax1 vacuolar Ca accumulation, CAX2 Cd ?	Hirschi et al 1996, 2000
COPT1	Cu-influx protein	Williams et al. 2000
IRT1 (*ZIP transporter*)	Iron, possibly Mn, Zn and Cd uptake	Eide et al. 1996; Korshunova et al 1999
LCT1	Rb, Na, Ca, and Cd uptake	Schachtman et al. 1997; Clemens et al. 1998
Metallothioneins (MTs)	Cytoplasmic metal buffering	Zhou and Goldsbrough 1995; van Hoof et al. 2001; Murphy et al. 1997
MtZIP2* (*ZIP transporter*)	Zn uptake	Burleigh et al. 2003
NtCBP4, *N. tabacum*	Plasma membrane calmodulin-binding cyclic nucleotide-gated channel	Arazi et al. 1999
Organic acids; citrate, malate	Increased Al resistance correlates with citrate or malate release from roots	Ma and Furukawa 2003
PAA1 *(P-type ATPase)*	Cu transport to chloroplasts	Shikanai et al. 2003
Phytochelatins (PCs) pathway; AtPCS1	Cytoplasmic metal buffering, long-distance metal trafficking?	Vatamaniuk et al. 1999; Gisbert et al. 2003; Gong et al. 2003
RAN1 *(P-type ATPase)*	Cu transport	Hiryama et al. 1999
ZAT1**, ZTP1**, TgMTP1*** *(CDF family)*	Intracellular sequestration of Zn	van der Zaal et al 1999, Assunçao et al. 2001; Persans et al. 2001
ZIPI-4 *(ZIP transporter)*	Zn uptake	Grotz et al. 1998
ZNT1-2** (*ZIP transporter*)	High-affinity Zn and low-affinity Cd uptake	Pence et al. 2000; Assunçao et al. 2001

** Medicago trunculata; **T. caerulescens; ***T. geosingense*

The CPx-type heavy metal ATPases have been identified in a wide range of organisms and have been implicated in the transport of essential, as well as potentially toxic, metals like Cu, Zn, Cd, and Pb across cell membranes (Williams et al. 2000). They are considered to be important not only in metal sequestration for essential cell functions, but also in preventing the accumulation of these ions to toxic levels. A novel family of similar proteins, Nramp, has been implicated in the transport of divalent metal ions, particularly Fe and Cd (Thomine et al. 2000). Disruption of an AtNramps3 gene slightly increased Cd resistance, whereas over-expression resulted in Cd hypersensitivity in *Arabidopsis*.

Another important family is the CDF proteins. CDF transporters have been characterized in both prokaryotes and eukaryotes and can transport across membranes divalent metal cations such as Zn, Cd, Co, Fe, Ni or Mn (Montanini et al. 2007). Some of the CDF family members are thought to function in catalyse efflux, and some are found in plasma membranes whereas others are located in intracellular membranes. van der Zaal et al. (1999) have reported that the protein zinc transporter of *Arabidopsis thaliana* (ZAT1) may have a role in zinc sequestration. Elevated zinc resistance was observed in transgenic plants over-expressing ZAT1 and these plants showed an increase in the zinc content of the root under conditions of exposure to high concentrations of zinc. However, ZAT1 is not confined to root tissue; protein analysis conferred that ZAT1 was constitutively expressed throughout the plant and was not induced by exposure to higher levels of zinc.

To-date, 15 members of the ZIP gene family has been identified in the *A. thaliana* genome. Different members of the ZIP family are known to be able to transport iron, zinc, manganese, and cadmium. Pence et al. (2000) cloned the transporter ZNT1, a ZIP gene homolog, in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. They found that ZNT1 mediates high-affinity Zn uptake as well as low-affinity Cd uptake. In recent studies, another group of transporters ATP-binding cassette (ABC transporters) have shown to be implicated in a range of processes including polar auxin transport, lipid catabolism, disease resistance, stomatal function, xenobiotic and metal detoxification (Kim et al. 2006; Rea 2007).

1.4.5 Oxidative stress mechanisms

When plants are exposed to different metals, they try to adjust themselves accordingly through producing certain proteinicious compounds or concentrating into certain structures. Different detoxifying mechanisms including cell wall sequestration

(Wierzbicka, 1998), isolation of lead from the cytoplasm with a membrane (Małecka et al., 2008), synthesis of PCs in response to Pb (Piechalak et al. 2002) playing a role in lead sequestration into plant vacuoles (Piechalak et al. 2003), are activated. Another important reaction of plants is the production of reactive oxygen species (ROS) resulting in an unbalanced cellular redox status (Sharma and Dubey 2005; Pourrut et al. 2008). Redox modifications at cellular level might induce changes in proteins and nucleic acids or the peroxidation of bio-membranes. Plants also contain a complex antioxidant system to tackle ROS (Apel and Hirt 2004). This system is comprised of antioxidants such as reduced glutathione, ascorbate or α-tocopherol (Vitamin E) and antioxidant enzymes such as superoxide dismutase, catalase, peroxidases, ascorbate peroxidase and glutathione reductase. Recently, Pourrut et al. (2008) have reported an oxidative burst in roots of *Vicia faba* and induced lipid peroxidation in response to Pb uptake. They have also found that Pb accumulation in leaves caused lipid peroxidation and a strong decrease of photosynthetic pigments.

Metal homeostasis and tolerance in plants remained a major field of research in the last decade, with particular focus on As, Cd, Ni and Zn. However, our understanding of molecular mechanisms of Pb accumulation, tolerance and translocation in plants is very poor (Clemens, 2006). Current knowledge of the functions of PCs, MTs, metal chelators, transporters and oxidative stress mechanism would only be fruitful if the genes responsible for these compounds could be easily manipulated. Most of the work reported in literature is concentrated to model species Arabidopsis halleri and Thlaspi caerulescens. Recent projects on genomic scale profiling of nutrient and trace elements in A. thaliana (Lahner et al. 2003) are very promising. But the application of all these developments to species other than model ones, is hindered due to unavailability of tools required for gene function studies i.e. genetic transformation and regeneration. Keeping in mind the wide diversity of plants growing in extreme conditions, understanding the molecular and physiological bases would allow developing or engineering plants with desired set of characteristics for environmental remediation (Milner and Kochian 2008) and sustainable development.

1.5 Phytoremediation and Genetic Engineering

Production of plants with improved characteristics by genetic engineering, i.e. by modifying metal uptake, transport and accumulation as well as metal tolerance, opens up new possibilities for phytoremediation (Karenlampi et al. 2000). Phytoremediation using

non-transgenic plants (grasses, sunflower, corn, hemp, flax, alfalfa, tobacco, willow, Indian mustard, poplar, Pelargonium etc.) shows good potential, especially for the removal of pollutants from large areas with relatively low concentrations of unwanted compounds due to time period constraints. Multifold increase in accumulation and/or tolerance has been reported in genetically modified plants as compared to wild types, for some metal pollutants e.g. As (Dhankher et al. 2002), Hg (Che et al. 2003), Pb (Martinez et al. 2006) and Se (Pilon et al. 2003).

Some hyperaccumulators, such as *Thlaspi caerulescens*, can take up high levels of metals to their harvestable parts but their low biomass limits their efficiency (Meagher 2000; Nedelkoska and Doran 2000). Through genetic transformation, plants can be transformed either for improved metal uptake or increased biomass. For example, nicotianamine synthase gene (Higuchi et al. 1999) is involved in the formation of phytosiderophore—the metal binding amino acid—that increases the bioavailability of metals to plants. However, the most common strategy involves targeting the proteins involved in metal homeostasis (metallothioneins, phytochelatins and glutathione) for genetic manipulations (Clemens et al. 2002). Although such approaches typically involve the manipulation of plant enzymes responsible for the formation of phytochelatins and related compounds e.g. over-expression of glutathione synthetase (Zhu et al. 1999), gamma-glutamylcysteine synthetase (Dhankher et al. 2002), phytochelatin synthase (Li et al. 2004), manipulations with other enzymes have also been successful. These enzymes are considered to be responsible for the first phase in plant detoxification, the activation reaction of recalcitrant compounds in plants (Sandermann 1994).

1.5.1 Genes for phytoremediation

Genetic engineering-based phytoremediation strategies for elemental pollutants like mercury and arsenic using the model plant *Arabidopsis* are being tested. The success of the techniques will lead the way for the remediation of other challenging elemental pollutants like lead or radionuclides. However, the identification and characterization of the genes in native hyperaccumulators hold prime importance. The real effectiveness of any gene could only be tested in the parent plant. Some of the examples of genes/molecules involved in hyperaccumulation, transport and metal homeostasis have been presented in table 5.

Availability of biotechnological techniques and knowledge of the desired genes can help to develop effective phytoremediation techniques. Plants can be engineered with enhanced metal tolerance, capable to adjust their rhizosphere to increase mobility of

targeted element, able to modify speciation within plant system for better translocation and ultimately storage of toxic elements either in vacuoles or after transformation into less toxic forms through binding with organic acids and thiol-rich chelators (Meagher and Heaton 2005).

1.5.2 Gene function discovery

Recent developments in large scale genomics programs on model species (NCBI, Genome project) have laid the foundation towards exploring the structure, expression and mechanisms involved in the regulation of genes upon exposure to abiotic and biotic stresses. Gene isolation is a relatively high throughput technology these days the identification of their functions and regulation mechanisms are the major impediments. Availability of genome databases with known functions could help to assign putative function to newly discovered sequence. This could merely give an idea of the biochemical function of the coding sequence. The majority of genes isolated have therefore been attributed putative functions on the basis of *in silico* studies. The strategies other than *in silico* aimed at understanding gene function are grouped under the name of "functional genomics". These strategies are based on either spatio-temporal expression of the genes or the over-expression of the target gene. The spatio-temporal expression of the genes (mRNA and protein expression) is the response to the stimulus during developmental stages, or to biotic or abiotic stresses (Narusaka et al. 2004). However, these studies, only give an indirect proof of the role and function of the genes. The over-expression of the desired gene is achieved by using a strong plant promoter and adjoining activation sequences to drive high level and constitutive expression of a gene coding sequence. The effect is high steady state mRNA and protein levels. Any phenotypic changes could then be assigned to the native gene's function based on biochemical pathways that are altered in the transformants. However, over-expression of an endogenous gene can lead to co-suppression by RNAi mechanism, a possible approach for deciphering gene function. However, the use of these strategies is impossible without availability of the tools required for gene transformations i.e. genetic transformation.

1.5.3 Genetic transformation procedure

Plant transformation and regeneration systems have become indispensable tools (Busov et al. 2005) since its initial application about 25 years ago (De Block et al. 1984),

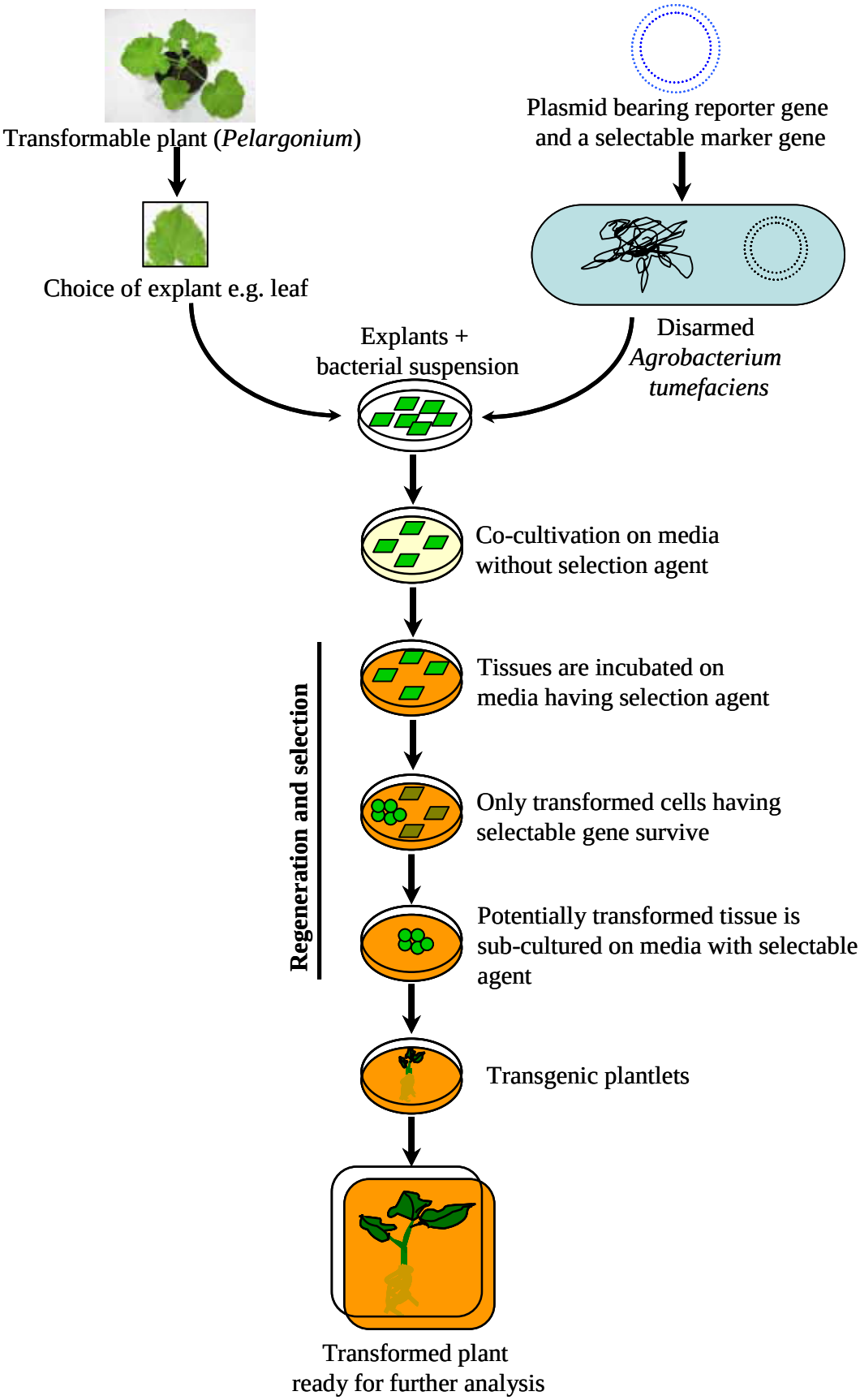

Figure 5: Schematic diagram of *Agrobacterium*-mediated transformation of plants.

to explain gene function and for crop improvement, either by modulating existing traits or introducing new ones. This allows the exploration of many aspects of plant physiology and biochemistry through analysis of gene function and regulation that cannot be studied by any other experimental method. Genetic transformation systems are based on the introduction of foreign DNA into plant cells, followed by the regeneration of such cells into whole fertile plants. A schematic diagram of the transformation protocol is presented in Fig 5.

The success of any plant genetic transformation strategy depends upon the availability of an efficient *in vitro* regeneration system coupled with appropriate selection regime and a method for introducing DNA into plant cells (Twyman et al. 2002). The efficiency of the regeneration system is greatly influenced by different factors particularly, the plant organ to be used as donor explants, the basic culture medium, growth hormones and their altogether interactions (Poulsen 1996). In addition to auxins and cytokinins, Thidiazuron (TDZ) has proven to be a highly effective regulator of plant morphogenesis. TDZ (N-phenyl-N'-l,2,3-thidiazol-5-yl urea) is a substituted phenylurea compound which was adopted for mechanized harvesting of cotton bolls (Murthy et al., 1998). Originally TDZ was classified as a cytokinin and induced many responses typical of natural cytokinins (Murthy et al. 1998). However, later research showed that TDZ, unlike traditional cytokinins, was capable of fulfilling both cytokinin and auxin functions involved in various morphogenetic responses of different plant species (Jones et al. 2007). TDZ has been mostly tested for inducing somatic embryogenesis through short period shock with it. Somatic embryogenesis in *Pelargonium* species has focused to a major extent on zonal (*Pelargonium x hortorum)* and regal (*Pelargonium x domesticum)* cultivars. Different explants such as hypocotyls and petioles (reviewed in Haensch 2007), hypocotyls and cotyledons (Murthy et al. 1999, 1996), have been used as starting material. TDZ demonstrated the ability to enhance the efficiency of somatic embryogenesis in a wide range of species including *Pelargonium sp.* (Murthy et al. 1998). However, the occurrence of true somatic embryos induced from hypocotyls on TDZ-containing medium in *Pelargonium x hortorum* and *Pelargonium domesticum* has been challenged. Histological analysis demonstrated that regenerated structures formed both through organogenesis and somatic embryogenesis were, indeed, shoot-like and leaf-like structures (Haensch 2004; Madden et al. 2005). On the other hand, TDZ was not necessary for inducing somatic embryos from petioles of *Pelargonium x domesticum* cv. Madame Layal (Haensch 2007) and in scented *Pelargonium sp.* ‘Frensham’ (KrishnaRaj et al. 1997). In

the latter case, the method proved to be an efficient tool for the development of transgenic scented *Pelargonium* plants (Bi et al. 1999). For genetic transformation, regeneration sytem is compulsory irrespective of the method of regeneration i.e. direct shoot formation, indirect through callus phase or embryogenesis. Genetic transformation is influenced by multiple factors particularly plant genotype, type of explant, bacterial strain, presence of phenolic substances like Acetosyringone in the culture and inoculation media to induce vir-gene, tissue damage, co-cultivation, antibiotics and the time of application (Boase et al. 1998; Opabode 2006; Liu et al. 2008; Moeller and Wang 2008).

1.5.4 Mechanism of genetic transformation

A DNA segment is genetically transferred by *Agrobacterium* to its host (plant cell), from its tumor-inducing (Ti) plasmid to the host-cell genome (Gelvin 1998). Engineered *Agrobacterium* strains by replacing the native T-DNA with genes of interest are the most efficient vehicles used today for the transformation process for the production of transgenic plant species (from Tzfira and Citovsky 2006). A set of bacterial chromosomal (chv) and Tiplasmid virulence (vir) genes encodes for proteins that forms the molecular machinery needed for T-DNA production and transport into the host cell. In addition, various host proteins have been reported to participate in the *Agrobacterium*-mediated transformation process (Tzfira and Citovsky 2002; Gelvin 2003). The vir region, located on the Agrobacterium Ti plasmid, encodes most of the bacterial virulence (Vir) proteins used by the bacterium to produce its T-DNA and to deliver it into the plant cell. In wild-type Agrobacterium strains, the T-DNA region (defined by two 25 base pair direct repeats termed left and right T-DNA borders) is located in *cis* to the vir region on a single Ti plasmid. In disarmed Agrobacterium strains with replaced native T-DNA, a recombinant T-DNA region usually resides on a small, autonomous binary plasmid and functions in *Trans* to the vir region (from Tzfira and Citovsky 2006). The step-wise process of genetic transformation is explained in Figure 6.

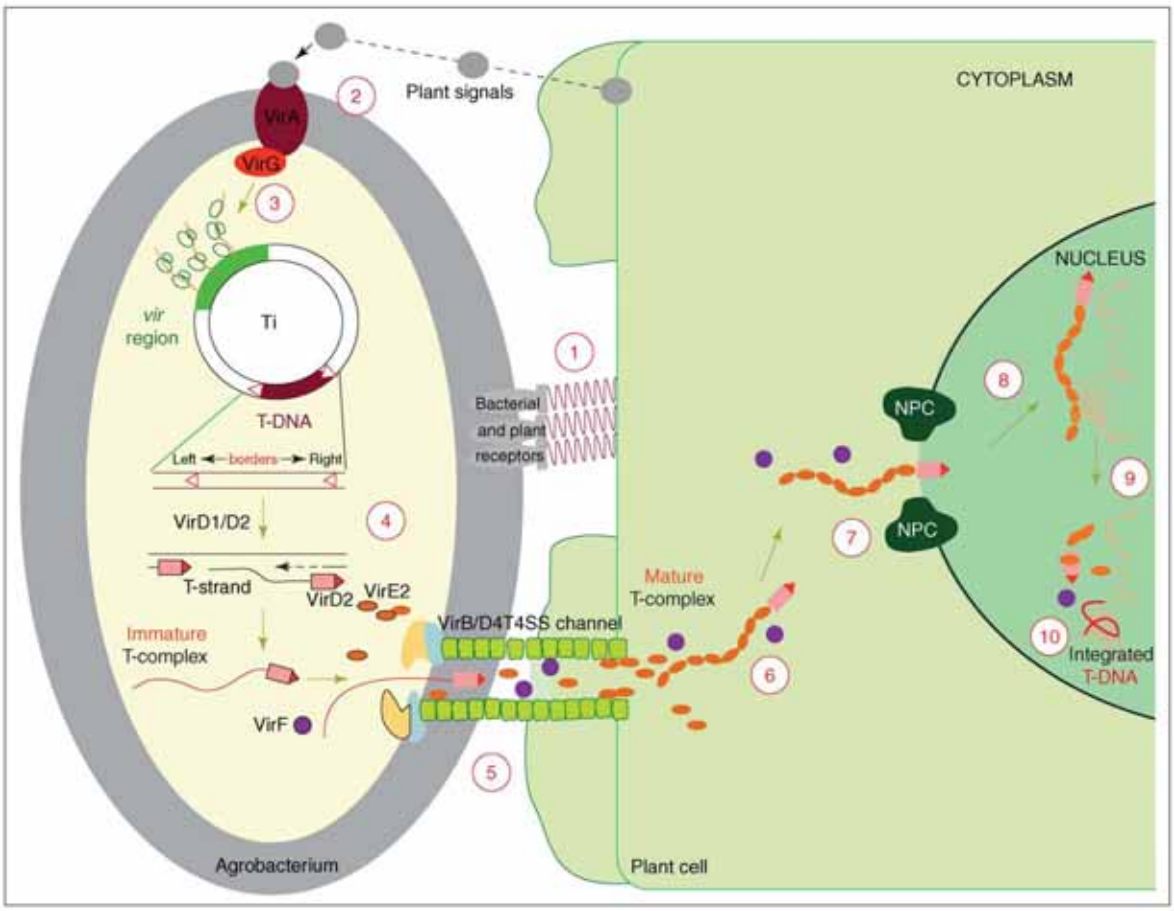

Figure 6: *Agrobacterium*-mediated genetic transformation (Tzfira and Citovsky, 2006). The transformation process comprises 10 major steps and begins with recognition and attachment of the Agrobacterium to the host cells (1) and the sensing of specific plant signals by the Agrobacterium VirA/VirG two-component signal-transduction system (2). Following activation of the vir gene region (3), a mobile copy of the T-DNA is generated by the VirD1/D2 protein complex (4) and delivered as a VirD2–DNA complex (immature T-complex), together with several other Vir proteins, into the host-cell cytoplasm (5). Following the association of VirE2 with the T-strand, the mature T-complex forms, travels through the host-cell cytoplasm (6) and is actively imported into the host-cell nucleus (7). Once inside the nucleus, the T-DNA is recruited to the point of integration (8), stripped of its escorting proteins (9) and integrated into the host genome (10).

1.6 Research approach and objectives

The phytoremediation, an environment friendly technique, offers a great hope for rehabilitation of the ecosystem as well as fulfilling food requirements of the ever growing world population through making contaminated land, culture-able. But its field application remains limited due to lack of effective hyperaccumulator plants, poorly understood mech-

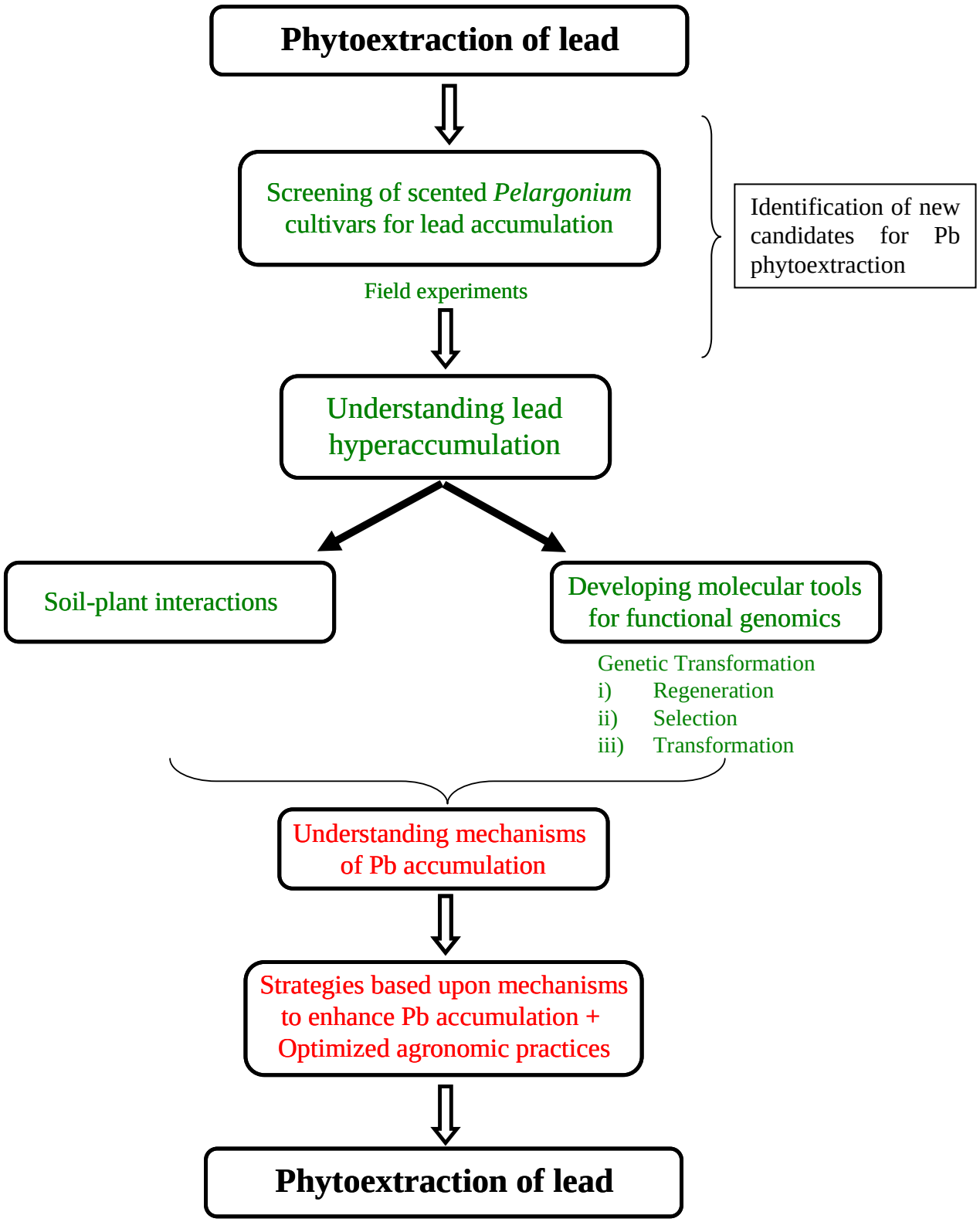

Figure 7: Schematic presentation of the research approach. The text in green colour indicates the objectives that were completed during this project. The portion in red colour denotes perspectives from this study.

-anisms and absence of respective agronomic practices. Identification of plants producing high biomass through rapid growth and accumulating elevated amounts of metals could help to reduce the time factor, a major limitation for plant based technology. If the plants of interest for remediation could help to overcome the costs e.g. energy production, essential oils etc, that would the ideal case. Another factor generally over-looked is the use of edible plants which could facilitate the entry of metals into food chain. So plants with least possibility of being eaten should be preferred for remediation purposes. We selected *Pelargonium* for phytoextraction purposes because they are high biomass producing plants. There is less threat of entry of the metal into food chain. Production of essential oil from the biomass could reduce the cost of remediation. Ultimately, the biomass could be used for energy production purposes. A schematic diagram of our research approach is given in Fig 7.

The objective of the present work was to assess scented *Pelargonium* cultivars for remediation of lead contaminated soils and subsequent developments of methods understand Pb accumulation. This work can be divided into two major parts. First part was concentrated on hyperaccumulation essays, phytoavailability and speciation of Pb. Second part was comprised of development of tools for understanding Pb hyperaccumulation. Stepwise specific objectives were:

i. Feasibility of lead phytoextraction by various *Pelargonium* cultivars in field conditions.
ii. Soil-plant transfer in relation to soil characteristics and genotype effects and Pb speciation in soil-plant system.
iii. Development of regeneration and transformation protocols for selected *Pelargonium* cultivars, a pre-requisite for gene function studies.

Chapter 2

Identification of lead hyperaccumulators

The project was started with collaboration of STCM, battery recycling industry. Six scented Pelargonium cultivars were grown on two Pb contaminated soils with contrasting characteristics, located in Bazoche and Toulouse, near STCM Plants. Six cultivars were grown in natural conditions without fertilization and irrigation for the feasibility assays of phytoextraction. The cultivars' performance was determined on the bases of Pb accumulation and the biomass produced. Further details and data are presented in the following publication.

2.1. *A field study of lead phytoextraction by various scented Pelargonium cultivars. Chemosphere 71:2187-2192.*

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.
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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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metal solutions, were capable of taking up large amounts of Pb over a 14 days treatment period while producing high biomass (Saxena et al., 1999). Most of the data available to-date regarding phytoremediation concerns hydroponics and pot experiments. In this context, field experiments on two contaminated contrasting soils were conducted to explore Pb phytoextraction capacity of six *Pelargonium* cultivars.

2. Materials and methods

2.1. Plant material

Six scented *Pelargonium* cultivars, propagated from cuttings of plantlets marketed by Heurtebise nursery, Clansayes, France, (http://www.pepinieres-heurtebise.com/), were grown in 0.5 l pots containing well-washed coarse Perlite, in the greenhouse, and regularly irrigated with nutrient solution containing 5000 µM KNO_3, 2000 µM $Ca(NO_3)_2$, 5000 µM KH_2PO_4, 1500 µM $MgSO_4$, 46 µM H_3BO_3, 9 µM $MnSO_4 \cdot H_2O$, 0.1 µM $MoNaO_4 \cdot 2H_2O$, 0.9 µM $CuSO_4 \cdot 5H_2O$, 15 µM $ZnSO_4 \cdot 7H_2O$ and 90 µM Fe–EDTA. The notations used in the present publication for the six scented *Pelargonium* cultivars are Attar, Atomic, Concolor, Clorinda, Rose, and Sweet, respectively, for *P. capitatum* cultivars Attar of Roses, Atomic Snowflake and Concolor Lace, *P. domesticum* cultivar Clorinda, *P. graveolens* cultivar Rose Geranium, and *Pelargonium sp.* cultivar Sweet Leaved.

2.2. Soil characteristics

Two top soils in the vicinity of active Pb recycling plants run by the company "Société de Traitement Chimique des Métaux" (STCM), contaminated by atmospheric fallout, were used for the field experiments. The first, (soil-B) is a calcic cambic soil, located near the factory of Bazoches in North-Western France, and surrounded by agricultural land. Lead contamination is present to a major extent, but other minor metal elements (Zn, Cu, Ni, As, Cr) have also been detected (Cecchi et al., 2008). The second, (soil-T) is an acidic soil, from urban Toulouse, located in South-Western France. Some physico-chemical characteristics of these two soils were determined by Lara Europe Analyses laboratory and are presented in Table 1. Soil-B is approximately 20 times less contaminated than soil-T, has a higher pH and is calcareous (2.1%). Organic matter (OM) concentration in soil-B is less than that in soil-T. Soil texture is also significantly different for the two soils.

2.3. Field experiments

The six *Pelargonium* cultivars were all field-tested in five replicates on both sites from May 2004 to July 2005. A density of 15 plants per m^2 was used on an experimental plot of 50 m^2 for soil-B and 10 m^2 for soil-T. The plants were grown under natural agro-climatic conditions, with neither fertilisation nor optimum irrigation so as to assess the feasibility of the remediation process in the natural context of an industrial site. In both cases, all above ground parts were trimmed off the plants after 150 days of growth i.e. in September 2004. Since *Pelargonium* does not tolerate temperatures below –6 °C, the plants grown in soil-B in North Western France, did not survive the frosts. Data for soil-B was from a single harvest. On the other hand, plants grown on soil-T under the warmer climate of South Western France, had two growing seasons. Shoots in soil-T were harvested 120 days after the onset of sprouting during the second season.

2.4. Shoot sample analyses

After harvest, the aerial parts of five replicated plants of each cultivar were weighed fresh. Lead adsorbed on to the leaf surfaces was determined according to the method as described by Ferrand et al. (2006). Shoots were then oven-dried at 80 °C for 48 h and dry weight determinations carried out. The dried plant material was ground to powder and 1 g of each sample was treated with a 1:1 acid mixture of trace metal grade HNO_3 and H_2O_2 at 80 °C for 4 h in a closed reactor for complete mineralization. Total Pb contents for the various *Pelargonium* samples were measured in solution by inductively coupled plasma optical emission spectrometry (ICP-OES), IRIS Intrepid II XDL/Thermo Electron Corporation. Standard reference materials were used to assess the accuracy of the acidic digestion procedure and measurements. Measurements on the reference samples (Virginia tobacco leaves, CTA-VTL-2, ICHTJ) demonstrated the validity of the assays: the concentration we found was 21.6 ± 1 mg kg^{-1}, for a certified value of 22.1 ± 1.2 mg kg^{-1}. Calculations were performed relative to shoot dry weight (DW).

2.5. Statistical analysis

The data obtained were subjected to analysis of variance (ANOVA) with two factors, using the software Statistica, Edition'98 (StatSoft Inc., Tulsa, OK, USA). For each bioassay, mean values with different letters represent a signif-

Table 1
Characteristic properties of the two soils

Soil	Clay (g kg^{-1})	Silt (g kg^{-1})	Sand (g kg^{-1})	pH_{H_2O}	N_{Total} (g kg^{-1})	P_2O_5[a] (mg kg^{-1})	Pb (mg kg^{-1})	OM
Soil-B	221	527	251	8.0	3.3	88	1830	5.0
Soil-T	112	337	550	6.0	3.8	95	39250	8.5

[a]Joret–Hebert method, OM = Organic matter.

icant difference ($p < 0.05$) as measured by the LSD Fisher test.

3. Results and discussion

3.1. Screening of Pelargonium cultivars for Pb accumulation potential

The *Pelargonium* cultivars grown on the two soils were screened for their Pb accumulation capacity. Under our experimental conditions, Pb adsorbed on *Pelargonium* leaves, as determined by a desorption procedure, was below the ICP-OES detection limit (0.05 mg Pb kg^{-1} DW) or represented less than 1% of the total shoot Pb. It was therefore ignored in all further calculations. The results presented in Table 2 indicate that there are significant differences in the total biomass in terms of dried matter of cultivars grown on contaminated calcareous soil-B as well as total Pb contents of different cultivars and lead quantity extracted per plant. According to Baker et al. (2000), shoot concentrations of Pb in hyper-accumulators should be in the range of 1000 mg kg^{-1} DW. The field experiments on soil-B showed the ability of Attar, Clorinda and Atomic *Pelargonium* cultivars to accumulate Pb in the same range. Schnoor (1997) reported that any plant useful for phytoremediation, should be vigorously growing, easily harvestable and exhibit a biomass of more than three tons DW ha^{-1} y^{-1}. Our results demonstrate that such is the case with the scented *Pelargonium* cultivars used in this study. On the basis of calculations, taking into account the planting density and number of cultures performed per year, the shoot dry weight produced was more than three tons DW ha^{-1} y^{-1} for all the *Pelargonium* cultivars and reached 45.3 tons ha^{-1} y^{-1} with Atomic cultivated on soil-T in 2005.

Comparison of Pb contents and extraction between Attar, Clorinda and Atomic *Pelargonium* cultivars grown on the two soils during 2004 is given in Fig. 1. Regardless of the cultivar, lead extraction was higher for *Pelargonium* plants, grown on soil-T. During the course of experiment, no morpho-phytotoxicity symptoms were observed on the *Pelargonium* cultivars cultivated on the two contaminated soils. The ability of the three *Pelargonium* cultivars to tolerate high concentrations of Pb on both calcareous and acidic soils satisfies the first prerequisite for use in phytoremediation. Taking high biomass into consideration, cultivars Attar, Clorinda and Atomic meet the two criteria to be qualified as Pb-hyperaccumulators.

Table 2
Biomass, total lead content in shoots and lead extracted per plant, for field cultures on soil-B

Cultivar	Shoot dry weight (DW) (g $plant^{-1}$)	[Pb] Shoots DW (mg Pb kg^{-1})	Pb extracted per plant (mg Pb $plant^{-1}$)
Attar	66 ± 13c	1467 ± 134c	96.7 ± 19b
Clorinda	56 ± 14c	1182 ± 336b	64 ± 15b
Atomic	28 ± 6ab	1107 ± 193b	30.5 ± 7b
Graveolens	39.5 ± 9.5b	435 ± 157a	17 ± 7a
Sweet	22.5 ± 6.5a	549 ± 76a	12 ± 1.5a
Concolor	16 ± 3a	468 ± 91a	8 ± 2.5a

For each bioassay, mean values with different letters are significantly different ($p < 0.05$) as measured by LSD Fisher test.

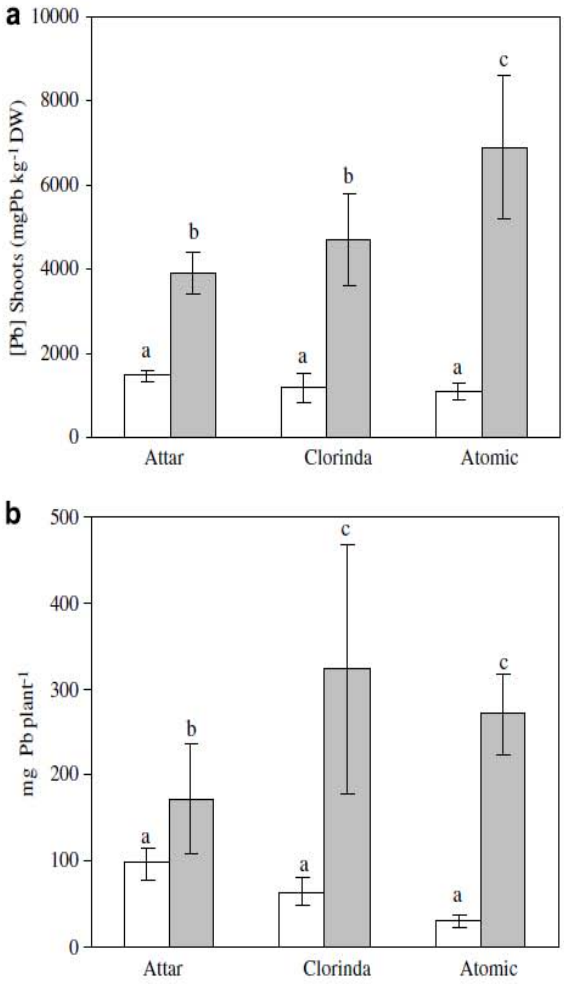

Fig. 1. Lead accumulation (2004) by three *Pelargonium* cultivars on two soils [B (□) and T (■)]. (a) Total Pb contents in shoots (mg Pb kg^{-1} DW). (b) Lead extracted per plant (mg Pb $plant^{-1}$).

3.2. Factors influencing the transfer of lead from soil to shoots

Data in Table 2 shows that the cultivar is determinant for the soil–plant transfer of Pb. The six cultivars grown on soil-B, could be divided into two groups for their Pb accumulation potential, the first one accumulating more than 1000 mg kg^{-1} DW (Attar, Clorinda and Atomic) and the second group (Rose, Sweet and Concolor) accumulating, on average, three times less. The differences observed in Pb accumulation between the various cultivars of *Pelargonium* can be accounted for by variations in the genetic background. Under similar pedo-climatic conditions, differences in the net Pb accumulation levels observed between the 6 cultivars could be the consequence

of complex signalling and molecular dialogues between biotic and abiotic components of the rhizosphere together with those of the root cells, which will together influence metal speciation, modify metal availability and translocation (Ma and Nomoto, 1996).

Under our conditions of field experimentation in soil-T, Pb concentration in aerial parts was often greater than 3000 mg Pb kg^{-1} DW. These values are close to those of Krishna Raj et al. (2000) where plants were treated daily with 7.55 mM $Pb(NO_3)_2$ solutions (corresponding to 1564 mg Pb l^{-1}) over a 14 day-period. According to Huang et al. (1997), Pb concentrations in the soil solution were usually less than 0.1% of total Pb in the solid phase of the soil; i.e. 1.8 and 39 mg l^{-1} for soil-B and soil-T, respectively. In the field, plants were able to accumulate for much longer times (in comparison with hydroponic conditions) but were exposed to much lower concentrations. *Pelargonium* cultivars could favour the transfer of Pb from the solid phase of contaminated soils to their soil-solutions.

The soil type significantly influenced Pb content (Fig. 1a) of *Pelargonium* cultivars and the quantity of lead extracted per plant (Fig. 1b). The transfer factor (TF): ratio of lead concentration in plant to soil, was calculated for the three cultivars on the two soils. TF for the less contaminated soil-B were higher compared to soil-T. Attar had maximum values of TF (0.8), followed by Clorinda (0.65) and Atomic (0.6) on soil-B. For soil-T, the values of TF were ordered: Atomic (0.18) > Clorinda (0.12) > Attar (0.1) and the values were similar for both growing seasons. For a given plant biomass and total metal concentration in the soil, the higher the value of TF, the greater the phytoremediation efficiency. As soil-T presented a very high lead concentration in comparison with soil-B, the ratios between the values of total shoot Pb contents measured for the two soils (soil-T/soil-B) were also calculated. These ratios were: 6.2, 4 and 2.7, for Atomic, Clorinda and Attar *Pelargonium* cultivars, respectively, i.e. greater Pb concentrations in the more acidic and more contaminated soil-T compared to that in soil-B. According to Acidic pH is reported to favour the solubility of Pb (Sanchez-Camazano et al., 1994). The three cultivars also differed in Pb extraction per plant (Fig. 1b) and the cultivar accumulating maximum Pb in both soils was not the same. Attar accumulated maximum Pb per plant on soil-B and Atomic, the minimum. On soil-T, Clorinda extracted of the most Pb per plant followed by Atomic and Attar, respectively. Plant responses to metals can differ strongly as complex interactions exist, particularly in the rhizosphere, between soil components and metals (Dumat et al., 2006).

Fig. 2 compares the capacity of Pb extraction per plant on soil-T, over two years (2004 and 2005). The efficiency of Pb extraction increased threefold during two growing seasons for Atomic and remained unchanged for Clorinda and Attar. This change could be the result of biomass variation. For Atomic, dry shoot biomass was 12.4 and 45.3 tons ha^{-1} y^{-1} DW in 2004 and 2005, respectively, while the shoot biomass of other two cultivars did not significantly differ between the two growing seasons (24 and 15 tons ha^{-1} y^{-1} DW, respectively for Clorinda and Attar).

3.3. Phytoremediation efficiency under field experimental conditions

Fig. 3 represents the estimated time to clean contaminated top soils (0–10 cm) by the three hyper-accumulator cultivars grown on soil-B in 2004 and, soil-T in 2004 and 2005. Only the top 10 cm were considered because the lead pollution was mainly found in this layer (Cecchi et al., 2008): at 30 cm depth the soil Pb concentration was reduced 10-fold. Lead availability to plants is controlled by its interactions with the soil components and speciation (Dumat et al., 2001 and 2006), the more labile metal fraction being more readily absorbed by the plant roots (Bermond et al., 2005). The metal extracting potential increases with increasing biomass as observed for Atomic in 2004 and 2005. Moreover, in the rhizosphere we postulate that *Pelargonium* is able to displace the solid-solution equilibrium and favour further absorption of lead. But only few kinetic data on phytoextraction of metals (Tongtavee et al., 2005) are available in the literature and none concern the absorption of lead by *Pelargonium*. We therefore estimated the time needed for total lead extraction using the equation described below.

Assuming that Pb phytoextraction follows a linear pattern, the quantity of Pb extracted per hectare and per year (Q_{Pb}: kg Pb ha^{-1} y^{-1}) can be expressed as

$$Q_{Pb} = (10^{-3} \times b_{DW} \times D) \times (10^{-6} \times [Pb]_{DW}) \times c \quad (1)$$

In this equation, b_{DW} corresponds to the dry weight of plant biomass per plant (g $plant^{-1}$ DW); D is the density of plants per hectare = 150,000 plants ha^{-1}; $[Pb]_{DW}$: total Pb concentration measured in shoots (mg Pb kg^{-1} DW); c

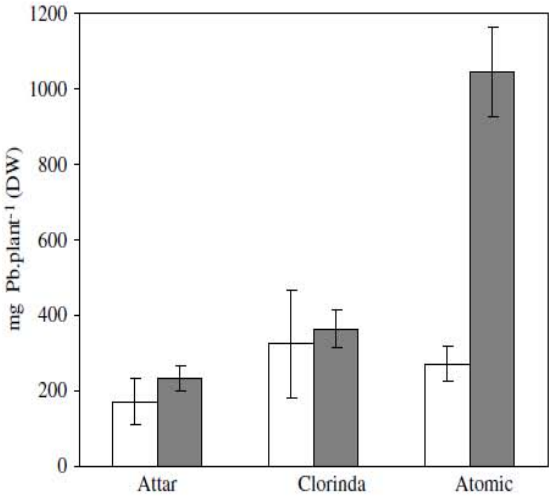

Fig. 2. Comparison of Pb extraction per plant (mg Pb $plant^{-1}$) on soil-T during two growing seasons. First season (□) from May to September 2004 and second (■) from April to July 2005.

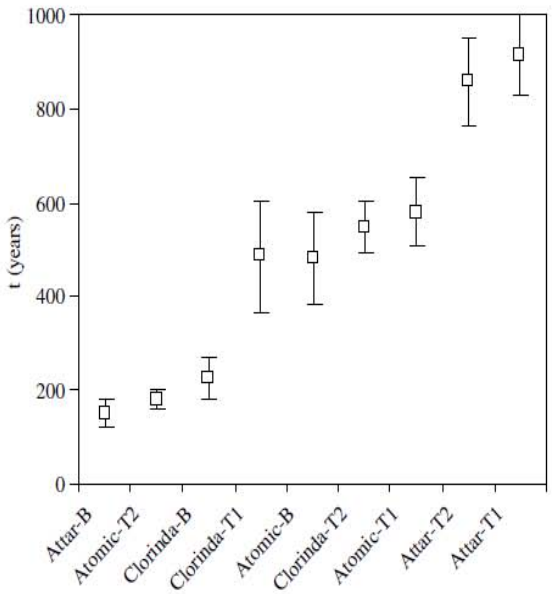

Fig. 3. Phytoremediation efficiency assessment of *Pelargonium* cultivars with respect to time required for cleaning contaminated soils (-B stands for culture on soil-B, -T1 and -T2 correspond to cultures on soil-T in 2004 and 2005, respectively).

is the number of crops per year: one for soil-B and two for soil-T. Using an average soil density of 1.2×10^3 kg m^{-3}, the weight corresponding to a top soil sheet of 1 m^2 surface would be 120 kg. Therefore, the quantities of Pb that correspond to one hectare of the contaminated top soils, termed S (total lead quantity in the top soil), are $S_B = 2.196$ tons and $S_T = 47.1$ tons, respectively, for soil-B and soil-T. The number of years necessary for the *Pelargonium* plants to perform total Pb extraction can be expressed by the following general equation:

$$t = S \times 10^3 / Q_{Pb} \quad (2)$$

The time estimated for total soil remediation ranges between 151 years for Attar grown on soil-B (minimum value observed) and 914 years for Attar on soil-T in 2004 (maximum value observed). The timing is site-specific and depends on future land use (Huang et al., 1997). However, *Pelargonium* cultivars could in any case reduce the available Pb fraction of contaminated soils and thus decrease the risk for the biosphere.

4. Conclusion

Our field experiments demonstrated the ability of several high biomass *Pelargonium* cultivars to hyper-accumulate Pb on both calcareous and acidic contaminated soils, without showing morpho-phytotoxicity symptoms. The estimated time scale for soil remediation with the *Pelargonium* plants was 151 years for soil-B with Attar and 182 years with Atomic for the soil-T. So *Pelargonium* cultivars could provide efficient remediation of moderately Pb contaminated soils.

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2.2. Perspectives

Three scented *Pelargonium* cultivars demonstrated their ability to hyper-accumulate Pb in field conditions and produced considerably high biomass as compared to other hyperaccumulators reported in literature. The cultivars' performance was different on contrasting soils and over different seasons. However, during this field experiment, many questions remained unanswered e.g. what are the factors controlling uptake of Pb by scented pelargonium studied, role of soil pH, soil Pb contents, soil organic matter and carbonates amounts, cultivars' ability to modify rhizosphere, translocation and speciation etc..? Due to the complexity and labour intensity of field experimentations, we opted for controlled conditions assays in the greenhouse and growth chamber to understand different factors controlling soil-plant transfer of Pb at rhizosphere level.

For this purpose a special cropping device (Fig 8) for rhizosphere studies (Neibes et al. 1993; Guivarch et al. 1999; Chaignon and Hinsinger 2003; Uzu et al. 2009) was used.

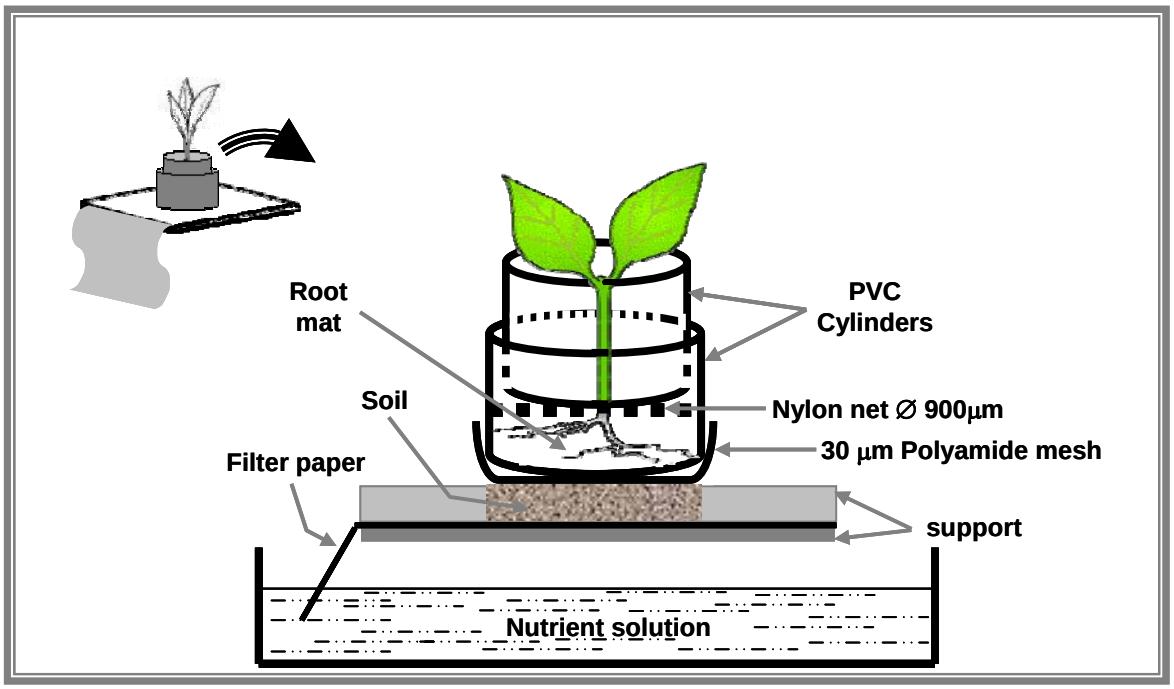

Figure 8: Cropping device used for rhizosphere experiments.

The device facilitates the separation of the roots from the soil at harvest. A small PVC cylinder is enclosed by a nylon net (diameter 900 µm) inserted into a larger cylinder, itself enclosed by a finer polyamide mesh (30 µm, Fyltis/Nytel, Sefar filtration). A space of 7-8 mm was left between the net and the finer mesh, where the roots could develop as a mat

named ‘root mat’. The soil (10 g) in round bottom plates (60 mm in diameter) is placed in contact with root mat through polyamide mesh. The soil is humidified by a filter paper present beneath the soil and dipped in nutrient solution on the other end.

On basis of field experiments, a Pb hyperaccumulator (Attar of Roses) and a non-accumulator (Concolor Lace) cultivars were selected to clearly distinguish plant effects for rhizosphere experiments. Two soils with contrasting characteristics were spiked with industrial particles in order to obtain three different levels of Pb i.e. control (0), ~ 500 and 1500 mg Pb kg^{-1} soil. These levels were selected on the basis of the fact that the field application of phytoextraction technique is potentially suitable to remediate moderately contaminated soils i.e. ~2000 mg Pb kg^{-1} as compared to highly contaminated soils i.e. ~40,000 mg Pb kg^{-1}. At higher concentrations, its applicability is hampered due to time required for phytoextraction. The detailed methodology and subsequent results are presented in the Chapter 3.

Chapter 3

Lead phytoavailability and speciation

In the previous study, the plants used were bought from Heurtebise nursery, Clansayes, France, (http://www.pepinieres-heurtebise.com/) and tested in field conditions for Pb phytoextraction experiments. For the subsequent experiments to understand Pb hyperaccumulation in soil plant system, the first step was to develop a method of producing rooted plants through cuttings from the stock plants, to avoid heterogeneity, in case we might have bought new plants from the nursery. For this purpose, different media were tested (Table 6). The experiments were repeated many times to have a reliable procedure for developing rooted plants, to be used for Pb phytoextraction essays at the Lab. The results presented in the table 3.1, are from two selected repeats which were better performing, after 30 day period. We started with hydroponics and then moved gradually to other substrates perlite, acid washed inert sand, organic soil, which are being used for potted plants. In hydroponics, we observed that with increasing Fe concentration, the percentage of rooted plants increased significantly (Table 6).

Table 6: Rooted plants (%) obtained from scented *Pelargonium* cuttings after 30 days culture on different media/substrates.

Medium	Additional treatment	Rooted plants (%)	
		Attar	Atomic
Hydroponics	5 mg L^{-1} Fe	26 ± 4^{e}	53 ± 5^{c}
	10 mg L^{-1} Fe	78 ± 7^{b}	75 ± 10^{b}
Perlite + Inert sand (1:1)	Hormone -	0	0
	*Hormone +	0	0
Perlite + Organic Soil (1:1)		38 ± 7^{d}	50 ± 6^{cd}
Organic soil		45 ± 8^{cd}	57 ± 4^{c}
Fertis®		100 ± 0^{a}	100 ± 0^{a}

More than 15 cuttings were grown each time. Results presented are from two replicates and different small alphabets show that the results are significantly different ($P<0.05$), measured by LSD Fisher test.

*Hormone de bouturage = 0.25% B-indole butyric acid (hormone being used in nursery for favouring root development).

Perlite-sand mixture was successfully used by KrishnaRaj et al. (1997) for Pelargonium sp. “Frensham”. However, in our case, it did not work and unexpectedly, no rooted formation in the presence of rooting hormones (hormone de bouturage), being used

regularly in commercial nursery. Then we tested the mixture of perlite and organic soil (25% OM) and, organic soil for development of rooted plants prior to Pb phytoextraction essays. The rooting efficiency was about 50%. All these rooting media tested could not provide a reliable procedure for having rooted plants as there were fluctuations in the efficiency each time (data not shown). Finally, we used Fertis®, which is low nutrient solid substrate. With Fertis®, the rooting efficiency was 100% and there was no cultivar dependency. So we used Fertis®, for having rooted plants, needed for experimentations in greenhouse and growth chamber (Phytotron). Detailed study focusing on Pb phytoavailability and its speciation in soil-plant system is presented in the following publication.

3.1 *Phytoextraction of lead by scented Pelargonium cultivars: availability and speciation. Submitted to Chemosphere.*

Phytoextraction of lead by scented *Pelargonium* cultivars: availability and speciation

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Abstract

Phytoavailability is one of the major factors to be considered for successful application of lead (Pb) phytoextraction. An experiment was performed using special cropping device, designed for rhizosphere studies, to estimate the available fraction of soil Pb, rhizosphere changes and Pb speciation in soil as well as in plant. Two contrasting scented *Pelargonium* cultivars were grown on two different soils and the soil-plant contact duration was two weeks. Both soils were spiked with characterized Pb particles emitted from recycling industry to achieve different levels i.e. 0, 500 and 1500 mg Pb kg^{-1} soil. Lead hyperaccumulator cultivar, Attar of Roses, significantly acidified its rhizosphere as compared to non-accumulator cultivar Concolor Lace. Dissolved organic carbon concentration in the rhizosphere was significantly higher for Attar of Roses as compared to Concolor Lace. Lead concentrations in the both cultivars were best correlated with $CaCl_2$ extracted Pb. Speciation studies with EXAFS and ESEM-EDS revealed that major part of Pb in soil was inorganic (mainly $PbSO_4$) whereas it was first complexed with organic acids and secondly associated with phosphorus within plant tissues.

Key words: Phytoremediation, rhizosphere, phytoavailability, speciation, pH, DOC.

1. Introduction

Lead contamination poses serious risks to human health causing irreversible disorders of nervous, digestive and reproductive systems, or anemia (Ahamed and Siddiqui, 2007) and, to plant vitality and metabolism (Chaney et al., 2007). High concentrations of Pb in the environment is the result of various anthropogenic activities (Sharma and Dubey, 2005; Dumat et al., 2001) and persistence of Pb. Half-life for Pb has been reported about 740–5900 years depending upon soil physical and chemical properties (Alloway and Ayres, 1993). Its mobility and bioavailability with respect to the chances of entering into food chain are determinant factors for environmental risk assessments (Zehl and Einax, 2005). Lead intake by humans can be due to the consumption of crop plants grown on contaminated soils, ingestion of contaminated soil, inhalation of soil particles and drinking water with high soluble concentrations (Alexander et al., 2006). In this context, Pb was recently classified as a substance of very high concern in the European REACH law (European Parliament Regulation EC 1907/ 2006 and the Council for Registration, Evaluation, Authorization and Restriction of Chemicals, 18 December 2006). Therefore, its uses might be strongly justified by industries in terms of cost-benefits analysis. Nowadays, battery manufacturing is the principal use of lead and the batteries represent 70% of the raw material for lead recycling industries reaching 160,000 t per year in France (Cecchi et al., 2008; Uzu et al., 2009).

Cleaning up of contaminated soils without disturbing natural equilibrium or producing new problems is an important environmental challenge. *Ex-situ* decontamination using physico-chemical techniques is labour intensive, expensive, and could affect biological properties of the soil. *In situ* decontamination by phytoextraction could be used as an alternative (Tanhan et al., 2007) but the time required to decontaminate remains a limiting factor (Keller et al., 2005). Low availability and mobility of Pb in soils could hamper the field application of the phytoextraction by augmenting the time period (Clemens, 2006) as compared to other elements like zinc (Dumat et al., 2006). It can however migrate through the soil with dissolved organic matter (Cecchi et al., 2008) or be mobilized by Pb hyperaccumulating plants (Arshad et al., 2008).

Phytoavailability of Pb is generally higher at low pH in the rhizosphere (Su et al., 2004). Depending upon plant species, root exudates can play a significant role in the bioavailability and transport of nutrients and contaminants (Lagier et al., 2000). The exudates compete for free metal ions to form soluble complexes and reduce metal

adsorption onto soil surfaces (Antoniadis and Alloway, 2002), and the resulting complexes could be taken up by the plant roots (Evangelou et al., 2004). The release of exudates from roots could alter dissolved organic carbon (DOC) contents of the rhizosphere that ultimately can influence metal uptake. However, the nature and amount of the organic acids excreted could strongly affect the mobility, bioavailability, degradation and phyto-toxicity of different metals (Lagier et al., 2000; Khan et al., 2006). According to Titeux and Delvaux (2009), larger mineralization and higher DOC release in the soil could promote metal release from exchange sites. Other factors including plant genotype, climatic conditions and agronomic management practices may influence the phytoavailability of heavy metals (Kabata-Pendias and Pendias, 2001).

Availability and transport from soil to root cells are only the first steps in metal accumulation. Effective translocation from roots to shoots needs symplastic pathway and active loading into the xylem (Clemens, 2006; Verbruggen et al., 2009). The complexation of metals with ligands in the xylem remains controversial. In xylem sap of *Arabidopsis halleri*, the dominant form of Cd was free Cd^{2+} ions (Ueno et al., 2008). According to Salt et al. (1999), the major proportion of Zn in the xylem sap of *Thlaspi caerulescens* was the free hydrated Zn^{2+} ions whereas Straczek et al. (2008) have observed Zn-organic acids complexes. In hyperaccumulators, nickel was transported in a complex form associated with histidine (Kramer et al., 1996). In the recent past, considerable advancements have been reported in understanding accumulation and translocation of Zn, Ni and Cd whereas very less is known about Pb in plant tissue.

In this context, the first objective of the present study was to evaluate the phytoavailability of Pb for scented *Pelargonium* cultivars on two contrasting soils by determining plant uptake, $CaCl_2$ exchangeable Pb, rhizosphere pH changes and DOC. The second objective was to observe Pb speciation in plant tissue using Extended X-ray Absorption Fine Structure (EXAFS-peeler) and Environmental Scanning Electron Microscopy─Energy Dispersive x-ray Spectroscopy (ESEM-EDS). EXAFS is well suited technique for Pb speciation assessments in plant samples because it is an element-specific probe sensitive to short range roder (Salt et al., 2002). It has been recently used by Straczek et al. (2008) for Zn speciation studies in tobacco roots. ESEM has become a powerful tool to study biological tissues as there is no need for special preparations as was required for high vacuum SEM.

2. Materials and methods

2.1 Soil material and spiking with enriched lead particles

Two uncontaminated top soils (0-30 cm) having contrasting characteristics (Table 1) were collected from vicinity of Toulouse (south-west of France), air-dried and sieved to 2 mm. These were named as Soil 1 (calcareous clay) and Soil 2 (sandy clay loam with acidic pH and low OM as compared to soil 1). These soils were spiked with well characterized Pb particles (Uzu et al., 2009) emitted from a secondary Pb smelter which currently recycles batteries, the Society for Chemical Treatment of Metals (STCM) located in the urban area of Toulouse (43°38′ 12″ N, 01°_25′34″ E). These particles contained 333,777 mg Pb kg^{-1} DW. Calculated amounts of the particles were added to the boxes containing 10 g of soil, to achieve final concentrations 0 (control), 500 and 1500 mg kg^{-1} of Pb and were thoroughly mixed as described by Uzu et al. (2009). These levels were selected on the basis of previous field experiments where different cultivars showed potential to remediate moderately contaminated soils i.e. ~2000 mg Pb kg^{-1} as compared to highly contaminated soils i.e. ~40,000 mg Pb kg^{-1} (Arshad et al., 2008). After spiking, the soils were turned over weekly for a month before using for culture experiments. This period allows establishing natural equilibration of the various sorption mechanisms in the soil (Alexander et al., 2006).

2.2 Plant material and preparations for rhizosphere experiments

We selected two cultivars of *Pelargonium capitatum*; Attar of Roses and Concolor Lace on basis of their performance in the field experiments (Arshad et al., 2008). Attar of Roses is a Pb hyperaccumulator while Concolor is a non-accumulator cultivar. This contrast could help to explain plant effects on phytoavailability of Pb. Potted plants of both cultivars were obtained from the Heurtebise nursery, Clansayes, France, (http://www.pepinieres-heurtebise.com/) and were grown in the greenhouse. The scented *Pelargonium* cultivars Attar of Roses and Concolor Lace will be referred to as Attar and Concolor, hereafter.

Cuttings (12-15 cm in length) of both cultivars taken from the greenhouse grown plants were grown on Fertis®, a low nutrient substrate that favors root development of the cuttings, for four weeks to develop rooted plants and were irrigated regularly with tap water. After 4 weeks, the rooted plants were transferred to special cropping devices designed for rhizosphere studies (Neibes et al., 1993; Guivarch et al. 1999; Chaignon and

Hinsinger, 2003; Uzu et al., 2009). These devices facilitate the separation of the roots from the soil at harvest. The eight cropping devices containing one plant/device were placed in containers having 20 L of nutrient solution to promote root growth and develop sufficient volume of roots for soil-plant contact. The composition of nutrient solution was 5000 μM KNO_3, 5000 μM $Ca(NO_3)_2$, 2000 μM KH_2PO_4, 1500 μM $MgSO_4$, 46 μM H_3BO_3, 9 μM $MnSO_4.H_2O$, 0.1 μM $MoNaO_4.2H_2O$, 0.9 μM $CuSO_4.5H_2O$, 15 μM $ZnSO_4.7H_2O$ and 180 μM Fe–EDTA. After two weeks of culture in the hydroponics, root mat was developed and plants were ready for rhizosphere studies. The level of nutrient solution was maintained with distilled water. The step was performed in a greenhouse with a photoperiod of 14 h, temperature 25 ± 1/22 ± 1 °C day/night cycles with 60-70% relative humidity.

Table 1: Characteristic analysis of the soils used for rhizosphere experiments

Characteristic	Unit	Soil 1	Soil 2
Clay	g kg^{-1}	421	228
Silt	g kg^{-1}	343	265
Sand	g kg^{-1}	236	507
Textural class		Clay	Sandy clay loam
pH water		8.22	6.41
pH $CaCl_2$		7.41	5.39
Organic carbon	g kg^{-1}	18.3	5.86
Organic matter	g kg^{-1}	31.7	10.1
$CaCO_3$ total	g kg^{-1}	268	<1
N total	g kg^{-1}	1.97	0.72
C/N		9.28	8.09
CEC cobalthexamine	$cmol_{(+)}$ kg^{-1}	30	10.7
Pb Aqua Regia	mg kg^{-1}	17.7	17.1

2.3 Culture of plants on soil

Ten grams of control and spiked soils were added to detachable round plates of cropping devices having 60 mm internal diameter and resulting into 3 mm thick soil layer. At the same time, each soil was sampled to determine the initial pH and for chemical analysis. During culture stage, containers having 4 L of nutrient solutions were covered with aluminum paper and 5 plants were placed on the top and the soils were humidified through filter paper dipped in the nutrient solution. During this stage, the concentrations of KNO_3, $Ca(NO_3)_2$, KH_2PO_4 and $MgSO_4$ were reduced to $1/10^{th}$ and, to half for iron of the

original nutrient concentrations, to favor metal accumulation (by reduction of ions' competition). The nutrient solution was changed weekly over a culture period of 2 weeks. While the levels in containers were maintained with nutrient solution sufficiently to keep the filter paper dipped in the solution. Soil controls that correspond to the soils without plants on the round plates were also maintained. These were humidified in a similar manner with the same nutrient solution as for culture to evaluate the pH changes influenced by nutrient solution during culture stage. The pH of nutrient solution was 5.5-6.0. From soil controls, pH changes were measured after 0, 4, 8, 12, and 15 days during the experiment by collecting a representative sample. After 2 week period, shoots and roots were harvested separately and whole the soil was considered as rhizosphere soil. There were five replicates for all the assays.

2.4 Soil and plant analysis

Fresh soils were taken for pH_{CaCl2} and $CaCl_2$ extracted Pb determinations. A variety of methods have been reported, either single extraction or sequential extraction (Reviewed in Wang et al., 2004). Soil pH (AFNOR, 1994) and Pb_{CaCl2} were measured from a 0.01M $CaCl_2$ (1:5 soil/solution on dry basis). The mixture was shaken for five minutes and left during 30 minutes to stabilize before pH determinations in the supernatant. After pH measurements, clear supernatants were collected for Pb determinations and stored at 4°C before analysis. For soil solution extraction, humid soil was taken in Eppendorf® tubes and centrifuged at RCF 15000 during 15 min at 20 °C temperature (modified from Angeles et al., 2006). The supernatants collected were considered as "soil solution", for estimation of pH, Pb contents and DOC. The DOC was determined using a Shimadzu 5000A TOC Analyzer.

The plant parts were freshly weighed at harvest after 15 days culture period. The roots were washed with 0.01 M HCl to determine Pb bound to the outer root cell walls, called $[Pb]_{adsorbed}$, according to the method described by Ferrand et al. (2006). The roots and shoots were then oven-dried at 80°C during 48 hours and weighed again for dry weight (DW). Dried plant material was ground to powder form and 125 mg of each sample was mineralized in a 5:1.5 mixture of HNO_3 and H_2O_2 at 80°C for 4h. After filtration, the elemental concentrations were determined with an IRIS Intrepid II XDL ICP-OES spectrophotometer. The accuracy of the acidic digestion and analytical procedures was verified using known reference material (Virginia tobacco leaves, CTA-VTL-2, ICHTJ).

2.5 Bio-concentration factor (BCF) and Translocation factor (TF)

TF is the ratio of metal concentration in plant's aerial parts to the metal concentration in plant's root (Marchiol et al., 2004), i.e. TF = $[C_{shoot}]$ / $[C_{root}]$. BCF is the ratio of metal concentration in plant tissues at harvest to the initial concentration of metals in external environment (Zayed et al., 1998). TF and BCF were calculated from the results obtained through chemical analysis.

2.6 Lead speciation through ESEM and EXAFS

Secondary and backscattering electronic images and X-ray elemental maps of roots and leaves were obtained using an ESEM working in low-vacuum mode. The Quanta 200 FEI instrument was equipped with an energy-dispersive X-ray (EDX) Quantax (Brucker) system. ESEM was operated at 25 kV. Roots were put on carbon substrates and analysed without further preparation. Because of the ESEM configuration, light element detection (C, N, and O) was ambiguous. ESEM-EDX analyses were performed at the "Geosysteme" laboratory (UMR CNRS 8157) of the University of Lille.

EXAFS spectra were recorded at the Pb L_{III}-edge (13.055 keV) at the French National Synchrotron Facility (SOLEIL, St Aubin, France) on the SAMBA beamline equipped with a Si(111) double crystal monochromator (Belin et al., 2005). The bulk spiked soil was dried, ground in an agate mortar and pressed as 5 mm-diameter pellets. Fresh roots of *Pelargonium* cultivated on Pb contaminated soil were plunged in liquid N_2, ground using a mortar immersed in liquid N_2, pressed as 5 mm-diameter pellet, and transferred in a cryostat cooled with N_2 by keeping the material in frozen state at all times. This is crucial to avoid changes of Pb speciation. Various Pb reference compounds were recorded, including $PbSO_4$, Pb oxysulfate, hydrocerrusite, pyromorphite, aqueous Pb-glutathione (15 mM $Pb(NO_3)_2$ + 15 mM GSH + 30 mM ascorbic acid, pH = 3), and aqueous Pb^{2+} (10 mM $Pb(NO_3)_2$, pH = 6.5). The solutions were mixed with 30% glycerol to avoid the formation of ice crystals. Spectra were recorded in fluorescent mode using a silicon drift detector (RONTEC) or in transmission depending on Pb concentration, at 77K for the roots and aqueous reference and at room temperature for soil, particles and inorganic references. The EXAFS interference function $\chi(k)$ was extracted using the ATHENA program, version 9, a part of the IFEFFIT package (Ravel and Newville, 2005). Sample spectra were fitted by linear combinations using the standard spectra mentioned above and other mineral and organic Pb references recorded previously (Manceau et al. 1996; Sarret et al. 1998a, 1998b) and Pb-sorbed ferrihydrite provided by A. Scheinost

(Scheinost et al., 2001). For a given compound, the spectrum recorded at 77K had only slightly higher amplitude than the one recorded at 300K, so we could combine both type of measurements in the linear combination fits. Each spectrum was first fitted with one component, and an additional component was allowed if the fit quality was improved significantly, *i.e.*, if the normalized sum squares residual parameter ($NSS = \sum[k^3 \chi(k)_{exp} - k^3 \chi(k)_{fit}]2 / \sum[k^3 \chi(k)_{exp}]^2 100$) was decreased by at least 10%. Using this procedure, the spectrum for the soil and roots were correctly simulated by four components. Satisfactory fits were defined by *NSS* increase within 5% of that for the best fit. Using this criterion, one and five good fits were obtained for the soil and roots, respectively. For the roots, average and standard deviation of the proportions of lead species were calculated on these good fits.

2.7 Statistical analysis

Statistical analyses were performed following the ANOVA procedure with the test of least significant difference (LSD) using STATISTICA software (Stat Soft, 2005).

3. Results

3.1 Lead accumulation

3.1.1 Total lead concentrations in shoots and roots

Several factors influence Pb concentration in the plant including genotype, total and available fraction of Pb in soil, type of soil, plant's ability to mobilize the soil Pb. Results showing Pb concentrations in plant parts of Attar and Concolor cultivars have been presented in Fig 1. Attar cultivar accumulated 284 ± 39 mg Pb kg^{-1} DW in shoots when cultured on soil 1 containing 1500 mg Pb kg^{-1} soil, whereas concentration in Concolor's shoots were significantly ($P<0.05$) lower i.e. 53 ± 4 mg Pb kg^{-1} DW (Fig 1A). With increasing levels of Pb in soil, Pb concentrations were increased significantly in both cultivars as compared to the control. Attar and Concolor cultivars gathered 3707 ± 696 and 1493 ± 123 mg Pb kg^{-1} DW, respectively when cultured on soil containing 1500 mg Pb kg^{-1} soil. Shoot Pb concentration for Attar cultivar was 201 ± 15 mg Pb kg^{-1} DW in shoots when grown on soil 2 (Fig 1B), with 1500 mg Pb kg^{-1} soil. Concolor cultivar had very low concentrations in shoots i.e. 37 ± 8 mg kg^{-1} DW. Similar trends were observed for roots with concentrations 10-28 times higher as compared to that of shoots. In roots, the maximum concentrations were 4042 ± 518 and 1905 ± 169 mg Pb kg^{-1} DW for Attar and

Concolor cultivars, respectively. Shoot Pb concentrations were 10-50 times lower in comparison to that of roots for both cultivars cultured on soil 2.

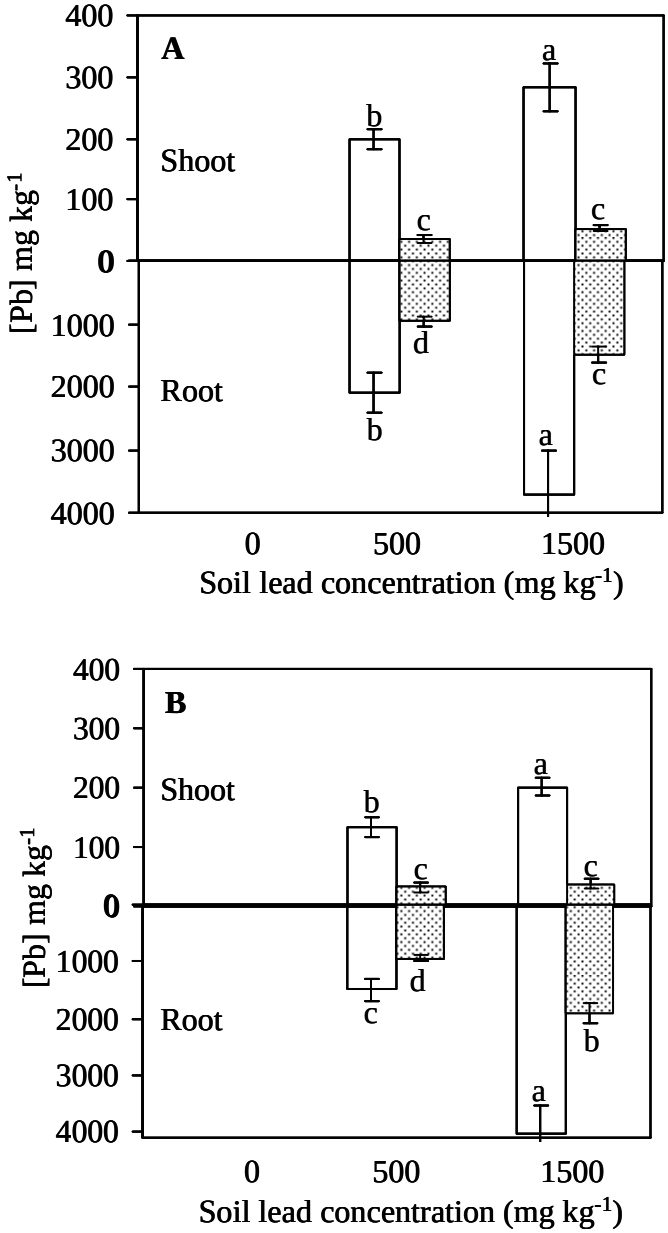

Figure 1: Lead concentrations in plant parts after two week culture on soils spiked with Pb-particles; A) culture on soil 1 and B) on soil 2. ☐ = Attar, ▨ = Concolor.

3.1.2 Lead uptake, translocation and bio-concentration

Lead uptake for both cultivars has been presented in Fig 2A. Total uptake was calculated from the concentrations and dry biomass using following formula;

$$\text{Pb uptake (mg)} = ([Pb_{shoot}] \times DW_{shoot}) + ([Pb_{root}] \times DW_{root}) \quad \text{[Eq 1]}$$

Lead uptake for Attar cultivar was 2.79 mg plant^{-1} when cultured on soil 1, whereas the maximum value for Concolor 0.5 mg plant^{-1} on the same soil and soil Pb level i.e. 1500 mg kg^{-1}. The uptake was decreased significantly on soil 2 as compared to soil 1 for Attar. However it did not differ significantly for Concolor cultivar on both soils.

Results regarding TF and BCF have been presented in table 2. TF values for Attar cultivar were higher at 500 mg kg^{-1} Pb as compared to 1500 mg kg^{-1} Pb irrespective of the soil type. TF values for Concolor were not changed (0.04) with increasing Pb concentration in soil 1. On soil 2, TF value at higher soil Pb concentration was slightly low (0.02) in comparison with low soil Pb (0.03). BCF was separately calculated for shoots (BCF_s and roots (BCF_r). BCF_s values for Attar cultivar were almost reduced to half when cultivated on soil 2 containing 1500 mg kg^{-1} Pb as compared to 500 mg kg^{-1} on both soils. Similar trends were observed for Concolor cultivar but BCF_s values ~5 times lower as compared to that for Attar cultivar. All the BCF_r values were higher than the BCF_s values for both cultivars. BCF_r values at 500 mg kg^{-1} soil Pb were higher than the values obtained at 1500 mg kg^{-1} soil Pb and were never reached two folds for both cultivars on both soils.

Table 2: Lead translocation and bio-concentration factors for *Pelargonium capitatum* cultivars.

Soil	Soil [Pb] mg kg^{-1}	Attar			Concolor		
		TF	BCF_s	BCF_r	TF	BCF_s	BCF_r
Soil 1	500	0.10	0.40	4.19	0.04	0.07	1.92
	1500	0.08	0.19	2.47	0.04	0.04	1.00
Soil 2	500	0.09	0.27	3.00	0.03	0.06	1.87
	1500	0.05	0.13	2.70	0.02	0.02	1.27

TF = Translocation factor; BCF_s & BCF_r = Bio-concentration factor (shoot & root).

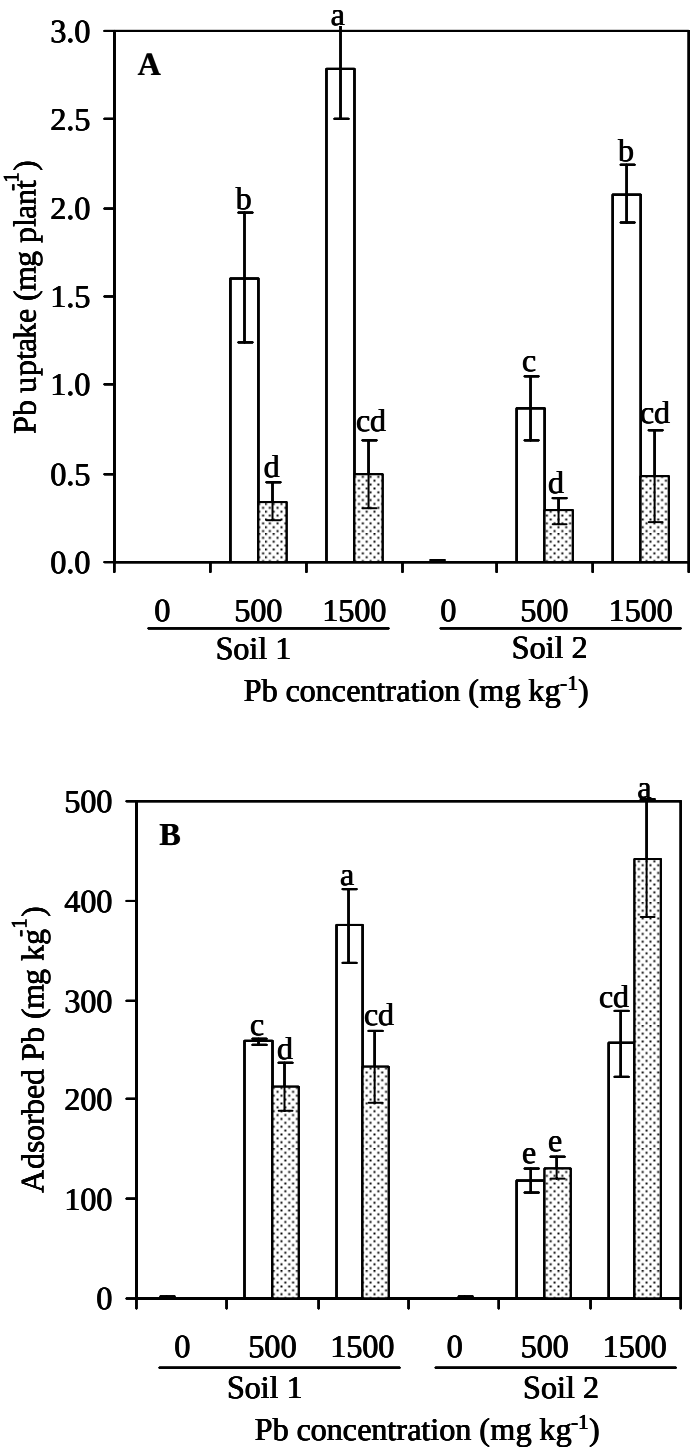

Figure 2: Lead absorption and adsorption by scented *Pelargonium* cultivars after two week soil-plant contact. A) Lead uptake, B) Adsorbed Pb. □ = Attar and ▨ = Concolor.

3.1.3 Lead adsorption

A part of total amount of metal taken up by plant may bind to cell wall substances (Greger and Johansson, 2004) but a major portion of Pb has been bound to the cell walls

(Wierbzicka, 1998; Uzu et al., 2009). Results presenting loosely bond Pb ($Pb_{adsorbed}$) to the root cell walls, extracted by washing with 0.01M HCl have been shown in Fig 2B. Considerable amounts of Pb were adsorbed to the root cell walls of both cultivars. Lead adsorption was significantly higher for Attar cultivar when cultured on soil 1 as compared to soil 2. The maximum adsorbed Pb concentration for Attar was 375 ± 37 µg g^{-1} DW_{root} on soil 1 containing 1500 mg Pb kg^{-1}. For Concolor cultivar, there was no difference in adsorption at 500 and 1500 mg kg^{-1} Pb cultivated on soil 1. Whereas on soil 2, $Pb_{adsorbed}$ was the highest i.e. 443 ± 59 µg g^{-1} DW_{root} for Concolor.

3.2 Plant induced rhizosphere changes

3.2.1 Soil pH

Three methods were used to measure soil pH: soil solution pH, pH $CaCl_2$ 0.01M (1:5 soil/liquid ratio) and pH H_2O (1:5). The results of pH changes have been presented in Fig 3, they can be interpreted in terms of ΔpH = (final pH - pH of the control soil (SC)---[Eq 2] Negative value of ΔpH will indicate acidification and vice versa. The pH of control soils for both soils was unaffected by the nutrient solution at the end of the experiment. Soil solution pH was changed up to -0.35 pH units by Attar cultivar on soil 1 whereas Concolor cultivar could not affect soil solution pH. In a similar manner, Attar cultivar acidified the soil and changed $pH_{soil\ solution}$ up to - 0.41 units of pH on soil 2 as compared to control soil without plant. Contrarily, Concolor cultivar alkalinised the soil resulting into + 0.7 $pH_{soil\ solution}$ units increase. The ΔpH were slightly different for pH_{H2O} as compared to $pH_{soil\ solution}$. The pH_{H2O} was decreased up -0.26 units by Attar cultivation on soil 1 while Concolor could not change the pH. On soil 2, the maximum ΔpH was -0.56 for Attar and +0.48 for Concolor cultivar. In case of pH_{CaCl2}, the maximum ΔpH -0.33 and -0.18 units for Attar and Concolor cultivars, respectively, cultivated on soil 1, as compared to control soil without plant. While the ΔpH on soil 2 were up to -0.61 and +0.45 for Attar and Concolor cultivars, respectively. The pH modifications observed for Attar cultivar were independent of the soil Pb contents. However, the pH changes induced by Concolor cultivar were dependent of soil Pb concentrations.

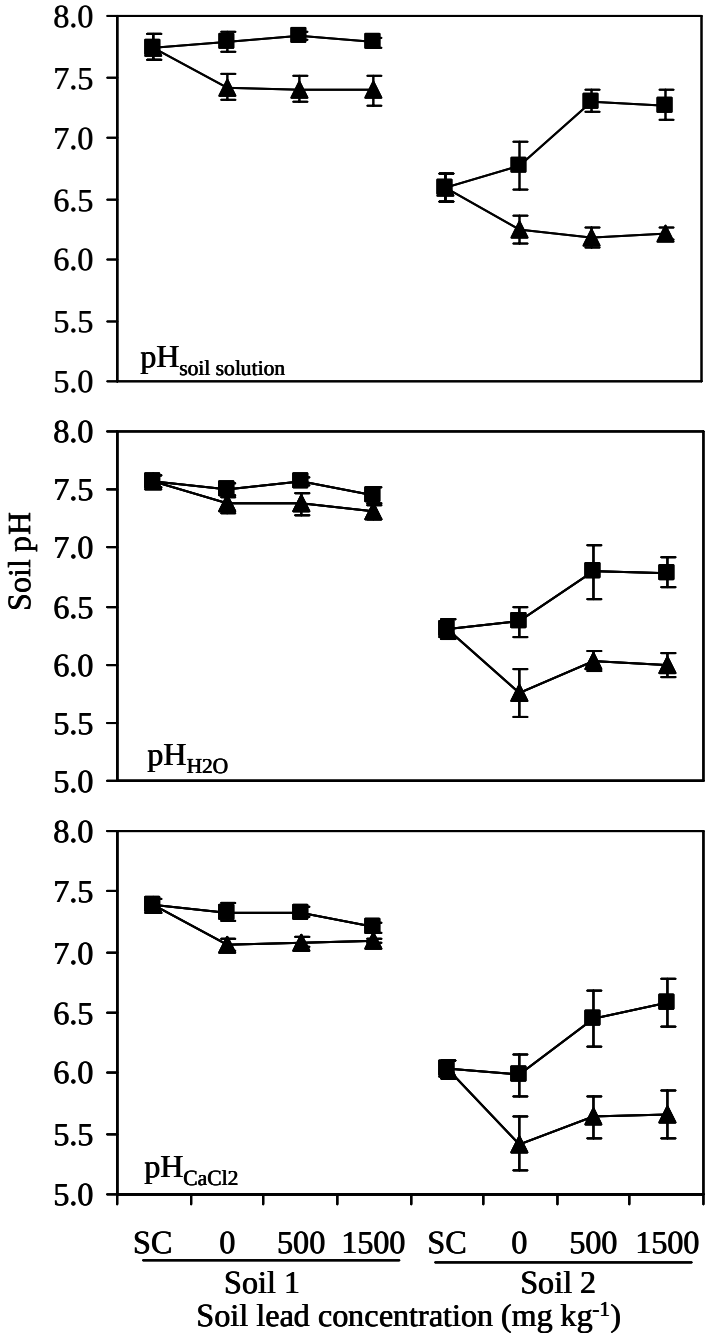

Figure 3: Effect of scented *Pelargonium* cultivars on rhizosphere pH. ▲ = Attar, ■ = Concolor, SC = control soil without plant.

3.2.2 Lead phytoavailable fractions in soils

To quantify the labile pool of nutrients/elements (readily available to the plants), various extraction methods have been used. Lead concentrations measured in soil solution, water and $CaCl_2$ extracted solutions to identify cultivar effects to mobilize soil Pb for uptake have been presented in Fig 4. The soil solution concentrations were higher for Attar

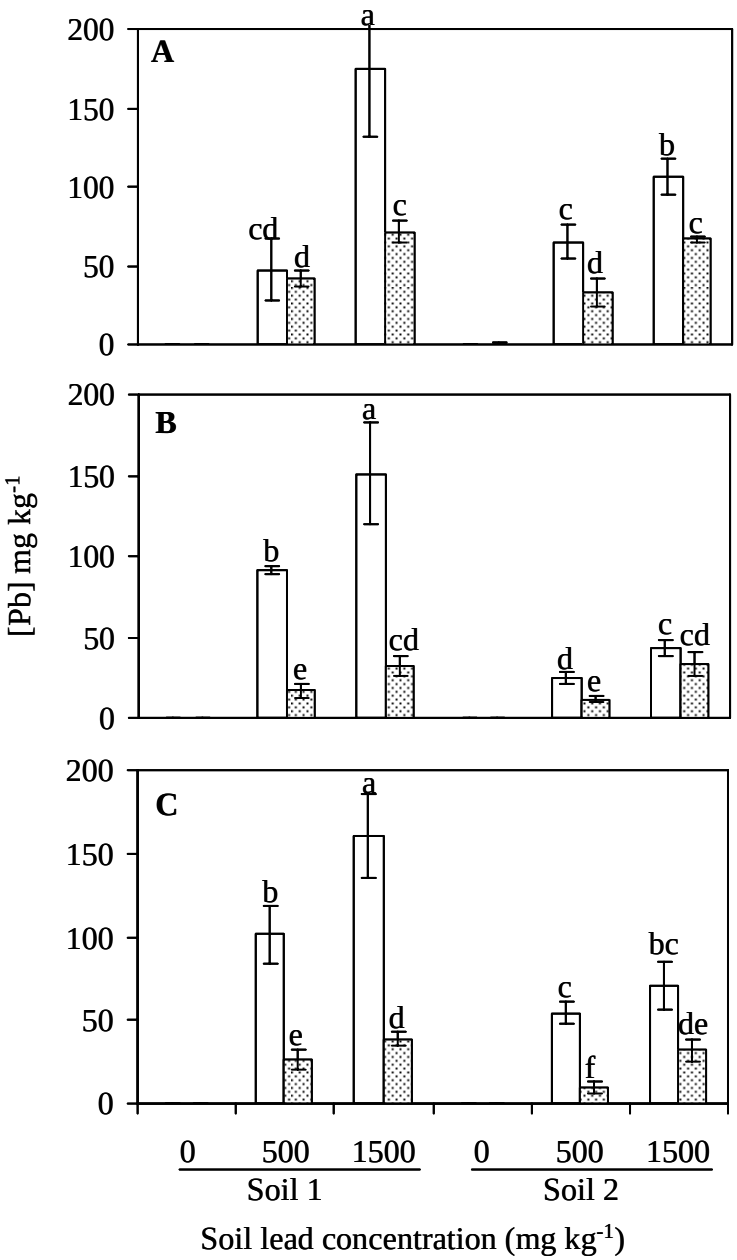

Figure 4: Effect of scented *Pelargonium* cultivars on plant available Pb contents in rhizosphere soil. A) $Pb_{soil\ solution}$, B) Pb_{H2O}, C) Pb_{CaCl2}. ☐ = Attar, ▨ = Concolor.

cultivar as compared to Concolor on both soils. The maximum $Pb_{soil\ solution}$ values for Attar were 175 ± 44 and 106 ± 11 mg kg^{-1} Pb, cultivated on soil 1 and soil 2, respectively. Whereas Concolor cultivar could mobilize to soil solution 71 ± 7 and 67 ± 2 Pb, mg kg^{-1} cultured on soil 1 and 2, respectively. Water extracted Pb concentrations were generally low as compared to soil solution except for Soil 1 in contact with Attar cultivar. The highest Pb_{H2O} concentration was 151 ± 32 mg kg^{-1} followed by 92 ± 3 mg kg^{-1} for Attar cultivar grown on soil 1 containing 1500 and 500 mg kg^{-1} of Pb, respectively. All other

values were below 50 mg kg^{-1} Pb in water extracted solutions. The trends were similar for $CaCl_2$ extracted Pb. The highest Pb_{CaCl2} concentration was 160 ± 25 mg kg^{-1} followed by 101 ± 17 mg kg^{-1} for Attar cultivar grown on soil 1 containing 1500 and 500 mg kg^{-1} of Pb, respectively. The Pb_{CaCl2} concentration reached 71 ± 14 mg kg^{-1} for Attar cultivar grown on soil 2 containing 1500 mg kg^{-1} of Pb whereas the values were below 40 mg kg^{-1} Pb for Concolor cultivar irrespective of the soil type and Pb contents.

3.2.3 Dissolved organic carbon

Plants provide a major contribution to DOC in soil through root turnover and exudation (Khalid et al., 2007). However organic matter decomposition may also contribute to the pool of DOC in soil solution (Buckingham et al., 2008). Results regarding DOC in soil solution have been presented in Fig 5.

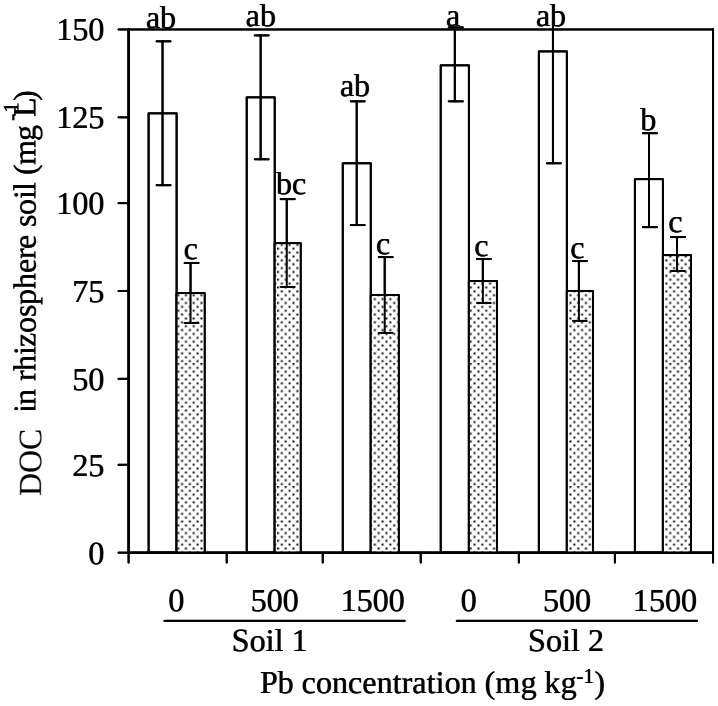

Figure 5: Dissolved organic carbon influence by scented Pelargonium cultivars during two week culture. Control soils without plant had 40 and 46 mg L^{-1} of DOC for soil 1 and 2, respectively. ☐ = Attar, ▨ = Concolor.

The base values of DOC measured on control soils without plant were 40 ± 4 and 46 ± 8 mg L^{-1} for soil 1 and 2, respectively. Significant increase in DOC concentrations was observed by plant culture on both soils. The maximum value 144 ± 32 mg L^{-1} of DOC in soil solution was recorded for Attar cultivar grown on soil 2 containing 500 mg kg^{-1} Pb.

The minimum value for Attar was 107 ± 13 on the same soil but at 1500 mg kg^{-1} Pb level. The values were significantly higher in the rhizosphere of Attar cultivar as compared to Concolor on both soils. The DOC value reached 89 ± 13 mg L^{-1} for Concolor cultivated on soil 1 at 500 mg kg^{-1} Pb and the lowest mean value was 74 ± 11 mg L^{-1}. Different concentrations of Pb in soil 1 could not affect the DOC concentrations in the soil solution of both cultivars. However, there was significant decrease in DOC value for Attar cultivated on soil 2 with 1500 mg kg^{-1} as compared to the control (0) with plant. But the DOC values for Concolor cultivar remained unchanged despite the different Pb levels in soil 2.

3.3 Lead speciation in soil-plant system

3.3.1 Environmental Scanning Electron Microscopy

Secondary and backscattering images and EDX spectra showing elemental composition were obtained from air-dried leaves and roots (Fig 6) of Attar cultivated on soil 1 having 1500 mg kg^{-1} Pb, during 2 weeks. In *Pelargonium* roots, the BSE images showed the precipitation of aligned solid particles having 10 µm length and 5 µm along the roots (Fig 6A). EDX analysis (Fig 6B) revealed that Pb, P and Cl are the major elements constituting such structure and can be attributed to the Pb-phosphate species (such as pyromorphites). Such precipitates are observed sparsely distributed along roots. Al, Si, Ca and Fe detected on the EDX spectra can be attributed to the soil particles which are concomitant to the Pb rich precipitates. Some fine particles (< 10µm) containing Pb with or without S were observed included within roots together with soil particles or scattered along roots (not shown). These particles can be attributed to Pb, PbO, $PbSO_4$ or PbS and probably originated from particle source as these species were identified in Pb source samples (Uzu et al., 2009). $PbSO_4$ and PbS are relatively soluble forms of Pb that could explain the low number of Pb rich particles observed within roots. Contrarily, there was no precipitation observed in leaves and Pb was evenly distributed.

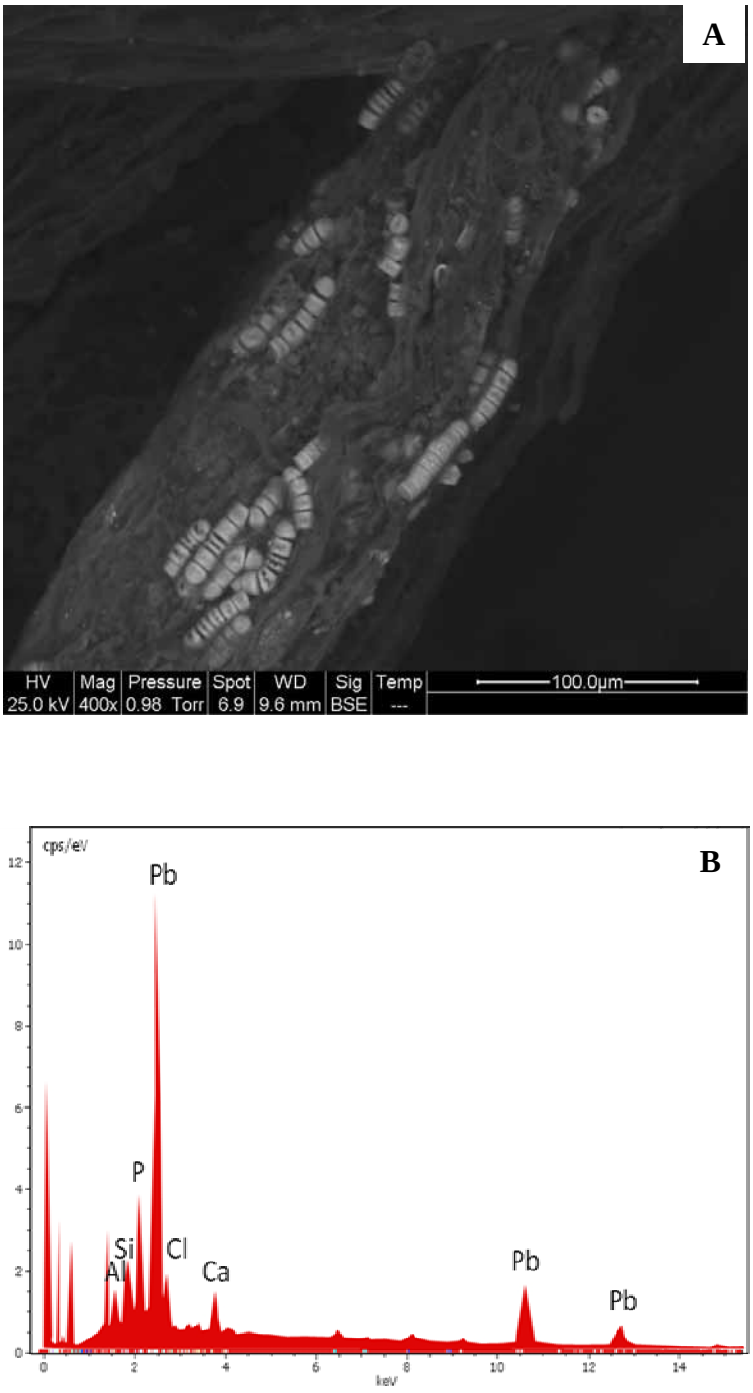

Figure 6. ESEM image and chemical analysis on the spectrum. A) BSE image of precipitates along roots. B) EDX spectra of precipitate performed by ESEM-EDX.

3.3.2 Extended X-ray Absorption Fine Structure (EXAFS) spectroscopy:

Figure 7 present EXAFS spectra of some reference compounds used in the linear combination fits and the spectra for the amended soil and for the roots with their fits. The EXAFS spectra for bulk soil and roots clearly differ by the shape of the second oscillation and position of the third and higher oscillations. Both spectra were correctly simulated by

four components. For the amended soil, the best fit was obtained with 30% $PbSO_4$ + 33% $PbO.PbSO_4$ + 47% αPbO + 12% Pb oxalate (NSS = 0.038). Other 4-component fits obtained were not satisfactory because NSS was increased by 10% and more, and did not reproduce correctly the shoulder on the second oscillation. Similarly, 3-component fits did not reproduce correctly the spectral features (NSS = 0.052), so the four Pb species including $PbSO_4$, $PbO.PbSO_4$, αPbO and organic Pb are likely present in the soil. The first three species have been identified in the original particles (Uzu et al., 2009). Organic Pb may result from the weathering of the particles and redistribution on the soil organic matter.

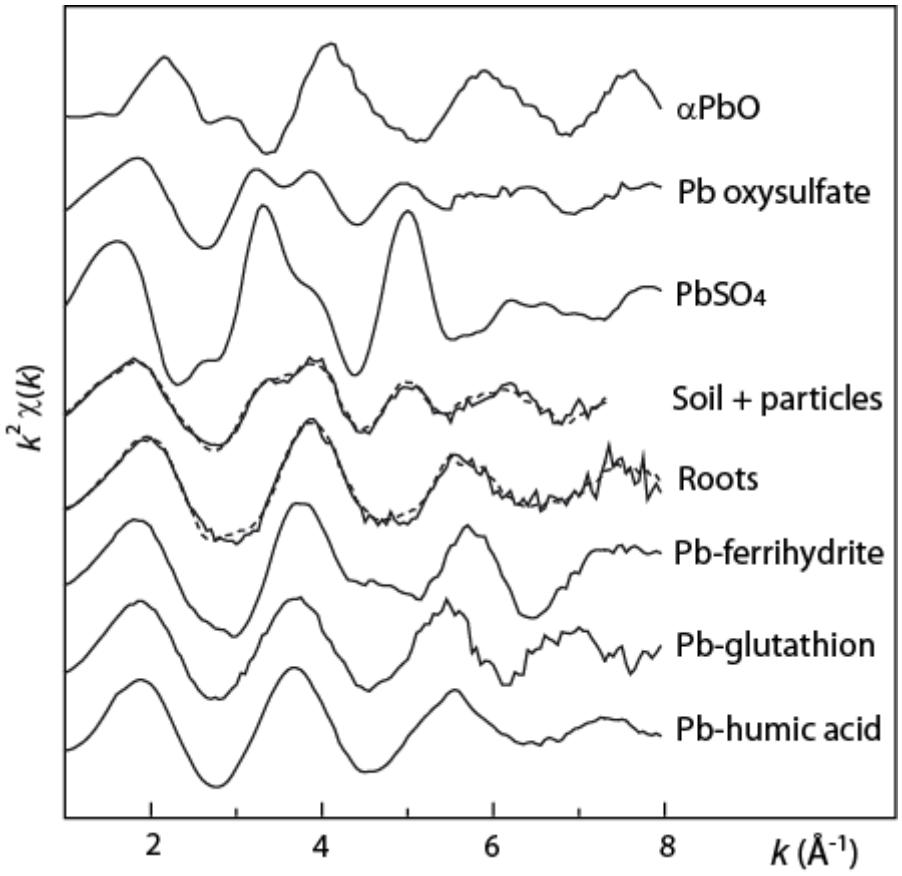

Figure 7: Pb L_{III}-edge EXAFS spectra for the bulk soil amended with Pb-containing particles, roots of scented *Pelargonium* cultivar Attar and representative Pb standard spectra used for the linear combination fits (dashed lines).

For the roots, five linear combinations were retained, and the following proportions were obtained: 52±5% αPbO + 33±4% Pb-sorbed ferrihydrite + 50±3% Pb-Thiols. In the five fits, Pb-Thiols were a combination of two references including Pb-glutathione and Pb-cysteine. If Pb-Thiols were excluded from the fit, NSS was increased by 25%. Therefore, the roots likely contain Pb bound to thiol groups. The sum of the three species is higher

than 100%. This may be due to a higher thermal disorder for the references recorded at 300K (αPbO and Pb-sorbed ferrihydrite) leading to a decreased amplitude compared to the root spectrum recorded at 77K, and/or to a higher structural disorder in the reference compounds. Pb-sorbed ferrihydrite may correspond to iron precipitates forming the root plaque as observed in aquatic plants (Hansel et al., 2001). No pyromorphite precipitates were detected in the roots by EXAFS, contrary to what was observed on sudax (Laperche et al., 1997). αPbO which was identified in the amended soil, likely correspond to mineral particles attached to the roots. Finally, Pb-Thiols may correspond to complexes present in the root cells, and result from a detoxification process involving S-containing ligands (cysteine, GSH, phytochelatins, metallothioneins).

4. Discussion

4.1 Phytoavailability of Pb in acidic and alkaline soils

Biomass (on dry weight bases) of scented *Pelargonium* cultivars Attar and Concolor cultivated on the two soils spiked with Pb particles was not affected with the presence or absence of Pb. Lead concentrations in plant parts and uptake were significantly higher for Attar as compared to Concolor cultivar (Fig 1 and 2). These trends are in good accordance with the field data presented by Arshad et al. (2008). During two weeks culture, shoot Pb concentration for hyperaccumulator cultivars, Attar, were reasonably high as compared to Hassan et al. (2008). They tested *Pelargonium zonale* for Pb extraction from artificially contaminated soil. The plants accumulated 54 mg kg^{-1} during 3 week pot cultures on soil containing 2000 mg kg^{-1} Pb. In our case, Pb_{shoot} concentration measured was 284 mg kg^{-1} only in two week soil-plant contact and the soil Pb level was 1500 mg kg^{-1}. Attar accumulated more that 134 mg Pb kg^{-1} DW giving whatever the Pb level or soil type (500 or 1500 mg kg^{-1} Pb).

Apart from Pb taken up by the plants, a considerable amount was adsorbed to root cell wall. The highest value of $Pb_{adsorbed}$ for Concolor (Fig 2B) cultivar also explained the low uptake and translocation (Table 2) in this cultivar compared to Attar. The adsorbed was calculated on % bases as follows;

$$\% Pb_{adsorbed} = [Pb_{adsorbed}]/ [Pb_{root}] \times 100 \quad \text{[Eq 3]}$$

The Pb adsorption was 6-12% for Attar whereas it was 14-23% for Concolor. The adsorption of Pb on to cell walls is an important phenomenon at soil-root interface. It strongly affects the translocation factor as Pb^{2+} binds to carboxyl groups at the root surface

(Seregin and Kozhevnikova, 2004) reducing absorbed quantities and rate of translocation to shoots (Pendergrass and Butcher, 2006; Piechalak et al., 2002).

The higher Pb accumulation for Attar in comparison with Concolor could be result of different factors. First, modification of rhizosphere pH: (1) Attar cultivar acidified the soil irrespective of the initial pH and soil Pb contents; (2) Concolor cultivar performed in a different way. Cultivated on soil 1, it either could not modify the pH or slightly acidified the soil whereas on soil 2, it alkalinized the soil (Fig 3). The purpose of applying different methods was to assess variations in soil pH and select the one method that could help to establish phytoavailability. The $CaCl_2$ (0.01M) is being considered as single extraction method the assessment of available fraction of nutrients and trace elements. However, soil solution pH and pH_{H2O} are good indicators of actual pH since no application of ions is involved, e.g. Ca^{2+} that could interfere with exchange sites. These pH modifications were exclusively resulted from plant action as only nutrient solution could not modify soil pH, either way, in soil controls maintained without plants. These results suggest that the plant could adjust its rhizosphere depending upon initial pH or exposure level. From the three methods used for pH determinations, the differences (acidification / alkalinization) in case of pH_{H2O} were lower as compared to soil solution and pH_{CaCl2}. So the Pb availability was not well predicted by pH_{H2O} which will be discussed later. Other factors including type of root exudates, mineral nutrition and genotype may play significant role in rhizosphere pH changes (Loosemore et al., 2003). Soil acidification generally favors mobilization rendering the ions available for plants (Hinsinger et al., 2003). Lin et al. (2004) and Kidd and Monterroso (2005) also observed that exchangeable Pb was much higher in the rhizosphere than in the bulk soil due to difference in pH.

Another parameter that could explain the differences in Pb uptake between the two cultivars is DOC amount in the rhizosphere. Comparatively higher amounts of DOC in the rhizosphere of Attar than that of Concolor cultivar (fig 5) might be the result of enhanced root exudation. DOC concentrations in soil were increase almost 3 times by Attar and 2 times by Concolor cultivar as compared to the controls without plant. The higher Pb contents in Attar could be due to high levels of DOC that directly indicate the presence of organic acids. Complementary analysis through ion chromatography of some samples for low weight organic acids (LWOAs) revealed that citrate and tartrate ions were the major LWOAs in the rhizosphere of *Pelargonium* cultivars (data not shown). These organic acids could increase Pb uptake (Wang et al., 2007). The significant increase in DOC and phenolics in soil solution as compared to unplanted control has been observed for 11 grass

species by Khalid et al. (2007). They have also reported enhanced biodegradability of DOC in the presence of plants that could potentially increase phytoavailability of elements in the rhizosphere. Titeux and Delvaux (2009) have reported that higher DOC release in the soil could promote metal release from exchange sites. The root exudates either in the form of DOC could affect metal speciation and behavior in the rhizosphere (Lin et al., 2004; Laperche et al., 1997; Welch 1995). This phenomenon has been particularly observed for calcareous soil by Chaignon and Hinsinger (2003). However slight decrease in DOC in the Rhizosphere of Attar cultivar cultivated on the soil containing 1500 mg kg^{-1} Pb might be the result of high Pb contents of the soil. The decrease of DOC in highly contaminated soil could result of reduction of organic matter mineralization in the soil as the phenomenon has been observed by Dumat et al. (2006) and Quenea et al. (2009).

The speciation of Pb in the soil might also influence its availability. Lead concentrations and uptake were generally higher on soil 1 than on soil 2. It might be explained as soil 1 is calcareous in nature containing 268 g kg^{-1} of $CaCO_3$, and the high contents of $CaCO_3$ may promote formation of Pb-CO_3 complexes that are comparatively soluble form of Pb in soil and can be taken up preferentially (Uzu et al., 2009). As pH influences metal solubility and transfer (Wang et al., 2006), the rhizosphere acidification could have displaced the equilibrium towards bicarbonates, which are less stable than carbonates (Sauvé et al., 1998).

4.2 Predicting phytoavailability

The total concentrations of metals in soils are not a good indicator of phytoavailable fraction of metals, due to complex chemical speciation of the metals in soil (Chen et al., 1996). We performed extractions for soil solution Pb, Pb_{H2O} and Pb_{CaCl2} measurements to predict Pb availability after two week culture on spiked soils. Shoot Pb concentrations were best correlated to $Pb_{CaCl2.}$

$$[Pb_{shoot}] = 0.0008 \times [Pb_{CaCl2}]^2 + 0.283 \times [Pb_{CaCl2}] + 5.51 \qquad (r^2 = 0.95) \qquad \text{[Eq 4]}$$

However, on soil 1, the Pb concentrations were independent of the method of extraction for Attar cultivar. Keeping in view the practical aspects, 0.01M $CaCl_2$ is a better option due to simplicity and possibility of pH measurements simultaneously. This extraction method has also been considered a suitable procedure for the determination of phytoavailable Pb (Pueyo et al., 2004; Uzu et al., 2009). Wang et al. (2004) have reported that the phytoavailability of trace elements was strongly correlated to extractable fraction by $CaCl_2$, total metal concentration in soils, soil pH, organic matter and cation exchange capacity.

4.3 Lead speciation in soil and plant

Several compounds with strongly different solubility and availability were observed with the two complementary techniques used (EXAFS and ESEM-EDS). The average Pb speciation through EXAFS revealed different forms in roots and the bulk contaminated soil. Conversion of Pb to organic species makes them less toxic, enhance plant's tolerance and increase translocation. Several studies have demonstrated an important role of organic acids in affecting plant tolerance to different elements (Reviewed in Wang et al., 2007). They have shown the binding of Pb(II) in wheat roots with carboxylic group in hydroponics through EXAFS. Zinc-organic acid complexing has been reported in tobacco roots by Straczek et al. (2008).

Because of the ESEM configuration, light element detection (C, N, and O) was ambiguous. However, that technique permitted to highlight minor inorganic lead species like phosphates and sulphates. Formation of pyromorphites in the roots of Pelargonium could help the plant to avoid from toxicity by the presence of Pb whereas other forms i.e. binding with sulfate could favor the translocation of Pb. However, formation of stable complexes could also help to increase the cell stock of P that is potentially useful to tackle toxicity effects of Pb in the cells. As experiments were performed only over two weeks, Pb-P complex might be there to handle sudden exposure to Pb and might be dissolved over the time increasing translocation of Pb in field for the long term experiments. The even distribution in the leaves may also help to decrease the extent of toxicity imposed by Pb. An increase in the concentration of phosphorus and sulphur has been observed in leaves of radish (Gopal and Rizvi, 2008).

5. Conclusion and perspectives

Two *Pelargonium capitatum* cultivars performed contrastingly to each other during two week soil-plant contact through special cropping devices. According to our results, higher Pb accumulation by Attar cultivar could be attributed to its potential to modify its rhizosphere by changing pH and DOC amounts. The increased concentration of DOC in the rhizosphere soil predicts the role of different organic acids. The BCFs and TFs were decreased with increasing soil Pb concentration. Lead concentrations in the both cultivars were best correlated with $CaCl_2$ extracted Pb. From the fits obtained by EXAFS, it can be concluded that Pb was bound to organic acids in roots. Its bonding with cysteine and GSH predicts the potential presence of Phytochelatins and Metallothioneins which play an

important role in metal accumulation and tolerance in plants. Lead phosphates were also observed with MEB-EDS. These low soluble compounds could be a storage form of Pb permitting to the plant to reduce Pb toxicity.

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3.2 Additional results

The particles used for soil spiking to obtain different levels of Pb in the rhizosphere experiments also contained 26900 mg kg^{-1} of Cd. Presence of Cd in particles resulted into 40 and 120 mg kg^{-1} Cd in soils containing 500 and 1500 mg kg^{-1} of Pb, respectively. So we also measured the concentrations of Cd in the shoots and root. The results are shown in Fig 4.2. Attar cultivar accumulated 48 and 62 mg Cd kg^{-1} in shoots when cultivated on soil 1 and 2, respectively (Fig 9a & b).

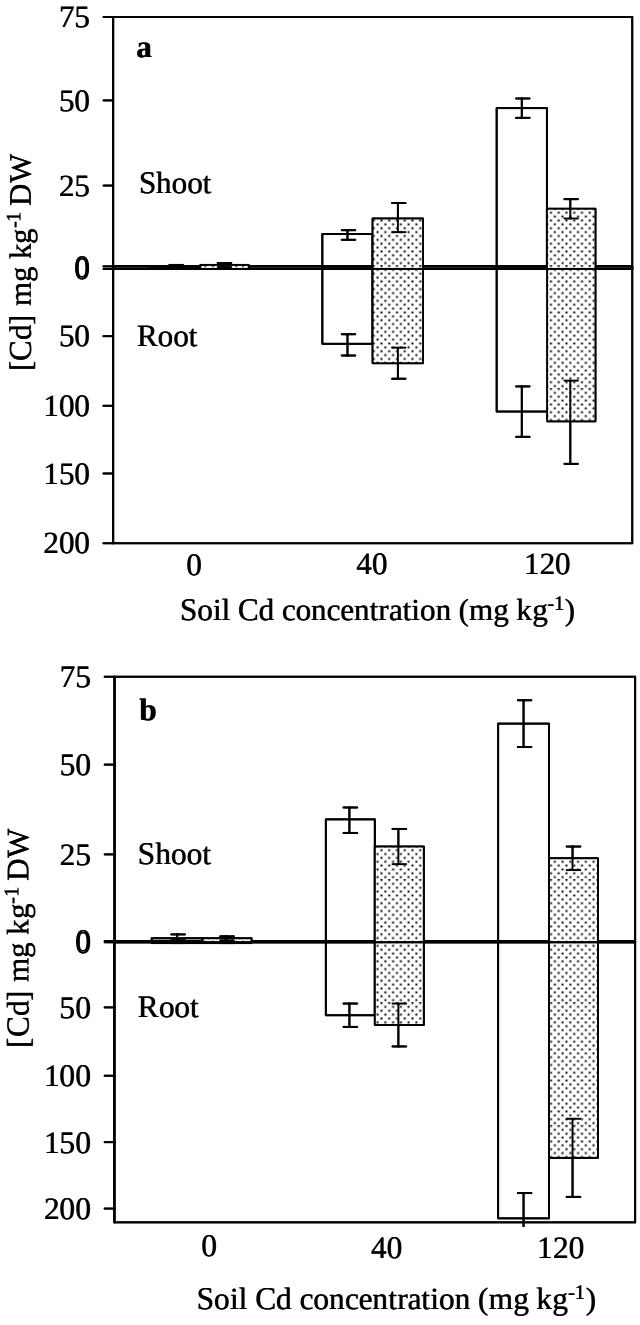

Figure 9: Cd concentrations in shoots and roots of scented *Pelargonium* cultivars. a) cultivated on soil 1, b) on soil 2. Attar (□), Concolor (▨)

The Cd concentrations in shoots for Attar cultivar were significantly high ($p<0.05$) as compared to that for Concolor at 120 mg Cd kg^{-1} soil. In general there was more accumulation on soil 1 as compared to soil 1, both in shoots and roots. There was no significant difference in root concentration of both cultivars on the same soil. However the root concentrations were almost two times for both cultivars when cultivated on soil 2 as compared to soil 1. This difference could be explained on the bases of soil pH. Acidic pH (soil 2) could favour the uptake of Cd in soil whereas Cd might be adsorbed on exchange site at alkaline pH (soil 1). According to (Kuo et al., 1985), an increase in pH results in increased adsorption of Cd to soil particles and reduces the uptake of Cd by plants. However, plants are capable of modifying phytoavailable pool of the elements in rhizosphere through exudation (Sterckemam et al. 2005).

These results showed the potential of scented Pelargonium cultivars for multielemnt accumulation. However, these results only gave an indication that these cultivars are capable of accumulating other metals. Detailed studies are needed to assess the possibility of multielement decontamination using scented *Pelargonium* cultivars.

3.3 General discussion

The results presented in Chapter 2 and in the first part of the current chapter, showed that Scented Pelargonium cultivars are capable of accumulation Pb without any symptoms of toxicity. Three *Pelargonium* cultivars; Attar, Atomic and Clorinda, accumulated Pb more than 1000 mg kg^{-1} DW or 0.1%, that is widely accepted concentration for the plants to be considered as hyperaccmulators. Moreover, the biomass was multifolds (up to 45 t ha y^{-1}) of 3 t ha y^{-1} (Schnoor, 1997) that the hyperaccumulator plants should have, at least. Potential of Attar cultivar for Cd accumulation makes it more attractive as most of the contaminated sites have multielement contaminations. Multielement accumulation is one of the most desired characteristics for plants to be used for phytoextraction purposes (Prasad and Freitas 2003). It is very promising to have plants with potential of multielement accumulation and high biomass but the identification of plants of this rare category might only be first step towards application of phytoextraction. Physiological parameters like availability, uptake, rhizosphere modifications, etc. could help to decide the feasibility of phytoextraction technique within given circumstances. But the major factor, limiting field application i.e. time could only be addressed with improved plant characteristics. Fig 10 shows a schematic diagram presenting possible options for the use of biotechnology for phytoextraction. Plants with improved accumulation can be obtained

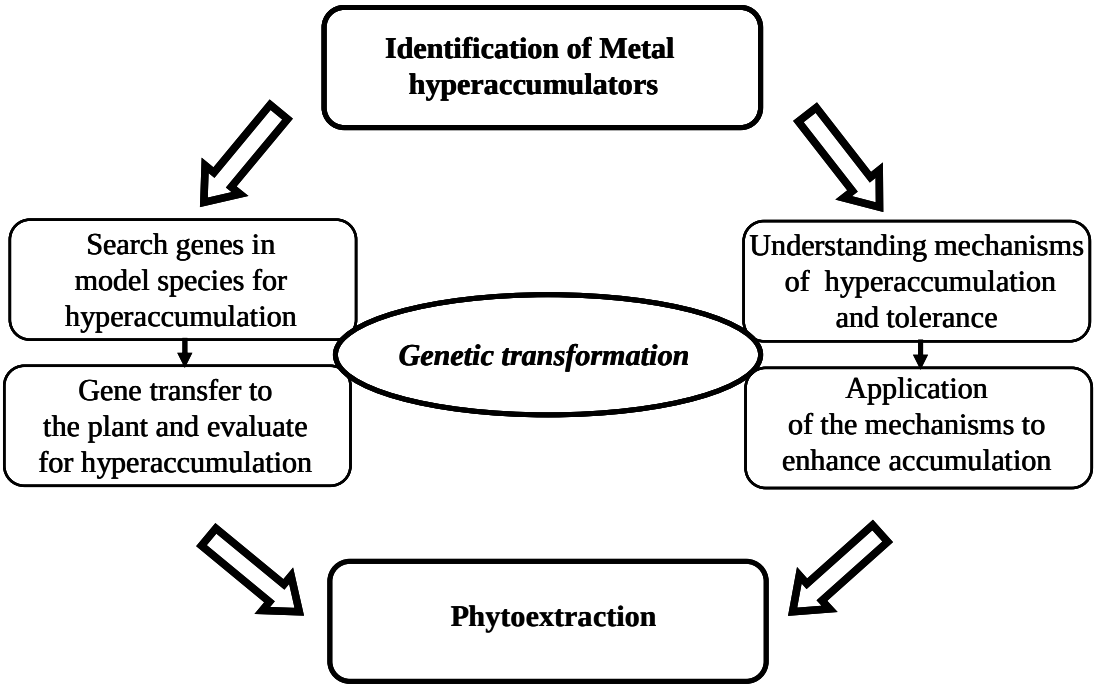

Figure 10: A schematic presentation of possible strategies for improved phytoextraction.

through gene transfer from model species to the target plants to be used for remediation. But for this option, biosafety remains a major concern for its field application. Other option could be the application of the mechanisms of hyperaccumulation, e.g. use of plant or bacterial metabolite to enhance accumulation. For both approaches, the knowledge of molecular mechanism and gene function is of critical importance. For which availability of tools is primary condition e.g. genetic transformation (Fig 10).

One of the major hindrances in plant genetic engineering is the fact that not all plant species are equally transformable. Even the availability of transformation procedure for a cultivar is not necessarily applicable to other cultivars of the same species. Genetic transformation systems are based on the introduction of foreign DNA into plant cells, followed by the regeneration of such cells into whole fertile plants. The success of any plant genetic transformation strategy depends upon the availability of an efficient *in vitro* regeneration system coupled with appropriate selection regime and a method for introducing DNA into plant cells (Twyman et al. 2002). We developed the regeneration and transformation protocols. The results and detailed discussions are presented in Chapter 4 comprising of two major parts; A. Regeneration and B. Transformation.

Chapter 4

Tools for functional genomics of lead hyperaccumulation

A-Plant Regeneration

A pre-requisite to the optimisation of genetic transformation in a species is to develop an efficient and reproducible system for the development of plants, from regenerated buds or embryos. Initially, we tested the competence for the two *Pelargonium* cultivars for their response towards somatic embryogenesis and morphogenesis. Using several published protocols as basis of our experimentations, (KrishnaRaj et al. 1997; Boase et al. 1998; Saxena et al. 2000; Hassanein and Dorion 2005), we could not have any embryogenic event and suitable medium for morphogenesis for the cultivars. We therefore focused on different regeneration schemes. The efficiency of the regeneration system is greatly influenced by different factors particularly, the plant organ to be used as explant, the basic culture medium, growth hormones and their altogether interactions (Poulsen 1996).

4.1(A) Choice of Explants

Different plant parts could serve as an appropriate explant e.g. leaf, petiole, cotyledon, hypocotyl, etc. The sources may also be different e.g. from greenhouse or *in vitro* grown plants. Two scented *Pelargonium* cultivars; Attar and Atomic were selected for regeneration protocol development on the basis of their performance for Pb phytoextraction in the field experiments. We tested two types of explants i.e. leaves and petioles. By using petioles as explants, cultured on different media (media are discussed later), we could not observe regeneration or callus formation. After that, we only focused on leaves as explants.

We obtained *in vitro* mother-plants by growing apical meristems on medium containing MS salts (Murashige and Skoog, 1962), 10 g L^{-1} sugar and 0.8% Agar as gelling agent.

We tested leaves from greenhouse plants and from *in vitro* plants simultaneously. The regeneration efficiencies (no. of explants forming shoots/ total no. of explants x100) for Attar and Atomic cultivars were 50 and 70% in preliminary essays on the medium containing 2 mg L^{-1} of BAP and NAA each added to MS. However, *in vitro* explants were very sensitive to antibiotics used for selection purpose and there was no morphogenesis at all. On the basis of these preliminary results, we used only leaf explants obtained from greenhouse plants. The effect of growth hormones, darkness and other relevant optimizations are presented in the following publication, which describes a highly efficient and reproducible regeneration system for two scented *Pelargonium* cultivars

4.2 (A) *High efficiency Thidiazuron-induced shoot organogenesis from leaf explants of lead hyperaccumulator scented Pelargonium capitatum cultivars. Submitted to Plant Cell, Tissue and Organ Culture.*

High efficiency Thidiazuron-induced shoot organogenesis from leaf explants of lead hyperaccumulator scented *Pelargonium capitatum* cultivars

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ABSTRACT

A pre-requisite to understanding gene function in plants is the availability of a genetic transformation protocol, itself dependent on an efficient regeneration system. The objective of present work was to optimise culture conditions for plant regeneration of two lead (Pb) hyperaccumulator scented *Pelargonium capitatum* cultivars (Attar of Roses and Atomic Snowflake). Two efficient shoot regeneration systems, from leaf disks of greenhouse-grown plants of both cultivars, have been developed. The first protocol consisted of basal MS medium supplemented by 2 mg L^{-1} each of BAP and NAA. The second one contained 10 µM TDZ in addition to 1 mg L^{-1} each of BAP and NAA during a pre-culture step of two weeks, followed by removal of TDZ from the culture medium. Regeneration efficiencies were close to 90% for both cultivars, with the induction of more than 100 shoots per explant. Regenerated plantlets were rooted on half-strength MS medium supplemented with 15 g L^{-1} sucrose and 1.5 mg L^{-1} of IAA. On organic soil, 100% of the i*n vitro* regenerated plants were successfully acclimatized in greenhouse conditions for both cultivars. In hydroponics, the survival efficiencies were 78 and 60% for Attar of Roses and Atomic Snowflake, respectively.

Key words: *Geraniaceae*, *in vitro* culture, regeneration, heavy metal, TDZ

Abbreviations:
BAP- N^6-benzylaminopurine
IAA- Indole-3-acetic acid
NAA- α-naphthaleneacetic acid
TDZ- Thidiazuron
MS- Murashige and Skoog

Introduction

Pelargonium species are commercially important crop plants, being used as source of essential oils in aromatherapy, perfumery, cosmetics, as insect repellents, anti-inflammatory agents and as bedding or pot plants. They have been classified into four groups: Scented (*Pelargonium capitatum, P. graveolens*), Zonal (*Pelargonium x hortorum*), Regal (*Pelargonium x domesticum*) and Ivy-leaf (*Pelargonium peltatum*). Scented and zonal pelargonium varieties are of highest market value, the latter being used as bedding plants. Improvement of *Pelargonium* species by conventional breeding is hampered by sterility, very low fertility or sexual incompatibility. Therefore, the use of biotechnological approaches is being increasingly considered as alternative techniques. To this end, *in vitro* regeneration systems of economically important species have been the subject of focus for the last 20 years.

Some cultivars of scented *Pelargonium* have been identified as Pb hyperaccumulators in greenhouse conditions (KrishnaRaj et al. 2000) and confirmed in field trials (Arshad et al. 2008). Considerable effort has been devoted towards understanding the physiological mechanisms underlying metal uptake, transport and sequestration in different plant species. Despite the progress made, the genetic and biochemical processes involved are still poorly understood (Yang et al. 2005), especially for Pb (Clemens 2006). Genetic transformation techniques have become powerful tools for cultivar improvement as well as for studying gene function in plants. The first step towards development of a genetic transformation protocol is the optimization of an efficient regeneration protocol, coupled to the availability of an appropriate selection regime. A regeneration system is dependent on the type of explants, the genotype, the basic culture medium, growth regulators and their altogether interactions (Poulsen 1996). Regeneration systems for *Pelargonium* species have mostly been described for Zonal (*Pelargonium x hortorum*), Regal (*Pelargonium x domesticum*) and Ivy-leaf (*Pelargonium peltatum*), (reviewed in Mithila et al. 2001; Haensch 2007), compared to fewer reports for scented *P. capitatum* and *P. graveolens* (Rao 1994; KrishnaRaj et al. 1997; Saxena et al. 2000; Hassanein and Dorion 2005).

Plant regeneration through indirect organogenesis was achieved in *Pelargonium x hortorum* after callus induction from anthers (Abo El Nil et al. 1976) and shoot tips of seedlings (Dunbar and Stephens 1989), followed by shoot and root differentiation. Adventitious shoot organogenesis of leaf disks of *Pelargonium x domesticum* occurred

often through a callus phase (Boase et al. 1998). Agarwal and Ranu (2000) used petioles and leaf explants of *Pelargonium x hortorum* and found a higher regeneration ability of petioles compared to leaves. Direct shoot regeneration systems were developed for *Pelargonium* x *hortorum* and scented *Pelargonium* (*P. captitatum* cv Bois joly and *P. graveolens* cv Grey Lady Plymouth) using leaf disks from *in vitro*-grown plants (Hassanein and Dorion 2005). Both direct and indirect organogenesis was established for *Pelargonium graveolens cv.* Hemanti (Saxena et al. 2000) contrarily to *Pelargonium graveolens* (L'Hert) which regenerated shoots only after an intervening callus phase (Rao 1994). All these studies did not make use of Thidiazuron (TDZ) in the culture medium.

TDZ has proven to be a highly effective regulator of plant morphogenesis. Originally TDZ was considered as a cytokinin and induced many responses typical of natural cytokinins (Murthy et al. 1998). However, later research demonstrated that TDZ, unlike traditional cytokinins, was capable of fulfilling both cytokinin (bud formation) and auxin (somatic embryogenesis) functions involved in various morphogenetic responses of different plant species (Jones et al. 2007). It has been shown that TDZ can initiate organogenesis in leaves and petioles of *Pelargonium zonale* and *Pelargonium peltatum* hybrids (Winklemann et al. 2005). They all regenerated by adventitious organogenesis. However, the response was genotype dependent with cultivars of *P. peltatum* expressing higher efficiencies than those of the *P. zonale* hybrids. In general, petioles were better than leaf explants for organogenesis. Adventitious shoot regeneration from petiole explants of *Pelargonium x hederaefolium* 'Bonete' (Wojtania et al. 2004) was also described both by organogenesis and somatic embryogenesis using high TDZ concentrations i.e. 9-18 µM (2-4 mg L^{-1}).

Our long term project aims at understanding plant Pb hyper-accumulation, specifically exploring gene function in Pb phytoextraction process by scented *Pelargonium* cultivars to develop improved phytoextraction technique. Genetic transformation would be used as a tool for gene function studies, depending on the availability of an efficient regeneration method. In preliminary studies, we screened more than 15 scented *Pelargonium* cultivars for their amenability to *in vitro* culture. Two *Pelargonium capitatum* cultivars, Attar of Roses and Atomic Snowflake were selected on the basis of better performances both for phytoextraction (Arshad et al. 2008) and to *in vitro* culture. We report here, optimized culture conditions of leaf disk explants that allow the development of regenerated shoots into mature flowering plants in greenhouse conditions. This is, to our knowledge, the first report of two equally efficient plant regeneration

methods by organogenesis, in the absence or presence of TDZ, for the scented *P. capitatum* cvs Attar of Roses and Atomic Snowflake.

Materials and methods

Plant material

Scented *P. capitatum* cultivars (Attar of Roses and Atomic Snowflake), propagated from cuttings of commercial plantlets from the Heurtebise nursery, Clansayes, France, were grown in pots containing special substrate for *Pelargonium*; a mixture of white peat, humic peat and clay granulate at pH_{CaCl2} 5.5-6.1 (Hawita Flor). The plants were maintained in a greenhouse and were regularly irrigated with tap water. Scented *Pelargonium* cultivars Attar of Roses and Atomic Snowflake will be referred to as Attar and Atomic, respectively.

Disinfection and culture conditions

The two latest fully developed leaves from greenhouse-grown plants aging about four months were harvested and washed with tap water for 30 min. The leaves were then dipped in 95% ethanol for 30 sec followed by immersion in filtered 2.5% Calcium hypochlorite during 20 min and rinsed thrice in double de-ionized sterile water. Sterilized leaves were cut into 0.25 cm^2 pieces, and 12 pieces were placed adaxial side down per Petri dish (94 x 16 mm) containing 25 mL of different regeneration media. More than 30 explants were cultured on each medium and all experiments were repeated thrice. Petri dishes were maintained in a culture room at 25°C with 70% relative humidity and a 14-h photoperiod (150 µmol m^{-2} s^{-1}) provided by 600W lamps (day light fluorescent lamp, Philips).

Regeneration media

Two types of regeneration media (RM) were tested to develop a high-output and time friendly tissue culture system. The first set of experiments contained different auxin and cytokinin combinations on MS based medium with 30 g L^{-1} sucrose (Murashige and Skoog 1962). Four growth regulators, BAP, NAA, Zeatin and Kinetin were chosen on the basis of previous reports on scented *Pelargonium* cultivars (Saxena et al. 2000; Hassanein and Dorion 2005). The different hormonal combinations resulted in eight regeneration media (Table 1). BAP concentrations ranged from 0.5 to 3.0 mg L^{-1} (2.2 to 13.2 µM) and those of NAA from 0.05 to 2.0 mg L^{-1} (0.3 to 10.6 µM). Only two levels of Zeatin, 0.5 and

1.0 mg L^{-1} (2.3 to 4.6 µM) and one for Kinetin 5.0 mg L^{-1} (23 µM) were tested. Explants were regularly examined to monitor morphogenesis responses. Those forming at least 10 shoots, on each explant were considered as responding explants and scored for regeneration efficiency calculations. In 2nd set of experiments, the effect of pre-incubation with MS medium supplemented by 1 and 10µM TDZ concentrations for two weeks, with subsequent transfer to RM3, RM5 and RM7 media, were assessed as described in Table 2. In 3rd set, 10 µM of TDZ were added to RM3, RM5 and RM7 during two week pre-incubation period prior to culture on these media (Table 2). For 2nd and 3rd set of experiments, the explants forming more than 25 shoots were only considered as responding and for regeneration efficiency calculations. All media were adjusted to pH 5.8 ± 0.05 and autoclaved (autoclave; SMI 134) during 20 min at 121◦C, solidified either by 0.8% bacto-agar (Fischer), 0.2% gelrite (Duchefa) or 0.3% Phytagel (Sigma).

Optimization of physical conditions

The effect of light and darkness as well as the type of gelling agent was tested for regeneration efficiency. Explants were either directly exposed to light conditions or cultured in darkness during 15 and 22 days respectively, before being exposed back to light conditions.

Elongation and rooting medium

After 7-8 weeks of culture, 16 regenerated buds were transferred to 900 cm^3 Vitrovent boxes (Duchefa, The Netherlands) containing 100 mL of elongation and rooting medium, consisting of half strength MS medium, 15 g L^{-1} of sucrose supplemented with 1.0, 1.5 or 2.0 mg L^{-1} of IAA and solidified by 0.8% agar.

Acclimatization of plants

Well developed and rooted plants were removed from Vitrovent boxes after 10 weeks, washed from the gelling agent, and acclimatized either in pots containing organic soil or in hydroponics (aerated non-circulating nutrient solution) in the greenhouse. They were maintained at 24-26 °C and 60-70% relative humidity with a 14-h photoperiod (45 µmol m^{-2} s^{-1}). Plants were placed in a closed mini growth-chamber, in the greenhouse during two weeks, after which they were opened every other day to allow gradual exposure to greenhouse conditions. Plants transferred to organic soil were regularly irrigated with tap water and every two weeks with the following nutrient solution (Uzu et al. 2009)

comprising macronutrients: 5 mM KNO_3, 5 mM $Ca(NO_3)_2$, 2 mM KH_2PO_4, 1.5 mM $MgSO_4$, and micronutrients: 46 μM H_3BO_3, 9 μM $MnSO_4.H_2O$, 0.1 μM $MoNaO_4.2H_2O$, 0.9 μM $CuSO_4.5H_2O$, 15 μM $ZnSO_4.7H_2O$ and 180 μM Fe–EDTA**.** In the case of hydroponic cultures**,** the nutrient solution was renewed every two weeks and the level was maintained with de-ionized water. Acclimatization efficiency, defined as the percentage of plants that survived the transition from *in vitro* to greenhouse conditions, was calculated on the basis of observations after one month.

Statistical analysis

Data obtained was subjected to analysis of variance (ANOVA) with two factors, 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.

Results and discussion

Optimization of shoot regeneration

A set of parameters such as gelling agent, light/darkness incubation period and regeneration medium having the best possible combination of growth hormones were optimized simultaneously to develop an efficient regeneration system. None of the gelling agents -Agar, Gelrite and Phytagel- tested had significant effects on regeneration efficiencies for both cultivars (data not shown). Eight regeneration media, presented in Table 1, were assessed for different light/darkness regimes. Results of the two best performing media for both cultivars are shown in Figure 1. Attar cultivar proved to be very light sensitive as explants rapidly showed signs of necrosis resulting in poor regeneration efficiencies of 7 and 2% on RM3 and RM5 respectively. On the contrary, organogenesis of Atomic was not severely inhibited by light on both media, nearing approximately 70% on RM3 and 50% on RM5. Both cultivars were very performing, when cultured on RM5 medium with a two week darkness pre-incubation period before being cultured back to light conditions. In this case, regeneration efficiencies reached 93% on the average for both cultivars, with at least 100 shoots randomly induced per explant. This efficiency is suitable for genetic transformation experiments as the number of cells involved in the morphogenic responses is very high. One additional week incubation in the dark reduced the organogenesis efficiency, in all cases. In our experiments, the effects of darkness were

more pronounced at higher concentrations i.e. 2 mg L^{-1} of each BAP and NAA, as compared to lower amounts of 1 mg L^{-1} of these regulators.

Beneficial effects and enhanced sensitivity to darkness at higher levels of auxins and cytokinins have been observed by Hassanein and Dorion (2005) but the optimal duration was 4-weeks in contrast to our results where 2-weeks darkness pre-incubation was optimum. This was also described in several other genera (Perez-Tornero et al. 2000; Zobayed and Saxena 2003) and could be explained by an inhibitory effect of light on hormone efficiency (Suzuki et al. 2004). All further experiments were thus carried out with a 15 day darkness pre-incubation period. During this phase of culture, explant size was not altered. Organogenesis was initiated one week after being put to light conditions with clear round smooth structures (Fig 2a) which developed into shoots 2 weeks later (Fig 2b), producing plantlets (Fig 2c) after a total of 7 weeks in culture.

Table 1: Effect of growth hormones on organogenesis potential of *P. capitatum* cultivars Attar of Roses and Atomic Snowflake with two week incubation in darkness.

Regeneration	Growth regulators (mg L^{-1})				Ratio	Shoot formation (%)	
Medium	BAP	NAA	Zeatin	Kinetin	BAP/NAA	Attar	Atomic
RM1	0.5	0.2	0.5	-	2.5	14.4 ± 1.9^{f}	0.0
RM2	1.0	0.5	1.0	-	2.0	21.1 ± 5.1^{e}	5.6 ± 3.9^{g}
RM3	1.0	1.0	-	-	1.0	75.4 ± 8.6^{c}	86.2 ± 3.3^{b}
RM4	1.0	0.5	-	-	2.0	24.4 ± 10.3^{e}	10.9 ± 1.8^{f}
RM5	2.0	2.0	-	-	1.0	89.4 ± 3.8^{ab}	96.6 ± 3.8^{a}
RM6	2.0	0.05	-	-	40.0	4.4 ± 2.6^{g}	30.3 ± 10.6^{e}
RM7	3.0	1.0	-	-	3.0	80.2 ± 5.4^{bc}	54.9 ± 5.9^{d}
RM8	-	2.0	-	5.0	-	0.0	0.0

RM = regeneration media containing MS salts, BAP = N^{6}-benzylaminopurine, NAA = α-naphthalene acetic acid. Mean values from three replications (30 explants per replication) with different letters are significantly different ($p < 0.05$) as measured by LSD Fisher test.

Regeneration efficiencies of eight media containing different combinations of auxins and cytokinins are presented in Table 1, which are the mean values of 3 replications performed with two week darkness regime. RM 3, 5 and 7 were better than others for both cultivars. In the case of Attar, regeneration efficiency on RM 5 (89.4 ± 3.8%) was significantly different to that of RM3 (75.4 ± 8.6 %), whereas RM7 (80.2 ± 5.4%) was not significantly different to RM3 (75.4 ± 8.6%) and RM5 (89.4 ± 3.8%). In the case of Atomic cultivar,

RM5 (96.6 ± 3.8%) had a significantly higher regeneration efficiency than RM3 (86.2 ± 3.3%) and RM7 (54.9 ± 5.9%). Both cultivars performed better on RM5, the regeneration scheme being independent of the cultivar, a highly desired characteristic sought, when developing regeneration system for any species.

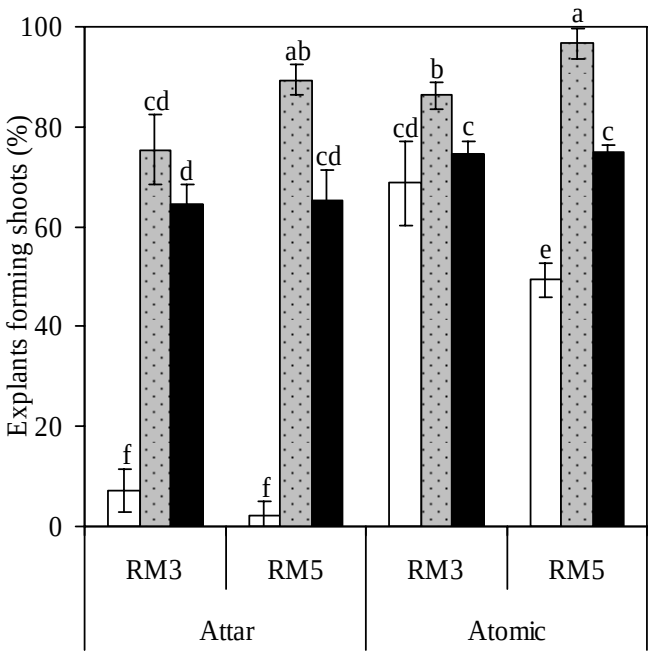

Fig 1: Influence of darkness on the regeneration efficiency of *P. capitatum.* Direct exposure to light (□), 15 days (▨) and 22 days (■) of darkness.

For both cultivars, the media containing only BAP and NAA, with either a ratio of 1 or 3, performed better than those with ratios of 2 or 40. Addition of zeatin to medium containing a BAP/NAA ratio of 2, did not improve the regeneration efficiency contrarily to the results from Hassanein and Dorion (2005) where the combination of zeatin and BAP was necessary to achieve high regeneration frequency. In their case, direct shoot regeneration with 100% of explants inducing buds that developed into shoots was obtained from *in vitro* leaf disks of *P. capitatum* cultivar Bois Joly on MS/2 medium supplemented with 0.5 mg L^{-1} NAA, 1.0 mg L^{-1} BAP and 1.0 mg L^{-1} zeatin. The difference of medium and regeneration efficiency could be attributed to the origin of explants. For the present experiments, the source of explants were greenhouse grown plants whereas Hassanein and Dorion (2005) used leaves as explants from *in vitro* grown plants. NAA proved to be most effective compared to IAA and, 1.0 mg L^{-1} each of BAP and zeatin were better than 2.0 mg L^{-1}. In our study, kinetin was most detrimental, when substituted for BAP leading to browning and death of explants. Agarwal and Ranu (2000) tested different media

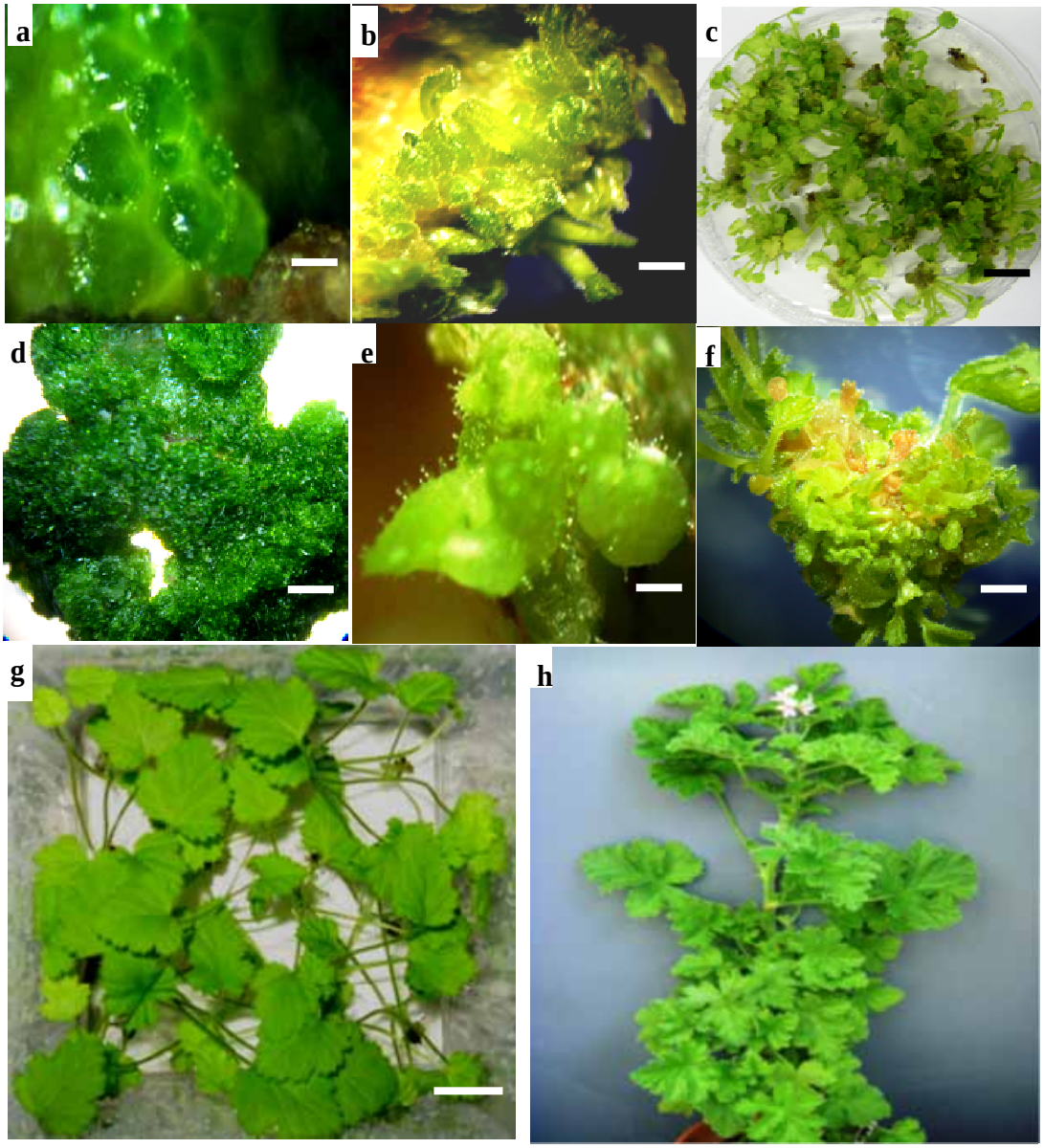

Fig 2: Shoot regeneration on leaf blade explants from *P. capitatum* cv. Attar of Roses without (a-c) and with TDZ (d-f). a) Shoot bud initiation on 2 mg L^{-1} BAP and NAA from leaf explants after 21 days culture. Bar = 2 mm. b) Development of buds into shoots after 5 weeks in culture. Bar = 2 mm. c) *In vitro* shoots developed on regeneration medium after 7 weeks in culture. Bar = 1 cm. d) Expansion and greening of explants after two week darkness incubation on 10 µM TDZ plus RM3 followed by 2 week culture on RM3. Bar = 2 mm. e) Onset of organogenesis, 6 weeks after culture initiation. Bar = 2 mm. f) Shoot development after 8 weeks in culture. Bar = 2 mm. g) Rooting of *in vitro* shoots on half strength MS medium supplemented with 1.5 mg L^{-1} IAA after 12 weeks of culture initiation. Bar = 2 cm. h) Acclimatized plant in the greenhouse, 18 weeks after experiment initiation.

containing a range of BAP (0.22–1.1 mg L^{-1}) concentrations in combination with 0.35 mg L^{-1} of IAA by using leaves as explants for three cultivars of *Pelargonium x hortorum*. They observed responses in a narrow range of BAP (0.22-0.56 mg L^{-1}) and above 0.56 mg L^{-1} of BAP, regeneration was completely suppressed. According to Saxena et al. (2000), the regeneration efficiency for *Pelargonium graveolens cv* Hemanti was lower from leaf segments than with petioles. The maximum number of shoots per leaf explant was achieved on medium containing 5.0 mg L^{-1} of Kinetin and 1.0 mg L^{-1} of NAA, contrarily to our results where kinetin decreased the regeneration efficiency.

Keeping in view the dual ability of TDZ as morphogenesis inducer (Jones et al. 2007), its effect on the response of both cultivars was tested in selected combinations with or without auxins and cytokinins. Different TDZ concentrations (1-20 µM) were tested. The two cultivars were sensitive to 15 and 20 µM, resulting in rapid tissue browning and inhibition of morphogenesis (results not shown). Finally, two concentrations (1 and 10µM) were retained for further experimentations (Table 2). Explants were pre-incubated during two weeks as prolonged exposure to TDZ (3-4 weeks) caused necrosis and death of primary shoot and callusing of primary root in most of the seedlings of pigeon pea (Singh et al. 2003). During the first two weeks in culture on regeneration media containing TDZ in darkness, explants generally expanded, swelled and thickened, showing intense cell division as in the work reported on lentils (Chhabra et al. 2008). Before sub culturing on TDZ free-medium, explants were cut into two or three (depending on the final size) in order to allow direct contact with the medium. They became dark green after 2 weeks in the light (Fig 2d). Morphogenesis started after 6 weeks resulting into small outgrowths (Fig 2e) which developed into shoots after 8 weeks in culture (Fig 2f). There was neither callus formation nor embryogenesis at any stage. KrishnaRaj et al. (1997) have also reported that there was no somatic embryo formation by TDZ application in scented *Pelargonium sp.* 'Frensham'.

The best regeneration efficiencies on culture media without TDZ pre-incubation were 89.4 and 96.6% on RM5 for Attar and Atomic respectively (Table 2, Exp 1) where the number of shoots per explant rarely exceeded 25 (Table 3). The shoots were not randomly distributed on the explants. This kind of regeneration systems could not be effective for developing a transformation protocol. So TDZ was tested to improve the number of shoots per explant. Increasing TDZ concentrations during the pre-incubation period, followed by culture on MS medium without BAP and NAA, resulted in increasing regeneration efficiencies in terms of number of shoots per explant for both cultivars (Table

2, Exp 2-3 and Table 3), showing that TDZ would have a cytokinin action, probably by increasing the level of endogenous cytokinins (de Melo Ferreira et al. 2006). However, increasing BAP/NAA ratio by increasing BAP concentrations, together with increasing TDZ concentrations resulted in different responses for the two cultivars. For Attar, increasing the TDZ concentration from 1 to 10 µM, followed by culture on RM3, did not alter significantly the regeneration efficiency when compared to culture on RM3 without the TDZ pre-incubation period. Doubling BAP and NAA concentrations without modifying the BAP/NAA ratio of 1, (RM3 to RM5) or tripling the BAP/NAA ratio, resulted in an overall decrease in the regeneration efficiency with 1 and 10 µM TDZ during pre-incubation period when compared to no pre-incubation with TDZ in terms of number of explants forming shoots. The response of Atomic cultivar was fairly much the same, except when pre-incubation with 10 µM TDZ was combined with culture on RM7. In this case, the regeneration efficiency was the same as when culture was performed only on RM3. These results show that the response of TDZ is determined by the ratio of BAP/NAA as well as by the absolute concentrations of BAP in the medium. Depending on the cultivar, TDZ can regulate the endogenous levels of auxins and/or cytokinins and modulate the morphogenic response. In the absence of growth regulators in the culture medium, TDZ in the pre-incubation medium would act as a cytokinin. Increasing too much the concentration of BAP in the medium, together with TDZ concentration, leads to a switch in the role of TDZ as a cytokinin-like growth regulator resulting in a decrease in regeneration efficiencies. In this case, TDZ could regulate the response by increasing the levels of endogenous auxins or its precursor, tryptophane (Murthy et al. 1995). This response however seems to be cultivar dependent, as increasing the BAP/NAA ratio together with BAP concentration (RM7) for Atomic, leads to a 4.5 fold increase in the regeneration efficiency with increasing TDZ concentration from 1 to 10 µM.

Pre-incubation of both cultivars on RM3 plus 10 µM TDZ followed by culture on RM3 resulted in higher regeneration efficiency (93%) with respect to culture on RM3 alone for Attar (75%) (Table 2, Exp 4 v/s Exp 1). These were the best results that could be reproducibly achieved with the two cultivars, each explant producing more than 100 regenerated shoots (Table 3). Results with higher ratios and concentrations of BAP/NAA were cultivar dependent. Pre-culture with 10 µM TDZ in combination with either RM5 or RM7 (2 and 3 mg L^{-1} of BAP respectively,) followed by culture on the same respective media, resulted in decreased regeneration efficiencies (65 and 34%) for Atomic. For Attar, pre-incubation with RM5 (2mg L^{-1} of BAP and NAA) as well as 10 µM TDZ, reduced the

Table 2: Effect of TDZ applied in pre-culture media on regeneration efficiency.

		Regeneration Efficiency (%)							
		Attar				Atomic			
Pre-culture Medium (2W)		Culture Medium							
Exp	Pre-incubation	MS	RM3	RM5	RM7	MS	RM3	RM5	RM7
1	None	0	75.4 ± 8.6^{c}	89.4 ± 3.8^{ab}	80.2 ± 5.4^{bc}	0	86.2 ± 3.3^{b}	96.6 ± 3.8^{a}	54.9 ± 5.9^{d}
2	MS+TDZ 1µM	23.5 ± 2.8^{g}	78.7 ± 4.6^{c}	21.5 ± 5.2^{g}	52.7 ± 10.0^{d}	22.3 ± 11.5^{f-h}	69.9 ± 11.9^{cd}	27.9 ± 12.9^{fg}	19.6 ± 6.4^{g}
3	MS+TDZ 10µM	51.1 ± 9.6^{d}	69.2 ± 12.8^{cd}	44.8 ± 0.9^{e}	32.0 ± 2.3^{f}	34.7 ± 7.3^{f}	54.4 ± 13.9^{d}	11.1 ± 1.9^{h}	87.5 ± 4.9^{b}
4	RM3+TDZ 10µM		93.5 ± 0.2^{a}				93.1 ± 6.5^{ab}		
5	RM5+TDZ 10µM			17.9 ± 14.6^{fg}				65.4 ± 9.4^{cd}	
6	RM7+TDZ 10µM				58.3 ± 16.1^{cd}				34.4 ± 9.4^{ef}

More than 30 leaf explants per assay were cultured on MS medium containing either 1 or 10 µM TDZ in the pre-incubation medium (Exp 2-3) followed by incubation on MS, RM3, RM5 and RM7, containing combinations of N^6-benzylaminopurine (BAP) and α-naphthalene acetic acid (NAA). In Exp 4-6, 10 µM TDZ was included in the pre-incubation medium containing RM3, RM5 or RM7 followed by culture on medium lacking TDZ. Mean values from three replications with different letters are significantly different ($p < 0.05$) as determined by LSD Fisher test.

W = weeks

regeneration efficiency with respect to conditions omitting hormones in the pre-incubation period (from 44.8 to 17.9%). However, pre-incubation with RM7 (3 mg L^{-1} BAP and 1 mg L^{-1} NAA) increased the regeneration efficiency (32 to 58.3%). This was not the case for Atomic where efficiencies increased 6 fold on RM5 (Table 2, Exp 3 and 5) and decreased 3 fold on RM7 (Table 2, Exp 3 and 6). These contrasting responses could be explained by genotype effects combined with the interplay between the actions of TDZ on auxin transport, synthesis or accumulation with *in situ* cytokinin concentrations, which would result in overall alterations in the endogenous hormone concentrations, leading to different regeneration efficiencies. RM3 (1 mg L^{-1} each of BAP and NAA), appeared the best in the presence of TDZ in contrast to RM5 (2 mg L^{-1} of BAP and NAA each) in the experiments without TDZ. This difference could be argumented as the lower levels of BAP and NAA in RM3 as compared to RM5 would be complemented by the TDZ-induced response on auxin synthesis and transport. Reduced efficiencies in the presence of TDZ for RM5 and RM7 may be due to negative effect of higher concentrations of growth regulators.

Table 3: Classification of the regenerated explants for some selected media in terms of number of shoots per explant.

Regeneration Medium	No of explants grouped on the basis of number of shoots per explant							
	Attar				Atomic			
shoots per explant	10-25	26-50	51-100	>100	10-25	26-50	51-100	>100
MS	-	-	-	-	-	-	-	-
RM3	65	7	-	-	64	17	-	-
RM5	80	12	-	-	83	14	6	-
RM7	73	15	1	-	37	15	-	-
MS + 10 µM TDZ→ MS	-	15	27	4	-	9	27	1
MS + 10 µM TDZ→ RM3	-	20	40	7	-	7	40	2
MS + 10 µM TDZ→ RM5	-	10	26	7	-	5	5	-
MS + 10 µM TDZ→ RM7	-	20	9	3	-	20	39	24
RM3 + 10 µM TDZ→ RM3	-	-	1	100	-	-	3	102
RM5 + 10 µM TDZ→ RM5	-	-	15	2	-	-	33	32
RM7 + 10 µM TDZ→ RM7	-	-	30	24	-	-	27	6

In the regeneration medium column, the arrow is directed towards the culture medium used after 2 weeks pre-incubation period in the presence of TDZ. For each medium, 90 or more explants were cultured. During data recording, the number of shoots per explant were counted and then were classified into different groups i.e. 10-25, 26-50, 51-100 and >100. For example, 65 explants produced more than 10 and less than 25 shoots per explants cultured on RM3 for Attar cultivar.

Shoot elongation, rooting and acclimatization of in vitro plants

After 7-8 weeks of culture initiation, shoots developed on regeneration media were sub-cultured on Elongation and Rooting Medium (ERM. Whatever the protocol used to develop shoots, they were elongated and rooted within 4 weeks (Fig 2g). Results concerning the influence of IAA on rooting efficiency are shown in Fig 3. The rooting efficiencies were 49 and 48% for Attar and Atomic cultivars respectively when plantlets were transferred to ERM supplemented by 1 mg L^{-1} IAA. The respective efficiencies for Attar and Atomic cultivars were 90 and 88% on medium supplemented by 1.5 mg L^{-1} IAA or 2 mg L^{-1} IAA. However, extremely dense rooting, enhanced root diameter and restricted growth were observed on the ERM containing 2.0 mg L^{-1} of IAA. We therefore chose 1.5 mg L^{-1} IAA for rooting in all experiments (Fig 2g).

Hassanein and Dorion (2005) has reported an optimum of 1 mg L^{-1} IAA for rooting of plantlets of scented *Pelargonium*. Boase et al. (1998) tested the effect of different NAA levels (0, 0.05, 0.1, 0.15 and 0.2 mg L^{-1}) in rooting medium for *Pelargonium x domesticum* cv. Dubonnet. The average root number increased with increasing concentrations of NAA but shorter roots with larger diameters were observed at higher NAA concentrations (0.1, 0.15 and 0.2 mg L^{-1}). In the present study, the trends were similar in spite of the different rooting hormone i.e. IAA which needed to be applied at 10 fold higher concentrations.

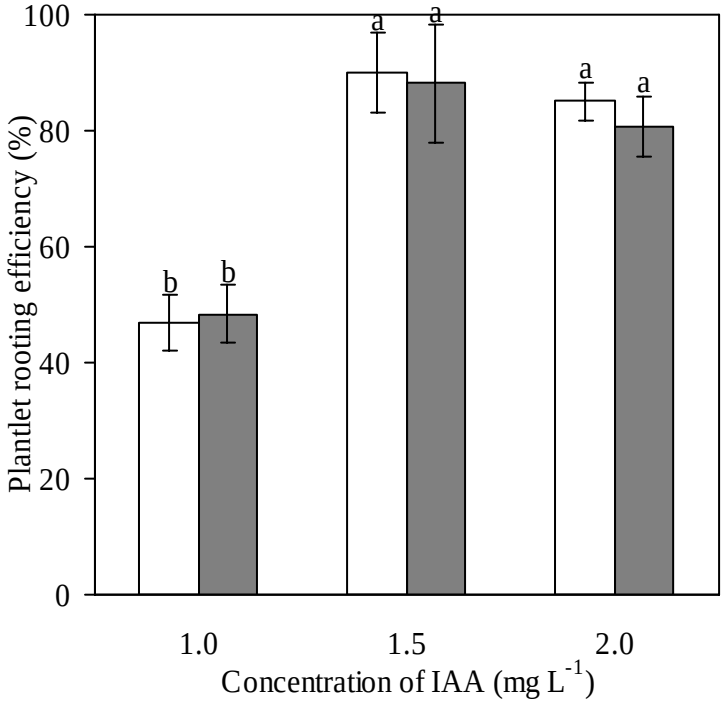

Fig 3: Effect of IAA concentration on rooting of regenerated plantlets of *P. capitatum* cultivars Attar (□) and Atomic (■).

Rooted plants of both cultivars were transferred to organic soil and hydroponics for acclimatization to greenhouse conditions. Acclimatization efficiency was 100% on organic soil for both cultivars. In hydroponics, the acclimatization efficiencies were 78 and 68% for Attar and Atomic cultivars, respectively. All the plants were maintained in greenhouse till flowering (Fig 2h). In spite of lower efficiencies of acclimatization in hydroponics, growth of plants was more vigorous and quicker in hydroponics and, flowering occurred earlier as compared to plants grown on organic soil. These efficiencies are higher than those reported by Agarwal and Ranu (2000) where only 25-50% of the shoots could be rooted and acclimatized from *in vitro* to greenhouse conditions (on mixture of vermiculite and perlite, 1:1). Hassanein and Dorion (2005) have reported 100% acclimatization efficiency for scented *Pelargonium* (on vermiculite and peat mixture, 2:1 v/v).

Conclusion and perspectives

Two regeneration systems from leaf explants of greenhouse plants of *P. capitatum* cultivars Attar of Roses and Atomic Snowflake have been developed. The first optimized medium was comprised of only BAP and NAA at concentration of 2 mg L^{-1} of each added to MS. The second optimized medium contains 10 µM TDZ in addition to 1 mg L^{-1} each of BAP and NAA during pre-culture of two weeks with subsequent removal of TDZ from culture medium. Fifteen days pre-incubation in darkness proved the best for maximum regeneration efficiency. The regeneration efficiencies were close to 90% or greater for both cultivars on both optimized media but in the presence of TDZ pre-incubation, the number of shoots per explants were about four times higher as compared to the medium without TDZ. These regeneration systems should be assessed for their amenability to genetic transformation. They could also be used to produce *in vitro* plants on a large scale on commercial basis.

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4.3 (A) General discussions

Different combinations of BAP, NAA, Kinetin, Zeatin and TDZ added to MS medium (Murashige and Skoog, 1962) comprising of macro- and oligo-elements, two regeneration media were optimized. The first one comprised only 2 mg L^{-1} of BAP and NAA added to MS media. The regeneration efficiency i.e. number of explants forming shoots/ total number of explants, was about 90% for both cultivars Attar and Atomic but the number of shoots per explant could rarely reach to 25 shoots per explants. This system should be tested for transformation experiments. To increase the number of responding cells which could help to develop better transformation system, we used two weeks pre-incubation in the presence of TDZ. The second optimized medium contained 1 mg L^{-1}, each of BAP and NAA with pre-treatment with 10 µM TDZ during two weeks. The regeneration efficiency was more than 90% for Attar and Atomic cultivars, coupled with high number of shoots per explant > 100 shoots per explant. This system could be more suitable for developing transformation protocol. The procedure and results for transformation essays using these two regeneration systems are presented in the following part of this chapter (4B).

B - Genetic Transformation

4.4 (B) Genetic transformation of lead-hyperaccumulator scented *Pelargonium* cultivars

Introduction

One of the major goals of plant biotechnology research is to gain in-depth knowledge of the function of each gene in a given plant species confronted to the biotic and abiotic stresses. Recent developments of ambitious genomics programs on model species including *Arabidopsis thaliana* (AGI 2000), *Lotus japonica* (Sato et al. 2008), *Medicago truncatula* (Bell et al. 2001), *Oryza sativa* (IRGSP 2005), (for further details and other species; NCBI, Genome project) have opened new avenues towards deciphering the structure, the expression and the mechanisms involved in the regulation of extensive sets of genes in response to environmental stresses. The outcome of these results could be expected to lead to spectacular spin offs in crop improvement and protection in the future.

Although nowadays, gene isolation is considered as being a relatively high throughput technology, the bottle neck remains identifying their function as well as the mechanisms underlying their regulation and expression. Putative functions of newly isolated genes are first assigned by comparing the target sequence with other sequences with known functions, from genome data bases. This method only gives an idea on the biochemical function of the coding sequence but cannot attribute a specific role in the organism's response to a stimulus. The majority of genes isolated have therefore been attributed putative functions on the basis of *in silico* studies. Other strategies grouped under the name of "functional genomics" have been developed with the aim of understanding the function of genes pinned by structural genetics.

Some strategies are based on either spatio-temporal expression of the genes or the over-expression of the desired gene. The spatio-temporal expression of the genes (mRNA and protein expression) is the response (in time and localisation) to the stimulus during developmental stages, or following different biotic or abiotic stresses, for example pathogen infection, heat, drought, salinity, or metal element exposure (Narusaka et al. 2004). However, these studies, only lead to indirect proof of the role and function of the genes. The over-expression of the desired gene is achieved by using a strong plant promoter and adjoining activation sequences to drive high level and constitutive expression. The effect is high steady state mRNA and protein levels. Any phenotypic changes could then be assigned to the native gene's function based on biochemical

pathways that are altered in the transformants. However, over-expression of an endogenous gene can lead to co-suppression by RNAi mechanism, and hence could also be an approach towards deciphering gene function. RNAi is a system within living cells that helps to control which genes are active and how active they are. Other strategies are summarised below.

Conventional methods of random gene inactivation have made use of chemicals Ethyl Methyl Sulfonate (EMS) or γ- irradiations which introduce mutations into populations. After phenotypic screening, genetic crosses can, through loci genetic mapping, lead to gene isolation. This strategy is known as "forward genetics" whereby starting from mutated populations; it is possible to work down to the gene by classical genetic mapping techniques combined to the use of molecular markers, provided these are available. With the advent of whole genome sequencing projects, a large amount of expressed sequences are available for gene function study. Therefore, instead of going from phenotype to sequence as it is the case with forward genetics, in "reverse genetics", the gene sequence is disrupted and the resulting phenotype and function are measured. Although in yeast, targeted gene inactivation by homologous recombination has enabled to unravel gene function in many cases (Cejka et al. 2005), this technology can unfortunately not be extended to plants. Indeed, in the latter case, DNA insertion is a random process and illegitimate recombination prevails. It is therefore impossible to control homologous recombination. Possible strategies to inactivate genes have first made use of expressing anti-sense RNAs (Kissil et al. 1999), transposon tagging (Anderson et al. 1997) and more recently RNAi constructs (Parizotto et al. 2004) based on the discovery of interfering RNA (Fire et al.1998). However, the use of these strategies imply that efficient methods of genetic transformation have first been optimised.

Genetic transformation has remained, for different plant species, an important area of research after the first successful foreign gene transfer and integration (De Block et al. 1984), leading to more than 144 plant species having been transformed to date (Busov et al. 2005). This tool is extensively being used in all areas of plant research aiming at improved productivity of safer and healthier products (Darbani et al. 2007). More recently, this area has been integrated in the field of environmental sciences e.g.phytoremediation, either for developing plants aiming soil clean up and phytomining (Doty 2008) or understanding mechanisms of metal uptake. Multifold increase in accumulation and/or tolerance has been reported in genetically modified plants as compared to wild types, for

some metal pollutants e.g. As (Dhankher et al. 2002), Hg (Che et al. 2003), Pb (Martinez et al. 2006) and Se (Pilon et al. 2003).

Considerable efforts for gene function discovery in metal accumulation and tolerance have been performed during the last decade, in identifying and exploring the role of Phytochelatins and Metallothioneins, metal transporters and chelators. *Arabidopsis halleri* and *Thlaspi caerulescens*, have become the favoured model plants to study hyperaccumulation, specifically relative to zinc and cadmium (Reviewed by Cobbett and Goldsbrough 2002; Yang et al. 2005; Clemens 2006; Milner and Kochian 2008; Memon and Schroeder 2009). Hyperaccumulation is widespread across different families and genera of plants from ferns to monocots and, more than 400 plant species capable of hyperaccumulating one or a few heavy metal elements at the same time have been reported (Prasad and Freitas 2003). Few reports have concerned mechanistic understanding of metal hyperaccumulation, largely because of the absence of molecular tools (genomic sequences, mutants) as well as the lack of a reproducible and efficient genetic transformation protocol for the most of the species. Recently, *Pelargonium* species have been indentified as Pb hyperaccumulator in greenhouse experiments (KrishnaRaj et al. 2000) and confirmed in field trials (Arshad et al. 2008). As demonstrated in Chapter 2, these cultivars can be qualified as hyperaccumulators for lead.

Our long term objective is to develop a model system to study molecular and physiological processes involved in lead absorption, transport and accumulation in scented *Pelargonium* species to improve Pb phytoextraction. As described in Chapter 3, we used special cropping devices adapted for rhizosphere studies (Neibes et al. 1993) to picture the mode of Pb absorption and translocation in the plant. As a follow up to these studies, we endeavour to decipher molecular mechanisms involved in Pb hyperacculation in scented *Pelargoium* cultivars. Therefore, we would anticipate pin-pointing targeted genes identified as major actors and identify their function in the process. As a pre-requisite to these studies, we set up a reproducible and efficient genetic transformation protocol for these cultivars. As stated in Chapter 4A, this is dependent on the availability of an efficient regeneration system but also on an effective selection regime. Indeed, the success of a reliable and reproducible transformation protocol relies on the ability to make two events coincide in the same cell; morphogenesis and transformation. This is shown and explained in the schematic diagram (Fig 1). Therefore, genetic transformation is merely a question of probability depending upon the availability of high numbers of cells competent for both the transformation and the morphogenesis event. However, a transgenic plant with proof of

insertion into the nuclear chromosomes does not necessarily mean that it will be a high expressor for the target transgene. Indeed, gene expression, whether related to endogenes or to transgenes, is subject to tight regulation at all levels of gene expression.

One of the limiting factors in transformation experiments is the screening and detection of the putative transformed events implying stable integration and expression of the transgene sequence. Although, the use of selectable marker genes has been the subject of major controversies, concerning the voluntary dissemination and commercialisation of transgenic plants (Miki and McHugh 2004), it remains an absolute requirement when setting up transformation protocols in the laboratory. The major objections towards the use of selectable marker genes have given way to the development of efficient methods to withdraw them from the selected events to be disseminated in the environment (Sundar and Sakthivel 2008). However, this will not be a subject of concern here, as transgenic plants developed within the scope of these studies, will solely be used for academic purposes, kept *in vitro*, and in confined environments.

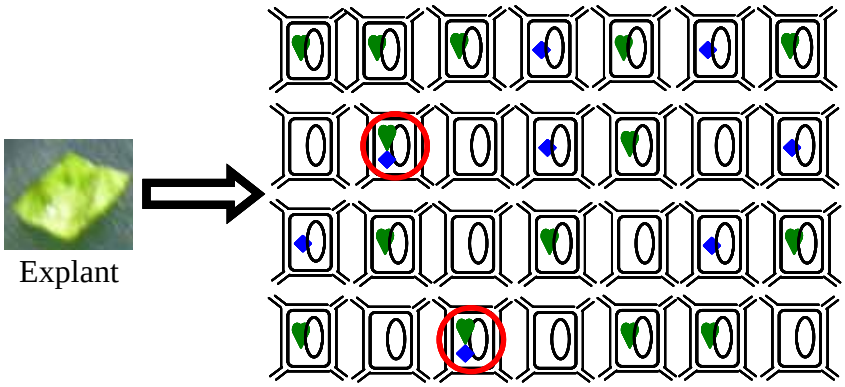


Figure 1: Schematic presentation of transformation and regeneration of plant cells.

◆ = Transformed cells ; ♥ = Cells with regeneration potential; ◆ + ♥ = Capable of transformation as well as regeneration (red encircled).

In this context, the objective of the present study was to develop an efficient transformation system for scented *Pelargonium* cultivars Attar and Atomic. To this end, two highly efficient regeneration systems (Arshad et al. submitted) for Pb hyperaccumulator cultivars Attar and Atomic were assessed for their suitability for genetic transformation. Various factors influencing the transformation efficiency have been addressed in the present work in order to achieve optimum transformation efficiencies for both cultivars.

Materials and methods

Plant material and explant preparation

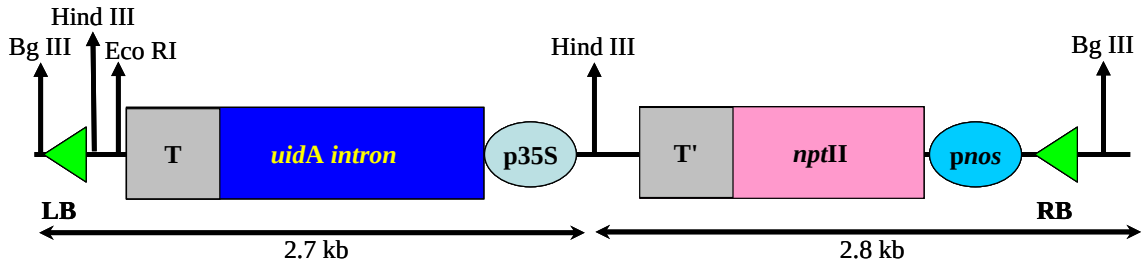
Scented *P. capitatum* cultivars (Attar of Roses and Atomic Snowflake), propagated from cuttings of commercial plantlets from the Heurtebise nursery, Clansayes, France, were grown in pots containing organic soil, in the greenhouse. They were regularly irrigated with tap water and fortnightly with nutrient solution. The two *Pelargonium* cultivars Attar of Roses and Atomic Snowflake will be referred to as Attar and Atomic, respectively. The two latest fully developed leaves from the greenhouse grown plants were harvested and washed with tap water for 30 min. Leaves were then dipped in 95% ethanol for 30 sec followed by immersion in filtered 2.5% calcium hypochlorite during 20 min and rinsed thrice with double deionized sterile water. These sterilized leaves were used as explants for transformation experiments.

A. tumefaciens strains and culture conditions

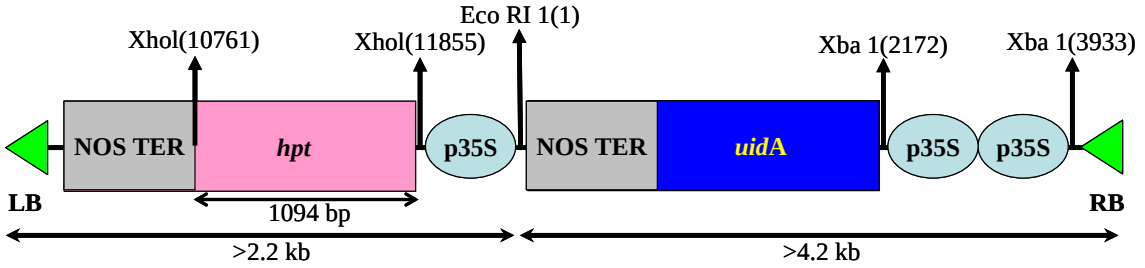
Two nopaline strains of *Agrobacterium tumefaciens* were used in this study: *A. tumefaciens* EHA105 (Hood et al., 1993) and C58 disarmed, both containing the disarmed Ti plasmid pTIBo542 (provided by S.B. Gelvin, Purdue University, USA). EHA 105 (Fig 2a) harbours the 14.7-kb binary vector p35S GUS INT (derivative of pBIN19, Vancanneyt et al., 1990) represented in Hewezi et al. (2002), and C58 disarmed (Fig 2b) contains the 12.8 kb pMDC162 binary vector (Curtis and Grossniklaus 2003) where the sequences between the attR1 and attR2 have been replaced by the double 35S CaMV promoter extracted from pMDC143 cloning vector (Curtis and Grossnicklaus 2003). In p35S GUS INT, *nptII* is under the control of p*nos* promoter. In pMDE162, *hpt* is under the transcriptional regulation of the cauliflower mosaic virus (CaMV) 35S promoter and nos terminator (Odell et al., 1985) and is adjacent to the left border of the T-DNA. *UidA* sequence is controlled by the double 35S CaMV promoter and the Nopaline synthase terminator sequence.

Cultures of *A. tumefaciens* were initiated from -80 ˚C glycerol stocks, and grown for 48H at 28 ˚C in liquid Luria–Bertani medium (10 g L^{-1} tryptone, 10 g L^{-1} NaCl, 5 g L^{-1} yeast extract, pH 7.2) containing 100 µM Acetosyringone (to enhance virulence) and antibiotics as follows: EHA 105 was cultured with 50 mg L^{-1} kanamycin, 50 mg L^{-1} rifampicin and 50 mg L^{-1}, ampicillin. C58 disarmed was cultured with 50 mg L^{-1} kanamycin (binary vector also contains kanamycin resistant gene), 20 mg L^{-1} gentamycin

and 50 mg L^{-1} rifampicin. Bacterial cultures were centrifuged at 4000g for 10 min at 4 ˚C and the bacterial pellet was re-suspended in inoculation medium containing 4 mM NH_4Cl, 5.5 mM $MgSO_4.7H_2O$, 12.5 mM MES at pH 5.6. The cells were centrifuged thrice to wash away the antibiotics. After centrifugation, the optical density of the culture was adjusted to 1.0 (OD_{660}) and 200 µM acetosyringone was added prior to inoculation.



a) p35S GUS INT



b) pMDC162

Figure 2: Schematic presentation of the T-DNA regions of the binary vectors used for transformation experiments. a) p35S GUS INT (derived from pBIN19, Vancanneyt et al. 1990) b) pMDC162 (Curtis & Grossniklaus, 2003). LB = left border, RB = right border, *npt*II = Neomycin phosphotransferase II, *Hpt* = Hygromycin phosphotransferase, *uid A* = GUS reporter gene, NOS Ter = nopaline synthase terminator, p35S = CaMV 35S promoter.

Infection and co-cultivation of Tobacco

Leaves of *Nicotiana tabacum* cv Xanthi of *in vitro* propagated plants were used as positive controls for genetic transformation to assess the functionality of the *Agrobacterium* strains. Mid ribs were removed, the remaining leaf blades were immersed in the bacterial inoculum, cut into 0.25 cm^2 sections and further wounded with the tip of the scalpel blade. Following a gentle shaking of the culture during 15 minutes, explants were blotted dry. Infected tissue was cultured with the adaxial surface in contact with the co-cultivation medium (MS medium without growth regulators). After 2 days of

cocultivation in the dark at 26°C, infected tissue was rinsed three times in inoculation medium containing 500 mg L^{-1} augmentin (SmithKline Beecham) to stop *A. tumefaciens* over-growing the tissues. Explants were blotted dry and placed the leaf adaxial surface in contact with the regeneration medium: MS medium containing 1 mg L^{-1} BAP and 0.1 mg L^{-1} NAA, 500 mg L^{-1} Augmentin and either 100 mg L^{-1} kanamycin or 25 mg L^{-1} hygromycin for leaves infected by EHA 105 and C58, respectively. Augmntin was present in the regeneration media throughout the culture period to check the growth of *Agrobacteria*. The regenerated shoots were transferred to half strength MS medium (in terms of macroelements only) without hormones containing 10 g L^{-1} of sucrose, respective antibiotics and augmentin. The shoots which developed into rooted plants in the presence of antibiotics were analysed for the presence *uid*A gene.

Infection and co-cultivation of Pelargonium

Sterilized leaves from greenhouse-grown *Pelargonium* plants, were immersed into bacterial cultures, cut into 0.25 cm^2 sections and the leaf lamina were further wounded with the tip of the scalpel blade as for tobacco. Infected explants were co-cultivated on different media: RM3, RM5 and RM5 + Acetosyringone 100 µM (AS). RM3 and RM5 were the regeneration media. The intention of adding Acteosyringone was to assess its on the transformation efficiency. Petri dishes were wrapped in aluminum foil and incubated in darkness at 25°C. After 72 h co-cultivation, explants were washed with liquid basal MS medium containing 500 mg L^{-1} augmentin and blotted on sterilized filter paper before transferring to regeneration medium with and without selectable markers. Petri dishes were cultured for two weeks in darkness and then exposed to light conditions in a growth chamber at 25°C with 70% relative humidity and a 14-h photoperiod (150 µmol m^{-2} s^{-1}) provided by 600W fluorescent lamps.

Regeneration media and selection

Two efficient shoot regeneration systems, previously optimized, from leaf disks of greenhouse-grown plants of both cultivars, were assessed for their amenability to genetic transformation. The first protocol consisted of MS basal medium supplemented by 2 mg L^{-1} each of BAP and NAA. The second one contained 10 µM TDZ in addition to 1 mg L^{-1} each of BAP and NAA during a pre-culture step of two weeks, followed by removal of TDZ from the culture medium. The effect of pre-conditioning explants on RM5 regeneration medium, in the presence of 100 µM Acetosyringone during 48H was also

assessed, prior to inoculation with Agrobacterium (Table 1). Each time, about 60 explants were cultivated and the assays were repeated twice.

The selected *Agrobacterium* strains had genes for hygromycin (C58 disarmed: pMDC162 2X35S GUS) and kanamycin (EHA 105: p35S GUS INT) resistance, as selectable markers. We evaluated the response of both cultivars to the two markers in view of assessing the best concentration for selection during the course of transformation experiments. The sensitivity of Attar and Atomic leaf explants to different levels of hygromycin and kanamycin was assessed by measuring the organogenesis potential on varying concentrations of these antibiotics. More than 30 explants of each cultivar were cultured on regeneration medium (RM5) containing 0, 2.5, 5, 10, 15, 20, 25, 30, 50, 75 and 100 mg L^{-1} of hygromycin or 0, 5, 10, 15, 20, 25, 30, 50, 75 and 100 mg L^{-1} of kanamycin. Our results (Chapter 4A) showed that the best medium for regeneration consisted of a 2 week preincubation period in the dark, on TDZ added to RM3 medium, followed by the removal of TDZ in the culture medium. We therefore tested the response of *Pelargonium* leaf explants to hygromycin and kanamycin using this regeneration system and the same levels of antibiotics were applied as for RM5. The sensitivity to these selectable markers was also assessed in combination with 500 mg L^{-1} augmentin, used to limit the growth of *Agrobacteria*. Augmentin added to the media was maintained through out the culture period. All the assays were repeated thrice. All the explants forming shoots (even 1 shoot) were considered responding explants and the number was recorded for regeneration efficiency calculations.

Elongation and rooting of shoots

Randomly distributed shoots on the explants were transferred to 100 x 20 mm Petri dishes (for 4 weeks) containing elongation and rooting medium (ERM) made up of half strength MS medium (MS/2 macro-elements), 15 g L^{-1} of sucrose supplemented by 1.5 mg L^{-1} of IAA, 500 mg L^{-1} augmentin, either hygromycin (10 mg L^{-1}) or kanamycin (15 mg L^{-1}) and solidified by 0.8% bacto-agar. From each cluster of shoots on the explants, only one shoot was picked to ensure independent events of transformation. For example, if there were 10 clusters or single shoots on a explant, only ten shoots were transferred to ERM. The surviving elongated plantlets were then shifted to 900 cm^3 Vitrovent boxes (Duchefa, The Netherlands) containing 100 mL of ERM supplemented by the same bacteriostatic and antibiotics.

GUS activity

Histochemical assays for GUS activity were performed to identify potentially transformed plants. GUS expression was determined on leaf parts and roots obtained from rooted plants under continuous selective pressure. Leaf and root explants were incubated in buffer containing 50 mM sodium phosphate buffer with pH 7.0, 10 mM EDTA at pH 8.0, 0.1% (v/v) Triton X-100 and 0.5 mg L^{-1} X-GlcA (5-bromo-4-chloro-3-indolyl β-D-glucuronide) (Jefferson et al. 1987). Tissues were vacuum infiltrated 3 times during 1 minute, with a quick release between each vacuum application followed by an overnight incubation at 37°C. Tissues were thoroughly cleared with 70% ethanol before observation.

Genomic DNA Extraction

Small-scale extraction of genomic DNA from 200 mg of young *in vitro Pelargonium* and tobacco leaves was achieved by using a modified version of the Dellaporta protocol (Dellaporta et al. 1983). Liquid nitrogen-frozen leaf tissue was first ground to a fine powder in a 2 mL Eppendorf containing 3 glass beads of 5 mm diameter using the Qiagen Retsch grinder. To this, was added 400 µL of extraction buffer (100 mM Tris pH 8.0, 50 mM EDTA pH8.0, 500 mM NaCl, 1% SDS , 0.5% Sodium Bisulfite and 0.1 mg ml^{-1} RNase). The mixture was homogenised by vortexing. After 30 minutes incubation at 65°C, 200 µL of 5 M potassium acetate was added, gently mixed by inversion and incubated on ice during 10 minutes. After centrifugation at 15000 g for 10 min at 4°C, the supernatant was collected in a 1.5 ml Eppendorf tube and 600 µL of chloroform/iso amyl alcohol (24/1 v/v) was added, mixed and centrifuged at 15000g for 10 min at 4°C. DNA in the supernatant was precipitated with 600 µL iso-propanol carefully mixed and centrifuged at 15000g for 10 min at 4°C. The supernatant was discarded and the genomic DNA pellet was washed with 70% ethanol, re-centrifuged and dried in an evaporator centrifuge for 2 minutes under low heat. The final DNA pellet was re-suspended in 50 µl of 10mM Tris-1mM EDTA, pH 8 and concentration was measured using a nano-drop spectrophotometer.

PCR screening of putative transformed plants

Preliminary screening of transgene sequences in putatively transformed plants was performed by PCR using β–glucuronidase (GUS) primer: 5’GTGAACAACGAACTGGA-ACTGGGC3’ and 3’ATTCCATACCTGTTCACCGAGG5’, which amplified a 1250 bp fragment. The reaction was performed in a 25 µl reaction containing 100 ng of DNA, 0.2

mM of dNTP, 0.2 µM of each primer, 1.5 mM of $MgSO_4$, 2.5µL of specific Taq polymerase buffer, 1 unit of KOD Hot start DNA polymerase from Novagen. The polymerase was activated at 95°C for 2 min, DNA was denatured at 95°C for 30 s, amplified during 35 cycles at 94°C during 30 s, 57°C for 60 s, 72°C for 90 s, followed by a final extension step at 72°C for 10 minutes. Amplified samples were resolved on a 1.2% agarose gel, followed by ethidium bromide staining and detection under UV light.

Results and discussion

Effect of antibiotics on shoot regeneration

An effective selection regime is very important for developing a transformation protocol. The selection strategies were developed using the antibiotics, hygromycin and kanamycin, for the two regeneration media. In the first instance, we tested regeneration medium containing BAP and NAA, each at 2mg L^{-1} together with augmentin, used as bacteriostatic agent. The presence of 500 mg L^{-1} augmentin in combination with either hygromycin or kanamycin did not affect the regeneration efficiency of both cultivars. With the application of augmentin during the development of a selection regime, the reponse of the explants and the lethal level for the antibiotics was not altered. Cultured on RM5 media containing hygromycin 10 mg L^{-1} or more, the explants turned brown quickly. There was no expansion of explants at 2.5 and 5 mg L^{-1} of hygromycin. The lethal limit (no shoot formation) was observed at 10 and 15 mg L^{-1} of hygromycin for Attar and Atomic, respectively (Fig 3a). For the transformation experiments, both cultivars were selected at 10 mg L^{-1} of hygromycin added to RM5. In the presence of kanamycin added to RM5 (Fig 3b), the explants remained green but there was no shoot formation at concentrations higher than 15 mg L^{-1} of kanamycin. There was no expansion of the explants irrespective of the cultivars. The shoot formation was completely inhibited at 15 and 10 mg L^{-1} of kanamycin for Attar and Atomic, respectively. Both cultivars were selected at 15 mg L^{-1} of kanamycin added to RM5, for the transformation experiments.

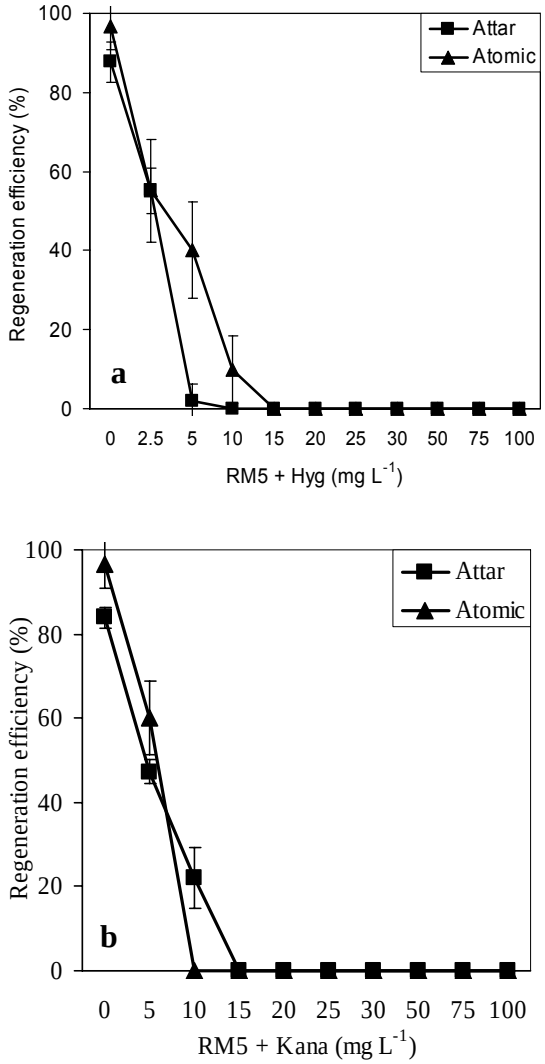


Figure 3: Antibiotic sensitivity of explants added to RM5. ■ = Attar and ▲ = Atomic cultivars. a) Hygromycin sensitivity, b) Kanamycin sensitivity. Results presented are the mean value from 3 replicates and the bars show standard deviation.

The second regeneration system involved the pre-incubation period of two week in the presence of TDZ. The application of 2.5 mg L^{-1} of hygromycin in the presence of 10 µM TDZ did not affect significantly the growth of explants of both cultivars. At 5 mg L^{-1}, the regeneration efficiency was reduced to less than 10% and 10 mg L^{-1} of hygromycin was lethal for the explants of both cultivars (Fig 4a and 4b). All the explants turned brown quickly when exposed to the hygromycin concentrations above 10 mg L^{-1}, without any expansion as was observed at 2.5 and 5 mg L^{-1}. According to some reports in literature (Opabode 2006), applying selection pressure too early can hamper the onset of morphogenic responses caused by the toxic effects of the selectable markers on the expression of genes involved in the morphogenic processes. Therefore, antibiotics were

applied after 1 and 2 weeks of the start of culture. Delaying the application of the antibiotics by one and two weeks, caused a shift in the lethal concentrations to 15 instead of 10 mg L^{-1} hygromycin, for both cultivars . This delaying period allowed the explants to expand prior to application of antibiotics. For all the assays for transformation, 5 mg L^{-1} of hygromycin was used for both cultivars, added to RM3, in the presence of TDZ to favour the explants to regenerate by reducing selection pressure.

The explants expanded at concentrations below 15 mg L^{-1} of kanamycin. They remained green for 8 weeks without any signs of morphogenesis. Shoot formation was completely inhibited at 20 and 15 mg L^{-1} of kanamycin for Attar and Atomic cultivars, respectively (Fig 4c and 4d). However, both cultivars had about 20% regeneration efficiency at 10 mg L^{-1} of kanamycin. The lethal limit of kanamycin was shifted up to 25 mg L^{-1} of kanamycin by one or two weeks delayed application. For transformation experiments, 10 mg L^{-1} of kanamycin was used, which is below lethal limit, to allow explants to initiate morphogenesis and transformation events. Newly regenerated shoots (on both media) from non-infected explants were allowed to develop on Elongation and Rooting medium (ERM) containing augmentin and either 10 mg L^{-1} kanamycin or 15 mg L^{-1} hygromycin. The concentrations used in ERM were higher than that were used during culture stage. At these concentrations, no shoot could elongate and, the development was completely inhibited when tested for non-infected regenerated shoots.

Hygromycin for *Pelargonium sp.* has only been reported once (Robichon et al. 1995) and 10 mg L^{-1} was sufficient to stop the growth of non-transformed explants. However, it has been used effectively for other plants including *Glycine max* L. (Olhoft et al. 2003; Liu et al. 2008), *Sesbania drummondii* (Padmanabhan and Sahi 2009), *Hordeum vulgare* (Bartlett et al. 2008), *Prunus domestica* L. (Tian et al. 2009), *Oryza sativa* (Twyman et al. 2002), *and Dichanthium annulatum* (Kumar et al. 2005). In our experiments, the two step application of hygromycin could be considered effective i.e. 5 mg L^{-1} in regeneration medium and 10 mg L^{-1} in rooting medium. Kanamycin, the second antibiotic used in current experiments, has frequently been used for genetic transformation of plants including *Pelargonium spp.* However, very high concentrations of kanamycin have been reported by various researchers as compared to that of our experiments. The optimized values for transformation experiments were 10 and 15 mg L^{-1} in regeneration and rooting media, respectively, for both cultivars. The reported concentrations from the literature for *Pelargonium spp.* were generally more than 50 mg L^{-1} in the regeneration and rooting medium (Boase et al. 1996; 1998; KrishnaRaj et al. 1997; Hassanein et al. 2005).

Kanamycin was considered very effective by Hassanein et al. (2005) for *Pelargonium capitatum* cv bois joly and *P x hortorum* whereas Robichon et al. (1995) have reported that the regeneration from cotyledon and hypocotyl of *P x hortorum* was not inhibited by its presence in the medium even at very high concentrations (400 mg L^{-1}).

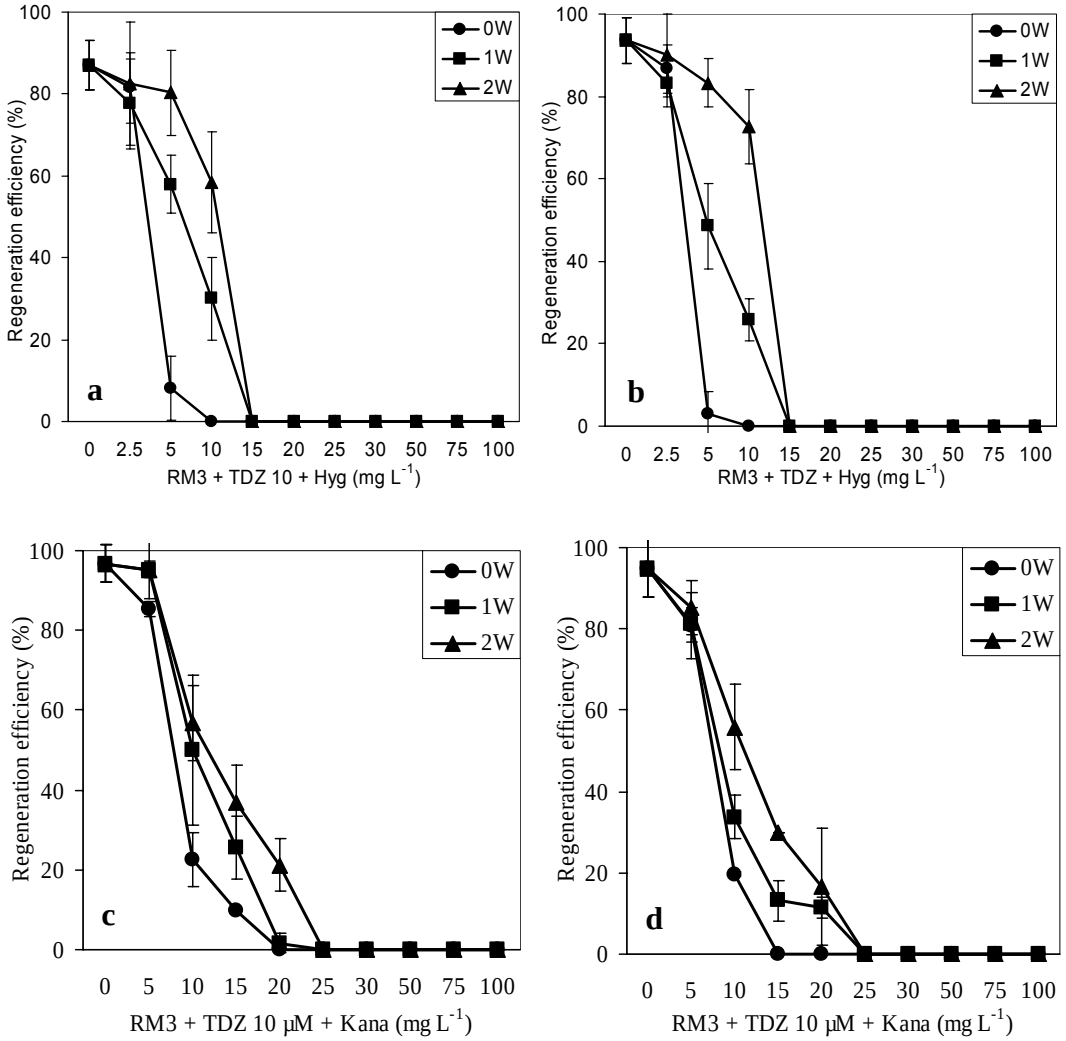


Figure 4: Response of *Pelargonium* cultivars to increasing hygromycin and kanamycin concentrations added to RM3 containing TDZ. Antibiotics were applied to the cultures at the start of culture (● = 0W), one week (■ = 1W) and 2 weeks (▲ = 2W) after the onset of culture. a) Attar of Roses; Hygromycin, b) Atomic Snowflake; Hygromycin, c) Attar of Roses; Kanamycin, d) Atomic Snowflake; Kanamycin. Results presented are the mean value from 3 replicates and the bars show standard deviation.

Another important factor that could strongly influence the transformation efficiencies is delaying the antibiotics' application which could help the explants to alleviate the sufferings from the combined stress of *Agrobacterium* and antibiotics (Liu et

al. 2008). According to our results, the delayed application of antibiotics, particularly, for Hygromycin, had no beneficial effects to get more number of rooted plants. The delayed application of kanamycin favoured the development rooted plants after infection in some cases. The best results were obtained when the antibiotics were applied after co-cultivation. The same time period was considered optimum by Hassanein et al. (2005) for *Pelargonium spp*. In contrast to these results, the increase in transformation efficiency have reported in maize with resting of 4-day (Zhao et al. 2001), 14 day (Olhoft et al. 2003) and 7 day (Liu et al. 2008) for soybean cultivars.

Assesment of capability of Agrobacteriun strains to transfer T-DNA

The functioning of *Agrobacterium* strains was tested by performing transformation of tobacco cultivar Xanthi. We obtained antibiotic resistant plants and some of them were GUS and PCR positive. However, these plants should be verified by Southern blotting. The detection of GUS activity in tobacco plants indicated that the strains were functional and capable of transforming T-DNA.

Production of transgenic Pelargonium

To develop a transformation system for scented *Pelargonium* cultivars, three strategies were assessed and are presented in Table 1. Stategy A differs from B by the pre-conditioning but the regeneration medium for both strategies is RM5. In stategy B, explants were pre-conditioned during two days on regeneration medium RM5 containing 100 µM Acetosyringone. This pre-conditioning period was followed by *Agrobacterium* infection. Many variants of the inoculation process have been tested and they concern a second round of wounding in the inoculum, with or without Acetosyringone. Strategy C is different with respect to co-cultivation medium (RM3) and regeneration medium. Regeneration medium contained TDZ that was removed after two weeks of culture start. The first strategy (A) did not produce any rooted plant for Attar cultivar, whatever the bacterial strain used for infection. Delaying the application of antibiotics did not favour to develop rooted. For Attar cultivar, seven rooted plants were obtained only when selection application was delayed by 3 weeks, after infection with EHA105 (Table 1, A4).

Table 1: Different strategies assessed for obtaining transgenic plants

Strategy	Pre-conditioning	Inoculation	CCM	Selection	RM	Rooted plants			
						Attar		Atomic	
						C58	EHA105	C58	EHA105
A	No	Explants cut in bacterial culture with AS200 and injured with scalpel.							
A1			RM5	After co-cultivation	RM5	0	0	0	0
A2			"	After 1 week	"	0	0	0	0
A3			"	After 2 weeks	"	0	0	0	0
A4			"	After 3 weeks	"	0	0	0	**7**
B	Yes: Explants injured and cultured on RM5 48H.								
B1		Explants were dipped in bacterial culture without AS and re-injured.	RM5	After co-cultivation	RM5	0	**9**	0	0
B2		2 mL of bacterial culture without AS, were added to petri dishes. No re-injury of the explants.	RM5 + AS100	"	"	0	0	0	0
B3		2 mL of bacterial culture with AS200 was added to petri dishes, No re-injury.	RM5 + AS100	"	"	0	0	0	**16**
C	No	Explants cut in bacterial culture with AS 200 and injured with scalpel.							
C1			RM3	After co-cultivation	RM3 + TDZ	**4**	**23**	**107**	**133**
C2			"	After 2 weeks	**"**	0	0	**1**	**34**

CCM = Co-cultivation medium; RM = Regeneration medium; AA = Ascorbic acid (0.1%); AS = Acetosyringone (100 µM).
Pre-conditioning: The treatment of explants prior to infection with Agrobacterium.

Pre-conditioning with 100 µM Acetosyringone (Strategy B) during 48 hours, generally increased the morphogenic response (10% more) of the explants as compared to that from strategy A, in the presence of selection. After culture on ERM in the presence of selection agents, no rooted plant was obtained for Attar and Atomic cultivar infected with Agrobacterium strain C58. Different results for both cultivars were attained after infection with EHA105 bacterial strain. Nine rooted plants of Attar were produced (Table 1, B1) and 16 plants of Atomic (Table 1, B3). These results showed that the presence of AS in the co-cultivation medium could not favour the development of rooted plants. For Atomic cultivar, the presence of AS could be important in developing rooted plants as we obtained 16 rooted plants when AS was present both in inoculation and co-cultivation media.

The third strategy (C) in the presence of TDZ 10 µM produced significantly high number of rooted plants for both cultivars as compared to two previously described methods. Delaying the application of antibiotics to culture medium did not favour the production of rooted plants. The efficiencies were higher for Atomic as compared to Attar cultivar. Four and 23 rooted plants were obtained for Attar after infection with C58 disarmed and EHA101, respectively (Table 1, C1). For Atomic cultivar, 107 and 133 rooted plants were obtained after infection with C58 disarmed and EHA105, respectively (Table 1, C1). On the basis of results from these three strategies, C1 method (Table 1) could be considered as an effective one for producing antibiotic resistant plant that could be putative transgenic plants.

The T-DNA transfer and its integration into the plant genome is influenced by many plant and bacterial factors including plant genotype, type of explant, bacterial strain, presence of phenolic substances like Acetosyringone in the culture and inoculation media to induce vir-gene, tissue damage, co-cultivation, antibiotics and the time of application (Boase et al. 1998; Reviewed in Opabode 2006; Liu et al. 2008). The two day pre-culture on regeneration medium supplemented with 100 µM AS promoted the production of kanamycin resistant rooted plants in some cases (Table 1, method B1 and B3). For Atomic cultivar, the presence of AS in pre-conditioning, inoculation and co-cultivation seemed important (Table 1, method B3), even without re-injury of the explants at the time of bacterial infection. The effects of wounding on transformation efficiencies in various species are controversial. Barik et al. (2005) has reported enhanced efficiencies in grasspea with wounding whereas in tea, transformation was best achieved using uninjured somatic embryos (Mondal et al. 2001). The presence of 100 µM AS in all the three phases i.e. pre-

conditioning, inoculation and co-cultivation, has been reported by Boase et al. (1998). However, they have not presented comparative results, explaining the role of AS.

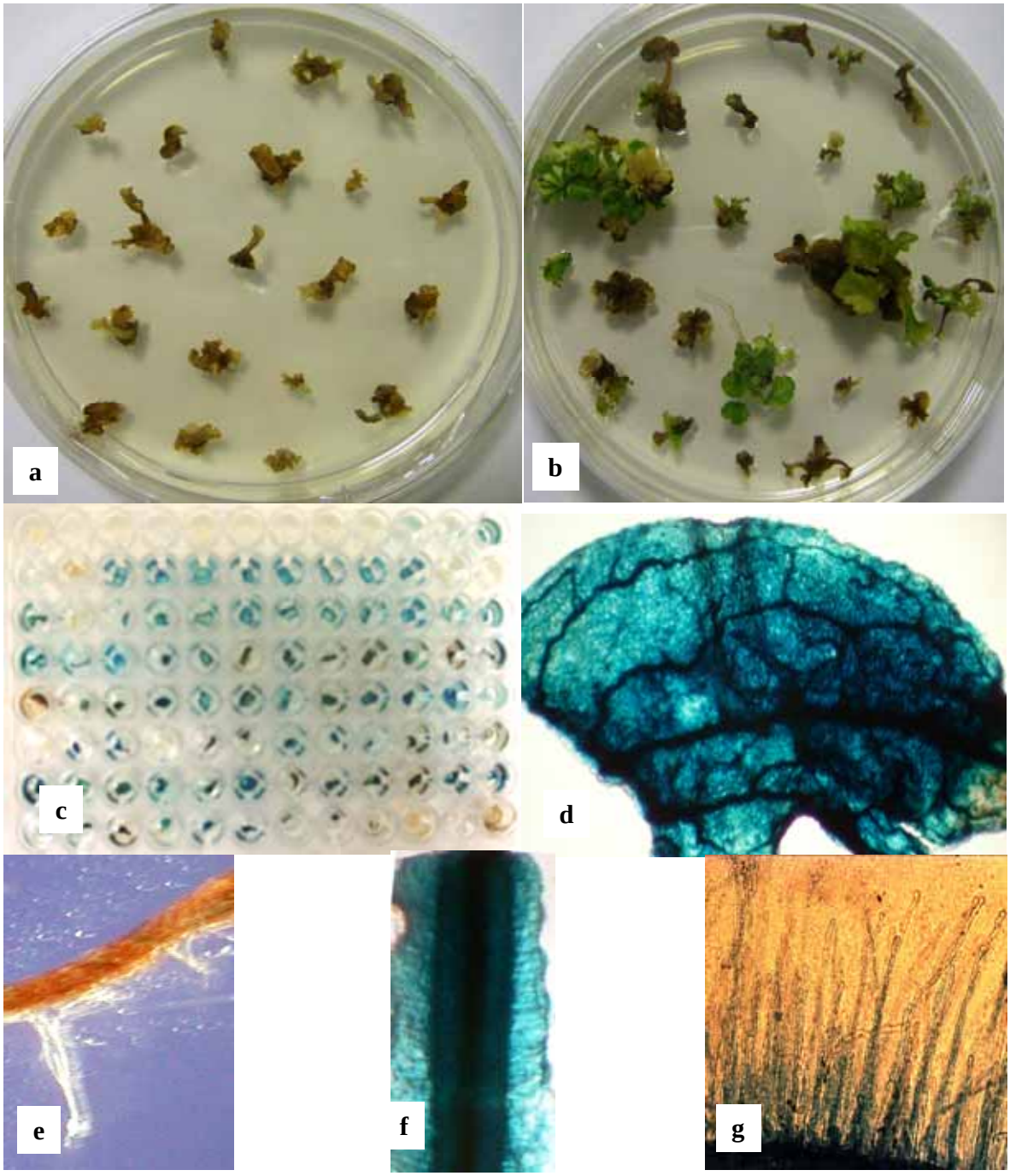


Figure 5: Selection of regenerated shoots on rooting medium containing 10 mg L^{-1} of hygromycin and GUS expression in different organs of plant (Atomic cultivar). a) non-infected control, b) Shoots regenerated from infected explants, c) histochemical GUS expression, leaf parts, d) Leaf part magnified 20x, e) root from non-infected control 30X, f) GUS expressing root, 30x g) Root hairs expressing GUS, 100x.

Histochemical GUS assay

After 3 week selection on ERM, many shoots gradually turned brown and died except for putative transformants (Fig 5a & b). The portions of leaves and roots from all the rooted healthy plants were subjected to GUS test (Fig 5c, d, e, f, g). The tissues which turned blue from rooted plants were considered as GUS positive. The results concerning potential transformation efficiencies after infect with C58 are summarised in Table 2. For Attar cultivar, all the shoots regenerated in the absence of selection agent turned brown. In the presence of hygromycin, only 26.5% explants formed shoots. Of the 394 shoots transferred to ERM, four rooted plants were obtained which were also GUS positive. For Atomic cultivar, three rooted plants were developed from the explants cultured in the absence of selection agent. Of which only one plant expressed GUS. In the presence of selection, 73% of the explants were regenerated. From the shoots transferred to ERM, 9.7% shoots developed into rooted plants. Eighty-two plants were GUS positive out of 107 rooted plants for Atomic cultivar. All the GUS positive plants also expressed GUS in the roots. The large number of shoots which were not capable of rooting was due to the application of lower level of hygromycin than lethal limit to promot regeneration. The presence of 10 mg L^{-1} of hygromycin in the rooting medium was lethal for the shoot which were not potentially transformed.

Table 2: Putative transformants after infection with disarmed *Agrobacterium* strain C58.

	Attar		Atomic	
Hgyromycin	-	+	-	+
a. No of explants cultured	88	117	97	104
b. Explants forming shoots	73	31	81	76
c. Shoots cultured on ERM	1770	394	2108	1106
d. Hyg^R rooted plants	0	4	3	107
e. Gus expressing plants-leaf	0	4	1	82
f. Gus expressing plants-root	0	4	0	82
g. PCR^+	0	2	0	20
T.E (%) = (g/c *100)	0	0.5	0	1.8

Hyg^R = Hygromycin resistant; PCR = Polymerase Chain Reaction: Amplification of *uid*A gene was perfomed to obtain the fragment of about 1250 bp.

There was also high number of escapes due to low concentration of kanamycin (10 mg L^{-1}) which turned brown with the application of higher levels (15 mg L^{-1}) in the rooting medium (Table 3). For Attar cultivar, only 4.5% of the shoots transferred to rooting medium could survive. For Atomic cultivar, the root forming shoots were 15.9%. All the rooted plants after infection with EHA105 were subjected to GUS test and no GUS positive rooted plant was observed, neither leaves nor roots, for both cultivars despite the considerable high number of kanamycin resistant rooted plants 133 (Table 3).

Table 3: Kanamycin resistant rooted plants and histochemical GUS expression, after infection with Agrobacterium strain EHA105.

	Attar		Atomic	
Kanamycin	-	+	-	+
a. No of explants cultured	70	90	100	99
b. Explants forming shoots	65	39	100	55
c. Shoots cultured on ERM	1917	511	2205	836
d. $Kana^R$ rooted plants	0	23	24	133
e. Gus expressing plants	0	0	0	0

$Kana^R$ = Kanamycin resistant

Comparing the results from infection with two different strains, the GUS expression oly in case of C58 disarmed strain could be attributed to the presence of double p35S promoter on pMDC162 vector whereas in case of EHA105 (p35S GUS INT), there is only single promoter. The difference could also be due the absence of intron on pMDC162 vector. For the time being, another hypothesis could be made for obtaining large number of kanamycin resistant plants on the basis of the observations by Robichon et al. (1995), the plant might have developed tolerance the antibiotic and they are not transformed. However, to confirm all these hypotheses, we absolutely need to perform Southern blotting. Only in the presence of the results of Southern blotting, we can argue and explain the present results clearly.

PCR screening

The DNA of Hyromycin-resistant plants of both cultivars was subjected to PCR analysis. DNA was also extracted from the non-infected controls of *Pelargonium* obtained through regeneration. The plasmid containing *uid*A gene was used as positive control. The results are shown in Fig 6. There were no bands for the negative controls. Out

of 4 rooted and GUS^+ plants of Attar cultivar, two were PCR^+ (Table 2). For Atomic cultivar, *uid*A bands were detected for 20 plants of the 82 tested. PCR positive plants can be called as putatively transformed. On the basis of PCR results, the transformation efficiency (No of PCR+ plants/ No of shoots regenerated on selective medium x100) could be 0.5% and 1.8% for Attar and Atomic cultivars, respectively (Table 2) after inoculation with C58 strain. However, it is known that PCR could also amplify bacterial sequences that are still present in the primary transformants. We performed several controls (such as amplification of sequences outside the T-DNA border) to ensure that the positive PCR responses do not result from bacterial sequences.

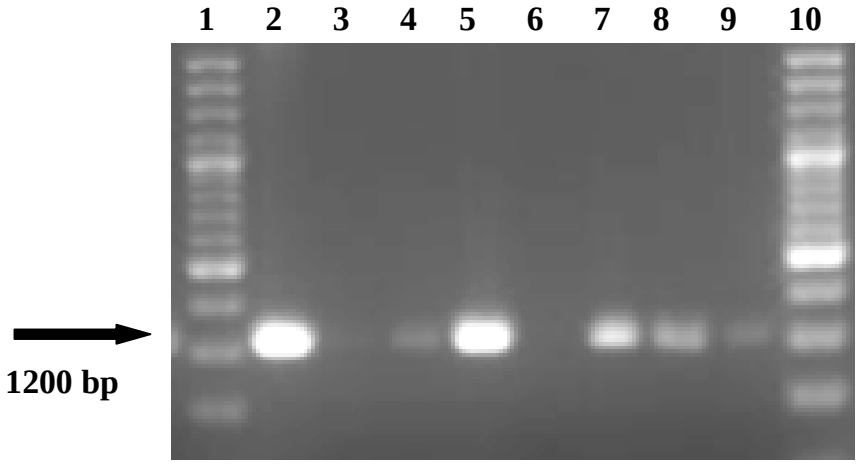


Figure 6: PCR screening for putatively transformed *Pelargonium* (Atomic) plants. 1 & 10: Markers for molecular weight, 2: GUS (*uidA* gene), 3: Non-infected *Pelargonium* (negative control), 4-9: Hygromycin resistant *Pelargonium* (Atomic) plants.

Without confirmation of all the antibiotic resistant plants through Southern blotting, it is difficult to explain or justify the results. We can make various hypotheses on the basis of bibliography. The absence of GUS activity in some of the rooted plants on selection medium could be explained by different factors including genomic position effects or gene silencing due to DNA methylation or to multi-copy insertions (Suzuki et al. 2001). Antibiotic resistant plants might be transgenic for respective marker genes, *nptII* or *hpt* and not for *uidA* (GUS) gene, which is also known as partial transformation (Robichon et al 1995; KrishnaRaj et al. 1997; Kishimoto et al 2002; Cui et al. 2004; Hassanein et al. 2005). All the GUS^+ plants were not PCR^+ which could be the result of transitory expression and the stable transformation was not happened as the PCR analysis was performed after 4-6 weeks of GUS test.

Conclusions and perspectives

In the present study, two regeneration systems previously developed for two hyeperaccumulating cultivars Attar and Atomic have been assessed for their amenability towards genetic transformation using two *Agrobacterium* strains C58 disarmed and EHA105. Of the different inoculation methods tested, the various regeneration media, the different selection schemes, it appeared that there was a genotypic effect on the efficiency of transformants (based on rooted plants and Gus assays). Atomic Snowflake gave consistently higher number of GUS expressors in rooted plants on selective medium. Using C58, only TDZ containing regeneration system produced hygromycin resistant plants. Both regeneration systems tested for obtaining putative transgenic plants produced kanamycin resistant plants but TDZ containing regeneration system was much more efficient. This could be due to the ability of TDZ to retard the yellowing of leaf explants in *Pelargonium* (Mutui et al. 2005). More number of explants remained green for long time and the bacteria had more time to transform. However, this hypothesis should be investigated to indentify the exact role of TDZ in transformation of *Pelargonium* capitatum. PCR analysis of randomly selected hygromycin resistant plants showed uidA bands. So these PCR positive plants could be putatively transformed. Confirmation of these plants is required by southern blotting to verify transgene integration pattern and could explain the different expressions obtained with the use of the two strains. This is the first report of transformation system for *Pelargonium capitatum* cultivars Attar and Atomic and the second one, using leaves as explants, after Hassanein et al. (2005) for *Pelargonium capitatum* cultivar Bois joly.

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CONCLUSIONS AND PERSPECTIVES

Lead phytoextraction with *Pelargonium*

In field experiments, Scented *Pelargonium* cultivars demonstrated their ability to hyper-accumulate Pb both on calcareous and acidic contaminated soils, without morpho-phytotoxicity symptoms and producing high biomass. Three cultivars: Attar, Atomic and Clorinda, accumulated more that 1000 mg Pb kg^{-1} DW, a limit given by Baker et al. (2000) for Pb hyperaccumulators. All the three cultivars produced more than 3 t ha^{-1} y^{-1} which is considered by Schnoor (1997), suitable for the plants to be used for phytoextraction. One of the cultivars i.e. Atomic was capable of accumulating up to 6904 mg Pb kg^{-1} DW and producing more than 45 t ha^{-1} y^{-1} which seems very promising in the perspectives of Pb phytoextraction. The maximum quantity of Pb extracted by Atomic cultivar cultivated on the soil-T (acidic soil) was 258 kg Pb ha^{-1} y^{-1} which is 8 times higher as compared to *Thlaspi* (Reeves and Brooks, 1983). The estimated time scale for total soil remediation with the *Pelargonium* plants studied were 151 years for the soil-B (calcareous soil) and 182 years for the soil-T. Soil-T contained 20 times more Pb than Soil-B. Despite an effective extraction with *Pelargonium* cultivars, the time required to cleanup is more that a century which is a major obstacle in the field application of this technique. So a lot of work is needed to make this technique applicable at large scale. In the context of field experiments, some of the perspectives are proposed in the followings:

I) *Optimisation of agronomic practices:* As for the field experiments, no optimised agronomic practices were used, we believe that there is a potential to increase biomass as well as accumulation in aerial parts through optimised NPK application and irrigation. In the literature, only a few studies have been reported on nutrient management for phytoextraction purpose. Wu et al. (2004) observed significant increase in shoot Cu uptake by Indian mustard through application of N and P in combination. However, they observed the decrease in shoot Cu uptake with increasing concentration of P applied singly. According to Wai Mun et al. (2008), the biomass of Kenaf (*Hibiscus cannabinus* L.) plants exposed to different levels of Pb was increased about six times with the application of 80 g chicken manure pellets. Moreover, the application of organic fertilizer also increased significantly the bioaccumulation capacity of individual plant. In a field experiment, Marchiol et al. (2007) recorded 10-20 times increase in the biomass of *Sorghum bicolor*

and *Helianthus annuus* with application of NPK as compared to control but there were no increase in concentration of metals including Pb, in the aerial parts of these two species. For Pb phytoextraction, careful management of applied nutrients is absolutely necessary due to its interactions in the soil with other nutrients as well as soil characteristics. For example, application of P could lead to stabilisation by forming Pb-precipitates known as pyromorphites ultimately reducing availability for plant uptake (Ryan et al., 2001). Similarly, organic fertilizers could promote fixation of Pb in the soil and making it unavailable for plants (Marchiol et al., 2007).

II) *Use of chelates:* Another option may be the judicious use of chelators to increase uptake as has been demonstrated by Hassan et al. (2008). However, concerns about the fate and impact of chelators on the environment are increasing and their use in the field conditions is becoming controversial. According to Epelde et al. (2008), application of EDTA significantly increased root Pb uptake and root-to-shoot Pb translocation as compared to that with EDDS. In a soil polluted with 5000 mg Pb kg^{-1}, the addition of 1 g EDTA kg^{-1} soil led to a value of 1332 mg Pb kg^{-1} DW in shoots whereas a shoot Pb accumulation of only 310 mg kg^{-1} DW was observed with EDDS application. Moreover, the plants treated with EDDS produced lower biomass than those treated with EDTA. However, EDDS degraded rapidly and less toxic to the soil microbial community as compared to EDTA. According to Cao et al. (2008), EDTA and EDDS were capable of mobilising 85 and 67% of Pb, respectively. Moreover, the application of these two chelates allowed extracting organic- and sulphide-bound fraction, which is most difficult to remove with plants. These results from the literature are promising but still the careful management practices are to be optimised for the application of chelates at large scale.

III) *Economic valorisation:* In the current scenario where field application of Pb phytoextraction requires further research to achieve acceptable time frame for decontamination process, the use of plants which are ornamentals and non-edible could serve as immediate solution for the soils waiting for decontamination decisions. Another type of interest could be the plants which

are sources of essential oils. Zheljazkov et al. (2006) have demonstrated that the heavy metals were not removed from the tissues during the process of steam distillation and the aromatic products were free of the metals. These facts support the use of *Pelargonium sp.* for remediation purposes. Scented *Pelargonium* cultivars offer several additional economic advantages that could offset the cost of deploying phytoremediation and render it a viable approach for remediating large area of contaminated lands: (i) Planting and harvesting of *Pelargonium* plants can be accomplished using conventional farm equipments, (ii) These ornamentals could beautify unused sites in quarantine. (iii) Harvested biomass can be broken down to yield various industrial products such as commercial-grade organic acids and alcohols. However it remains to be demonstrated that metal ions would not be present in the extracted oil or could be separated from the biomass to enable it to be fragmented into safe industrial by-products for every day consumer use.

Phytoavailability and speciation of lead studied

The results of field study indicated that soil pH, soil Pb contents, OM and cultivar type were some of the important parameters for Pb phytoextraction and could be helpful the remediation time management. In rhizosphere experiments, we demonstrated that the hyperaccumulator cultivar, Attar, was capable of modifying rhizosphere pH and DOC, and increased accumulation was observed with increasing soil Pb contents in the range of lead concentrations tested. Lead in *Pelargonium* roots was likely bound to Thiol groups (Pb-glutathione and Pb-cysteine) which could result from a detoxification process involving S-containing ligands (cysteine, GSH, phytochelatins and metallothioneins). The potential of Attar cultivar for accumulating Cd was demonstrated and it could help to remediate soils polluted by more than one element. In the followings, different parameters are discussed in detail;

I) *Availability of Pb*: The phytoavailable fraction of a metal in a soil is a core parameter to be considered for effective phytoextraction technique. Changes in rhizosphere pH are of prime importance driving the availability of elements in the soil. With decreasing pH, increased release of Pb enhancing Pb^{2+} in soil solution has been observed (Badaway et al., 2002; Sukreeypongse et al., 2002). In our case, increased Pb uptake was positively correlated with

decreasing rhizosphere pH in case of Attar cultivar. More accumulation by Attar cultivar as compared to Concolor was also positively correlated with increased DOC contents in the rhizosphere. Changes only in rhizosphere pH and DOC could not be sufficient to explain the bioavailability of Pb. There might be microbial interactions in the rhizosphere which could play an important role in Pb mobility. For future research, the special cropping device used for the present study could be employed for screening of other *Pelargonium* cultivars. This could help to either find new cultivars with better Pb phytoextraction potential or to have cultivars with distinct capabilities of modifying their rhizosphere which might help for better understanding of rhizosphere interactions influencing the mobilisation and accumulation of Pb.

II) *Localisation of Pb*: During the rhizosphere experiments, we recorded very high concentrations of Pb in roots. So the questions was, whether Pb entered into the *Pelargonium* roots or not? Microscopic studies have demonstrated the presence of some dotted and granule like structure in cell wall regions. Cell wall shape was also irregular as compared to the control (Fig 11). This granule formation requires to be further studied for indentifying what is exactly present in these structures? Other questions that have been opened might be; at what concentrations, these changes in shape could happen? What are ultimate effects on plant growth and its ability to accumulate Pb? Is there any potential effect or changes in the levels of enzymes involved in photosynthesis? Finally, is it possible to use the *Pelargonium* cultivars for phytostablistaion of Pb? As higher Pb concentrations in roots of *Juglans regia* have also been reported by Marmiroli et al. (2005) and they have proposed potential use of this species in Pb phytostabilistaion. But for this purpose, long term experiments are required to assess cost-benefit ratios and feasibility.



a) Root cell not exposed to Pb (x40) b) Root cell exposed to Pb (x40)

Figure 11: Microscopic images of root cells of *Pelargonium capitatum* cultivar Attar. a) Control, b) exposed to Pb.

III) *Speciation of Pb:* The spectrometric (EXAFS) and microscopic (ESEM) studies revealed different species of Pb present in soil and plant roots. The identification of Pb-Thiols bonding through EXAFS indicates the presence of Phytochelatins like compounds which could play an important role in detoxification mechanisms. However, these results are preliminary which provide only the informations concerning the presence and kind of compounds. In a similar context, Marmiroli et al. (2005) have reported the binding of Pb with lingo-cellulosic structure forming Pb-O bindings in roots. Further studies are required to determine the exact role of theses S-containing compounds in *Pelargonium* during metal stress. How their concentrations are altered upon exposure to Pb? Quantification of the Thiols and determination of other products i.e. sugars, polyols etc. could also help to understand the modifications in the cell wall. The precipitate formation in the roots observed by ESEM is of particular interest in bioavailability of Pb to the plants. The chemical analysis of the precipitates strongly hinted that these are phyromorphites which are the least soluble forms of Pb. Similar structure have also been reported by Cotter-Howells et al. (1999). As in the past and also in our case, the data was obtained from single cultivar. The comparison between different cultivars, particularly in hyperaccumulator and non-accumulator could help to understand the role of these precipitates. The pyromorphites are actually phosphorus (P) rich compounds and their formation may influence the availability or deficiency of P to the plant ultimately affecting photosynthetic efficiency. Their formation procedure and the timings within plant roots, their effect of phytoavailability, possible role in detoxification, their chance of disintegration over long period, these are the questions to be addressed in future research! These understandings could play an important role to develop an optimized phytoextraction strategy.

A summary of the work performed related to soil-plant interactions using scented *Pelargonium* cultivars is presented in the following figure (Fig 12).

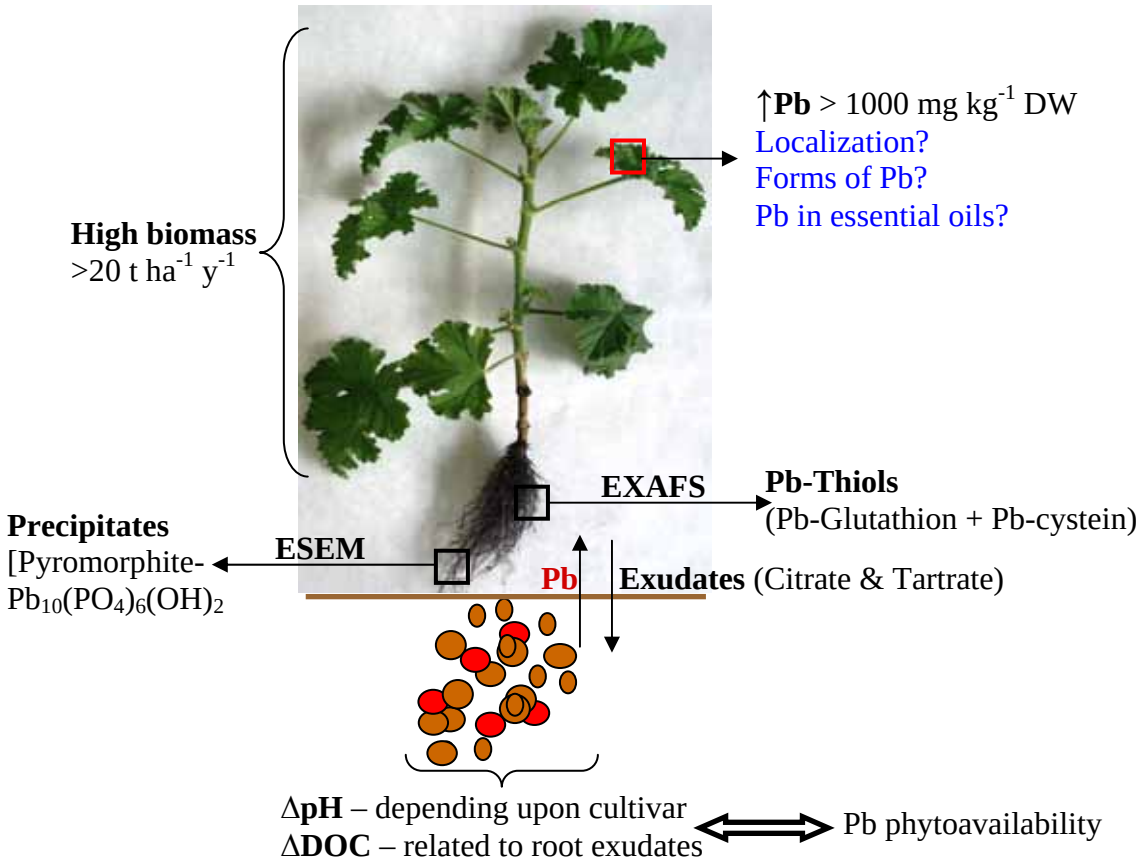

Figure 12: Schematic representation of the work focussed on the understanding of soil-plant interactions. Phrases in blue colour concern perspectives and phrases in dark colour concern the scientific conclusions of the present work.

Tools development for comprehension of Pb hyperaccumulation

Biotechnology approaches could offer the possibility of developing plants with improved agronomic properties, but its main assets concern their applications in fundamental research to decipher mechanisms involved in physiological processes. We have developed regeneration systems for two hyperaccumulating cultivars i.e. Attar of Roses and Atomic Snowflake. Using these protocols, selection regimes have been optimised and ultimately putative transgenic plants were obtained. A schematic presentation of the protocols developed is given in Fig 13.

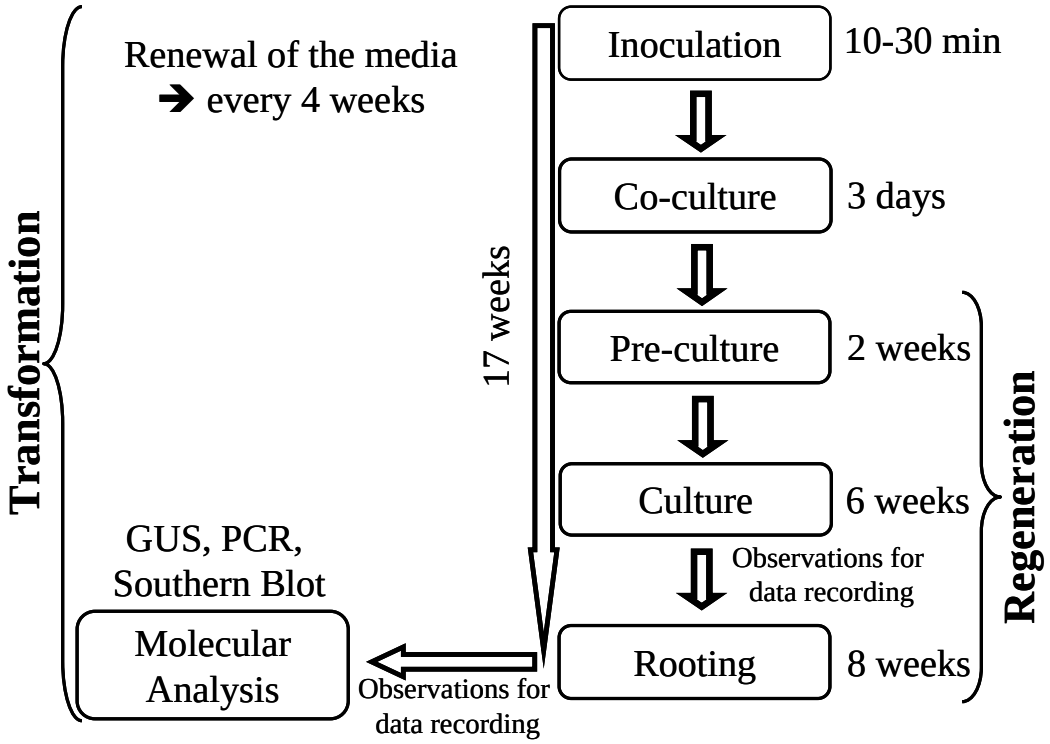

Figure 13: Schematic presentation of the optimized regeneration and transformation protocols.

The applications of these protocols and future research possibilities have been presented in the followings;

I) *Regeneration systems for Pelargonium cultivars:* An efficient regeneration system using two week pre-incubation with TDZ @ 10 µM and culture on medium supplied with BAP and NAA, 1 mg L^{-1} each. The number of shoots regenerated per plant was more than 100 which ensures the possibility of obtaining large number of plants within short time (about 18 weeks). This

system can be employed for phytoextraction by different ways. Firstly, it may help to develop a transformation system (chapter 4B). Genetic transformation is one of the well documented tools being used for understanding of molecular mechanisms and so would be the case with Pb hyperaccumulation mechanisms. Secondly, the large number of plants obtained through regeneration may have a mutant plant which might be sensitive to Pb exposure. If that is the case, it would surely ease the understanding of Pb hyperaccumulation as we will have the plants of same species and cultivar but with different response to Pb stress. It can be ideal thing for indentifying the genes involved in Pb hyperaccumulation just by comparing the mutants to the parent plants. Thirdly, as Pelargonium cultivars used in this study are male sterile and can only be propagated through vegetative means, so we can use this efficient regeneration system for obtaining large number of plants to be transferred to field conditions for phytoextraction purposes.

II) *Genetic transformation:* The regeneration systems developed earlier helped us to develop transformation systems for Attar and Atomic cultivars. The efficiencies for Attar and Atomic were 0.5 and 1.8 % calculated as; *No of PCR$^+$ plants/ number of total shoots regenerated x 100*, when inoculated with disarmed C58. However, the integration of *uid*A remains to be verified through southern blotting. Integration patterns will indeed, enable to correlate gene copy numbers to expression levels in these different events. The two aspects of this work will be to the benefit of a long-term project that our team has planned concerning the identification of the major actors involved in regulating hyperaccumulation in *Pelargonium*.

III) *Molecular mechanisms:* The availability of the tools developed during this project can help to understand molecular mechanisms involved in Pb hyperaccumulation. Cropping device used to assess plant induced changes in the rhizosphere will be applied for plant exposure to metal stress in controlled environments. Using a differential proteomic approach, identification of up or down-regulated genes during exposure of plants to the metal stress should be possible. During the preliminary steps, different proteins/enzymes produced during stress conditions could be indentified. In parallel to proteomics,

metabolomics could help to indentify different metabolites produced upon exposure to Pb stress. Comparing the results of both proteomics and metabolomics (Fig 14) may lead to the identification of a similar product or enzyme involved in the production of that product. Study of the potential role of the concerned product would help to unveil its role in Pb phytoextraction and probably, would serve a first step in understanding of molecular mechanisms of Pb hyperaccumulation by scented Pelargonium cultivars.

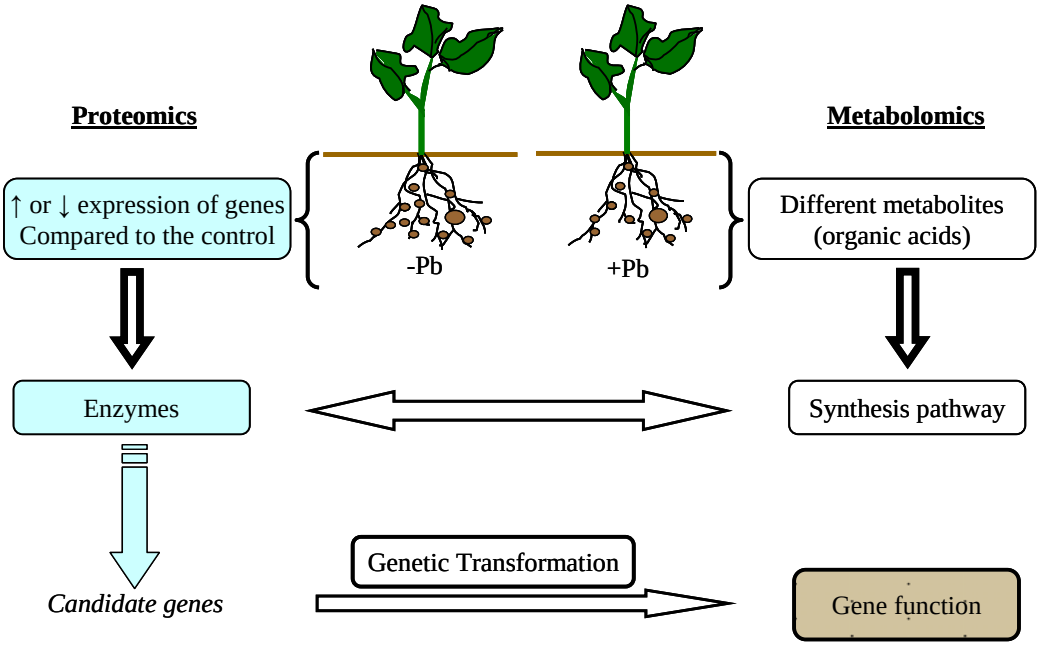

Figure 14: Methodology outline for studying gene function for Pb hyperaccumulation by scented *Pelargonium* cultivars.

Correlation with observations on Pb-speciation, localization as well as on the full spectrum of metabolites should result in the identification of sequences, described or not in protein data banks and which are impaired to one or more of the processes involved in hyperaccumulation. These studies should lead to the identification of coding sequences, which would then be expressed, or suppressed using RNAi constructs. Coupled to the use of tissue-specific promoters, these constructions should enable to dissect events during the initial steps (active transport across root plasma membranes) and later ones (translocation to shoots and specific compartment sequestration). The observed phenotypes with respect to the non transformed ones may shed some light on aspects governing hyperaccumulation in Pelargonium scented varieties. A brief scheme is presented in Fig 15 and describes

the correlations between the different parts of the present work within the scope of this ambitious project.

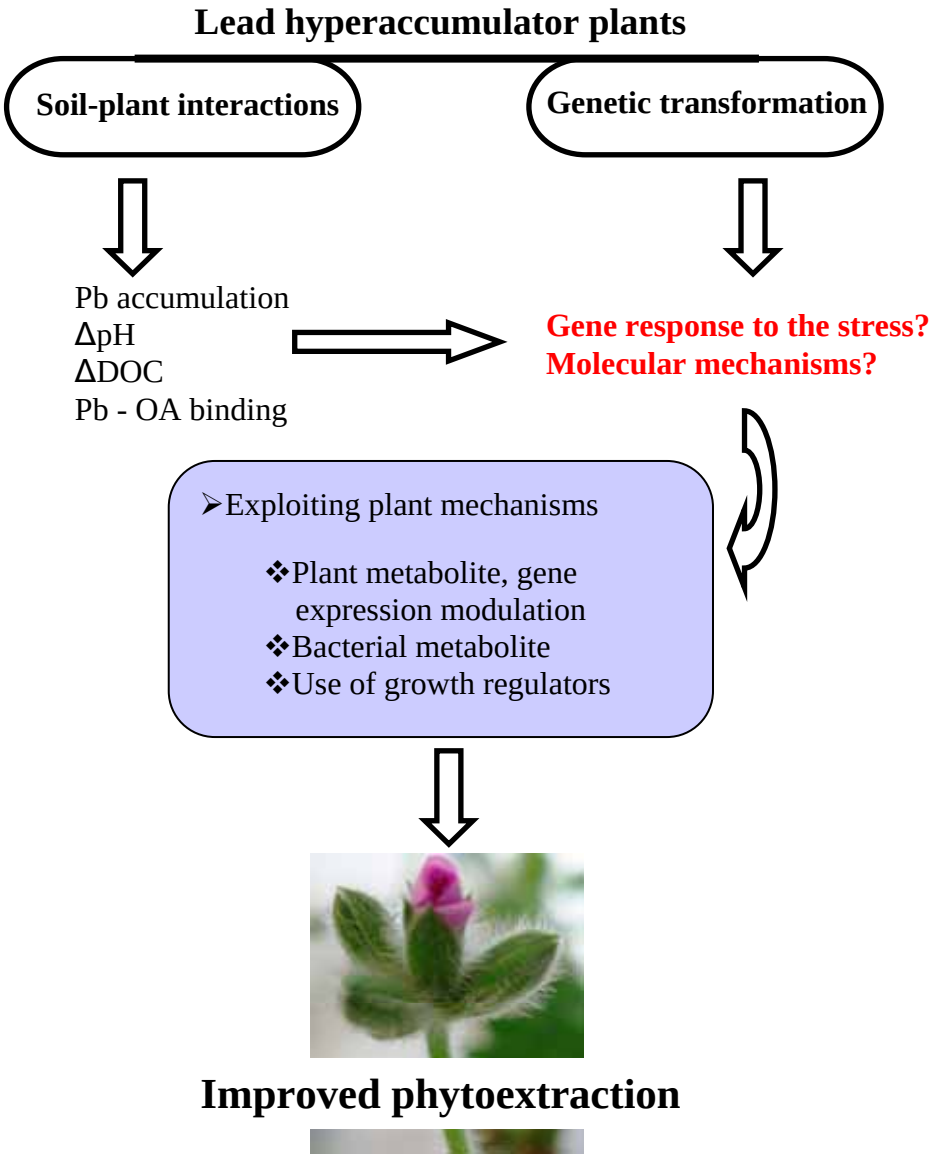

Figure 15: Prospects for development of improved phytoextraction technique using scented *Pelargonium* cultivars. OA = organic acids, DOC = dissolved organic carbon

The elucidation of genetic and molecular aspects of all these processes is a major challenge and considerable task and can successfully be addressed with model plants such as *Arabidopsis halleri* and *Thlaspi caerulescens*. It would involve analysis of natural genetic variation, genetic segregation in crosses in species with contrasting phenotypes, global analysis of the transcriptome by microarray analysis, mapping of gene networks and

QTL, mutant isolation, characterization of proteins and coding sequences with the hyperaccumulation phenotype, comparative analysis of regulation for specific genes, functional analysis by complementation in yeast mutants sensitive to metal stresses and also the demonstration *in planta* through the use of transgenic plants. In the long run, it is anticipated that the cellular and molecular mechanisms depicted would drive us to the use of natural additives to cultures that would promote metal extraction from soil.

Conclusions et perspectives (présentation synthétique) :

Cultivés en plein champs sur deux sols contrastés (calcaire et acide) contaminés, les *Pélargonium* odorants ont démontré leur capacité à accumuler le Pb dans leurs parties *aériennes, sans symptômes de phytotoxicité, et avec une biomasse élevée. L'utilisation de Pélargoniums* odorants pour l'assainissement a plusieurs avantages économiques qui pourraient jouer en sa faveur et rendre cette approche viable : (i) la plantation et la récolte des plants de *Pélargonium* peuvent être accomplies en utilisant des équipements classiques agricoles, (ii) ces plantes ornementales peut embellir les sites non utilisés en quarantaine, (iii) la commercialisation d'huiles essentielles, acides organiques et alcools qui pourraient être commercialisés après vérifier de leur qualité.

La quantité maximale de plomb extraite observée est 258 kg Pb ha^{-1} an^{-1} (Atomic cultivar sur le sol-T). Les temps estimés pour décontaminer les sols sont respectivement de 151 ans pour le Sol-B et 182 ans pour le sol-T. Ces résultats indiquent que le pH du sol et la teneur en Pb sont des paramètres qui influencent fortement l'efficacité de la phytoextraction. Nous avons démontré que le cultivar hyperaccumulateur, Attar, était capable d'acidifier sa rhizosphère et d'augmenter significativement la concentration en carbone organique dissout. Une augmentation de la phyto-accumulation du plomb a été observée avec l'augmentation de la teneur en Pb du sol, dans la gamme des concentrations en plomb étudiées. Le plomb dans les racines de *Pélargonium* est probablement lié à des thiols (Pb-glutathionne et Pb-cystéine), qui pourraient résulter d'un processus de détoxification impliquant des ligands contenant sulfure (cystéine, GSH, phytochelatines et metallothioneins). Le potentiel du cultivar Attar pour accumuler Cd pourrait offrir une possibilité de décontaminer les sols pollués par plus d'un élément.

Les temps de remédiation de l'ordre du siècle limitent cependant le développement de la phytoextraction. Ces durées de traitement pourraient être réduites en optimisant la fertilisation, l'irrigation et en utilisant des chélates comme cela a été démontré par Hassan et al. (2008). Toutefois, l'utilisation de chélates (type EDTA) dans des conditions de terrain est de plus controversée en raison de leur impact potentiellement délétère sur l'environnement.

L'identification des entités moléculaires liées au plomb dans différents tissus, est un des préalables à l'élucidation des mécanismes régulant les différentes étapes qui aboutissent au phénotype hyperaccumulateur. Le couplage de ces informations aux outils

des biotechnologies pourrait mettre en lumière, les gènes qui gouvernent l'absorption, la translocation et l'accumulation des éléments métalliques dans les plantes. Ceci permettrait d'aboutir à la découverte des voies métaboliques, impliquées et donc des enzymes et des produits qui régissent le phénotype résultant. Le pré-requis indispensable à ce type d'étude est l'outil de la transformation génétique, qui est le seul moyen de démontrer la fonction d'un gène et son implication dans le phénotype. Nous avons développé des systèmes de régénération pour deux cultivars hyperaccumulateurs : Attar of Roses et Atomic Snowflake qui ont débouché sur un protocole de transformation génétique efficace, de l'ordre de 35%, pour Atomic Snowflake, en nous basant sur le nombre de plantes ayant amplifié par PCR, une séquence spécifique correspondant au gène *uidA* par rapport au nombre total de plantes éprouvées. Mais il est connu que la PCR peut aussi amplifier des séquences bactériennes, encore présentes dans les transformants primaires. Bien que nous ayons effectué les contrôles nécessaires (amplification de séquences à l'extérieur des bordures du T-DNA) pour nous assurer que les réponses positives aux PCR ne résultaient pas de l'amplification de séquences bactériennes résiduelles, seules des expériences d'empreintes par la technique de Southern, permettra de démontrer l'intégration au niveau du chromosome nucléaire et de corréler le nombre de copies insérées à l'expression dans ces différents événements. Les deux aspects de ce travail pourront être mis à profit d'un projet novateur et ambitieux à long terme que nous prévoyons concernant l'identification des principaux acteurs impliqués dans la régulation d'hyperaccumulation chez les plantes et le *Pélargonium* en particulier.

Le dispositif de micro-cultures sera utilisé pour évaluer les changements induits par les éléments traces métalliques chez le *Pélargonium* au niveau de la rhizosphère. En utilisant une approche de protéomique fonctionnelle différentielle, l'identification des gènes surexprimés ou réprimés au cours de l'exposition des plantes au stress métallique sera étudiée. Les résultats obtenus sur la spéciation du Pb, la localisation, ainsi que sur tout le spectre des métabolites en conditions de non exposition seront comparés à ceux des conditions exposées aux stress métalliques. Ces différences devraient aboutir à l'identification des séquences de protéines, décrites ou non dans les banques de données et qui seraient impliquées dans l'hyperaccumulation. Ces séquences protéiques permettront de déduire l'identification de séquences nucléiques codantes, qui seront ensuite exprimées à l'aide de promoteurs performants, ou réprimées en utilisant des constructions RNAi. Couplé à l'utilisation de promoteurs tissus-spécifique, ces constructions devraient permettre de disséquer les événements survenus au cours des premières étapes (le transport

actif à travers la membrane plasmique racinaire) et des successives (translocation vers les parties aériennes et la séquestration dans le compartiment spécifique). Les phénotypes observés par rapport à ceux des phénotypes non transformés pourraient expliquer les aspects relatifs à hyperaccumulation dans les *Pélargonium* odorants.

L'élucidation des aspects génétiques et moléculaires de l'ensemble de ces processus est un défi majeur et une tâche qui ne peut être réalisée qu'avec les plantes modèles *Arabidopsis halleri* et *Thlaspi caerulescens,* hyperaccumulateurs de métaux. Ce projet comporte différentes stratégies faisant appel à de très nombreuses compétences transversales, à savoir : des analyses des variations génétiques naturelles, le criblage au niveau de la ségrégation des croisements des espèces avec des phénotypes contrastés, l'analyse globale du transcriptome à l'aide de bio-puces, la cartographie des réseaux de gènes et des QTL, l'isolement de mutants, la caractérisation de protéines et de séquences codantes du phénotype hyperaccumulateur, la recherche de promoteurs spécifiques, l'analyse comparative de la régulation pour les gènes spécifiques, l'analyse fonctionnelle par complémentation de mutants de levure sensible au stress métallique et également la démonstration *in planta* avec l'utilisation de plantes transgéniques. À long terme, la compréhension de ces mécanismes cellulaires et moléculaires devrait permettre d'aboutir à la caractérisation d'effecteurs qui pourraient être utilisés en tant qu'additifs naturels qui permettraient d'augmenter l'extraction des métaux du sol.

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ANNEXES

Annex I: Arshad et al. 2008. 4th European Bioremediation Conference, Chania, Crete, Greece. 3-6 Sep, 2008.

4th European BioRemediation Conference

ID 119

PHYTOEXTRACTION OF LEAD BY SCENTED *PELARGONIUM* CULTIVARS: FIELD AND CONTROLLED-CONDITION EXPERIMENTS

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ABSTRACT
Phytoremediation strategies are emerging as innovative tools for environmental restoration of metal-contaminated sites. The main objective of this project is to study phytoextraction of Pb by scented *Pelargonium* cultivars, with emphasis on Pb phytoavailability, transfer, cellular localization in different parts of the plants and its interactions with others metals. Field tests, rhizosphere studies and hydroponics experiments were conducted with different scented *Pelargonium* cultivars. In the field, 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. In rhizosphere experiments, both cultivars (Attar of Roses and Atomic Snowflake) tested, lowered their rhizopshere pH suggesting enhanced availability of lead for plant uptake. Results for short term hydroponics indicate that Pb concentrations are maximum in roots followed by stems, old and young leaves. Atomic Snowflake accumulated up to 8374 ± 1068 mg kg^{-1} of Pb in roots over a period of one week. Microscopic observations depict that accumulated lead was localized in cell wall areas.

Key words: Phytoextraction, *Pelargonium*, lead, hydroponics, rhizosphere, cyto localisation.

1. INTRODUCTION

Presence of high concentrations of lead in the environment poses serious risks to human health causing irreversible orders of nervous, digestive and reproductive systems, or anaemias [1] and to plant vitality and metabolism [2]. Anthropogenic activities induce high levels of lead in soils [3]. Battery manufacturing is the principal current use of lead and batteries represent 70% of the raw material for lead recycling industries reaching upto 160,000 t per annum in France [4]. *Ex situ* decontamination using physico-chemical techniques is labour intensive, expensive, and affects the soil physical and biological properties. Phytoremediation, the use of plant species to clean up soil and water, is gaining a lot of importance in recent times since it is a cost effective, promising and environmentally friendly technology [5]. 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 [6]. 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 [7]. 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 [8] and hyper accumulating plants often produce low biomass [9]. Current research on the development of phytoremediation is thus targeted towards the identification of new plant species [7]. Certain cultivars of scented Pelargonium have been reported as hyperaccumulators particularly, lead. *Pelargonium sp*. Frensham, exposed to a mixture of metals (lead + cadmium + nickel) accumulated in excess of 4.72% Pb + 0.44% Cd + 0.52% Ni per kg of root DW, as well as 0.17% Pb + 0.07% Cd + 0.14% Ni per kg of shoot DW within 14 days [10].

Three of the six cultivars tested in field conditions: *P. capitatum* cultivars Attar of Roses, Atomic Snowflake and *P. domesticum* cultivar Clorinda are hyperaccumulators of lead and were very effective in Pb-phytoextraction [5]. Finally two cultivars: Attar of Rose and Atomic Snowflake, were selected for further controlled experiments to evaluate the effect of the source of plants either from *in vitro* cultures or from directly rooted plants in organic soil, duration of the exposure to lead in hydroponics and to contaminated soil, total lead concentration, other metals and changes induced by the plants to rhizosphere soil in order to improve the knowledge of the parameters and mechanisms involved in the absorption of lead by *Pelargonium*.

2. MATERIALS AND METHODS

2.1 Plant and soil materials

Six scented *Pelargonium* cultivars (*P. capitatum* cultivars Attar of Roses, Atomic Snowflake and Concolor Lace, *P. domesticum* cultivar Clorinda, *P. graveolens* cultivar Rose Geranium, and *Pelargonium sp.* cultivar Sweet Leaved), propagated from cuttings of commercial plantlets were grown in 0.5 L pots containing organic soil, in the greenhouse, and regularly irrigated with nutrient solution. Soil (0 – 10 cm depth) was collected from the vicinity of an active Pb Recycling Plant were used for field and rhizosphere experiments. The soil located near the factory in North-Western France, is contaminated by atmospheric fallout and surrounded by agricultural land. Lead contamination is present to a major extent (1830 mg kg^{-1} of dry soil), but other metals and metalloids (Zn, Cu, Ni, As and Cr) have also been detected [4]. The soil was air–dried and sieved to 2 mm.

2.2 Experiments

2.2.1 Field experiments

The six *Pelargonium* cultivars were all field-tested in five replicates at a density of 15 plants per m^2 on an experimental plot of 50 m^2. Plants were grown under natural agro-climatic conditions, with neither fertilisation nor optimum irrigation so as to assess the feasibility of the remediation process in the natural context of an industrial site over a period of 150 days.

2.2.2 Rhizosphere studies

Modified cropping devices [11] specially designed for rhizosphere studies were used for these experiments. A 6-week period was necessary to allow for the development of a dense root system to cover the mesh in hydroponics. The nutrient solution was renewed fortnightly and level was adjusted with distilled water. After 6 weeks, the plants in cropping devices were put in contact with soil place in round plates (10 g of air dry soil per plate) in such a way that roots were separated from the soil only by a nylon mesh, therefore allowing the study of the soil rhizosphere. The concentration of KNO_3, $Ca(NO_3)_2$, KH_2PO_4 and $MgSO_4$ in the nutrient solution was reduced to $1/10^{th}$ and that of Fe–EDTA to half the concentration used during rooting stage in order to reduce the competition between nutrient elements and lead for their uptake by the plants. The culture period lasted 2 weeks. Soil in bottom plates without plants was considered as controls. These also allowed monitoring of pH changes due to nutrient solution. At the end of the culture, young leaves + petioles, old leaves, stems and roots were harvested separately and analysis for Pb and other metals was performed. The soil was collected to determine the rhizosphere pH and lead availability and study the soil solution. There were 5 replicates for each cultivar and control.

2.2.3 Hydroponics

Twelve cm cuttings, from plants of Attar and Atomic cultivars, were grown in 0.5 L pots containing compost soil. The cuttings were alternately irrigated with nutrient solution and tap water. Rooted plantlets were ready for use after 4 weeks. Sixteen plantlets were transferred to 20 L containers with nutrient solution for hydroponics. Three levels of Pb (0, 1 and 10 ppm) were applied to the nutrient solution over periods of one week. The nutrient solution level was maintained by regularly topping up with distilled water for the one week experiment. For the two weeks experiment, solution was renewed after one week. Containers were placed in green-house with a 14/10 h light/dark cycle, temperature 26/22 °C and 60/70% relative humidity. At the end of the culture, young leaves + petioles, old leaves, stems and roots were harvested separately and analysis for Pb and other metals (Ca, Fe, K, Mg and Na) was performed. There were 5 replicates for each cultivar.

2.3 Soil and Plant analysis

Moist rhizosphere soils were taken for pH_{CaCl2} and lead extraction by $CaCl_2$ performed in order to estimate the availability of lead. Soil pH [12] and $CaCl_2$–Pb were measured from a 0.01M $CaCl_2$ (1:5 soil/solution on dry basis) solution prepared in de–ionized water. After pH measurements, clear supernatants were collected for Pb determinations and stored at 4°C before analysis. For soil solution extraction, humid soil was taken in Eppendorf tubes and centrifuged at 15000g, during 15 min at 20 °C temperature. The supernatants were collected for pH and Pb content. Plant samples were weighed and oven dried at 80 °C for 48 h and then dry weight was measured. The plant samples were ground with grinder (ball grinder), then digested in concentrated HNO_3 (65%, specific density, 1.38) in DigiPrep. The analysis was performed by ICP-OES.

2.4 Microscopy

Root, leaf and stem segment of control and contaminated scented *Pelargonium* cultivars were cut into small pieces and prepared for microscopic observation using the protocol provided by Microscopy Platform (IFR40), Toulouse, France.

3. RESULTS AND DISCUSSION

3.1 Screening of Pelargonium cultivars for Pb accumulation potential under field conditions

Pelargonium cultivars grown on the contaminated soil were screened for their Pb accumulation capacity. The results presented in table 1 indicate that there are significant differences in the total biomass in terms of dry matter of cultivars grown on contaminated soil as well as total Pb contents of different cultivars and lead quantity extracted per plant. According to Baker et al. [13], shoot concentrations of Pb in hyper-accumulators should be in the range of 1000 mg kg^{-1} DW. The field experiments on soil-B showed the ability of Attar, Clorinda and Atomic *Pelargonium* cultivars to accumulate Pb in the same range. Schnoor [14] reported that any plant useful for phytoremediation, should be vigorously growing, easily harvestable and exhibit a biomass of more than three tons DW ha^{-1} y^{-1}. Our results demonstrate that such is the case with the scented *Pelargonium* cultivars used in this study.

Cultivar	Shoot dry weight (DW) g $plant^{-1}$	$[Pb]_{Shoots}$ DW mg Pb kg^{-1}	Pb extracted per plant mg Pb $plant^{-1}$
Attar	66 ± 13 c	1467 ± 134 c	96.7 ± 19 b
Clorinda	56 ± 14 c	1182 ± 336 b	64 ± 15 b
Atomic	28 ± 6 ab	1107 ± 193 b	30.5 ± 7 b
Graveolens	39.5 ± 9.5 b	435 ± 157 a	17 ± 7 a
Sweet	22.5 ± 6.5 a	549 ± 76 a	12 ± 1.5 a
Concolor	16 ± 3 a	468 ± 91 a	8 ± 2.5 a

Table 1: Biomass, total lead content in shoots and lead extracted per plant, for field cultures. For each bioassay, mean values with different letters are significantly different ($p < 0.05$) as measured by LSD Fisher test.

3.2 Changes in pH and phytoavailable Pb by scented *Pelargonium* cultivars in contaminated soil

The pH of the control soil was unchanged. On the other hand, pH and Pb concentrations variations were induced by Pelargonium cultivars (Table 2). Both cultivars Attar of Roses and Atomic Snowflake acidified the rhizosphere soil by changing pH up to 0.7 pH units. There was no difference between the cultivars concerning these two parameters. In both cases ($CaCl_2$ and centrifugation), the final concentrations of Pb in rhizosphere soil were strongly decreased by plant cultures as compared to the control soils. These results suggest that in the present experimental conditions *Pelargonium* were able to considerably reduce the labile fraction of lead. For longer time experiments, it can be supposed that a transfer of lead from the solid phase to the soil solution will be achieved.

	pH_{CaCl2}	Pb_{CaCl2} (mg kg^{-1})	$pH_{Soil\ Solution}$	$Pb_{Soil\ Solution}$ (mg kg^{-1})
Control	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
Atomic	6.78 ± 0.04	7.8 ± 2.3	7.3 ± 0.1	14 ± 3.6

Table 2: Effect of cultivars on soil pH and Pb concentration measured in 0.01*M* $CaCl_2$ and of extracted soil solution during two weeks soil-plant contact.

3.3 Lead accumulation in hydroponics and interactions with nutrient elements

Table 3 represents the concentrations in roots of lead and different metals when Atomic Snowflake cultivar exposed to different concentrations of lead provided in nutrients solution. Atomic Snowflake cultivar accumulated up to 8374 ± 1068 mg kg^{-1} in roots, 8.2 ± 1.7 mg kg^{-1} in stems, 2.3 ± 1.4 mg kg^{-1} in old leaves and 1.2 ± 0.8 mg kg^{-1} of Pb in young leaves over a period of one week. Similar trends were observed for Attar of Roses (data not shown). According to our preliminary results, Atomic Snowflake cultivar had greater concentrations of Pb, Ca, Fe and K and lower concentrations of Mg and Na as compared to Attar of Roses. In general, Ca concentrations were decreased in both cultivars at higher levels of Pb applied. Iron concentrations were increased at higher levels of Pb applied in nutrient solution. At 1 mg L^{-1} of Pb applied, K concentrations were higher in young and old leaves as compared to control and lower at 10 mg L^{-1} as compared to 1 mg L^{-1}.

Treatment	Concentrations in roots (mg kg^{-1})					
Pb mg L^{-1}	Ca	Fe	K	Mg	Na	Pb
Control	18584 ± 1684	9238 ± 1306	20908 ± 2847	5378 ± 594	859 ± 71	10.9 ± 0.9
1	19057 ± 3472	9933 ± 712	20055 ± 1315	4799 ± 1676	1010 ± 423	971 ± 315
10	15229 ± 2698	14332 ± 1224	18731 ± 3708	4949 ± 2548	927 ± 235	8374 ± 1068

Table 3: Effect of Pb on concentrations of different cations in roots of cultivar Atomic Snowflake in hydroponics during one week.

3.4 Localization of lead in hyperaccumulating Pelargonium cultivars

After one week of contact with lead in hydroponics, dense globular areas were observed in root cells around cell walls (Fig 1) as compared to control that potentially might be deposition of lead as observed by Panich-pat et al. [15] in roots of *Typha angustifolia* grown on lead contaminated soil. We will confirm our preliminary results with electron microscopie coupled with chemical analysis.

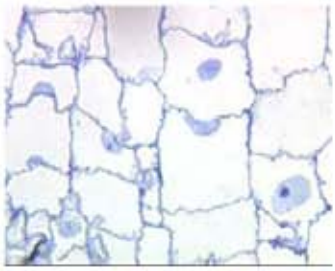

Figure 1 Micrograph of roots of scented Pelargonium cultivar Atomic Snowflake exposed to 1ppm of Pb observed in Optical microscopy optique after colouration (60x03)

4. CONCLUSION AND PERSPECTIVES

The difference of Pb concentrations in aerial parts of *Pelargonium* cultivars in field and hydroponics could be explained on the basis of period of exposure that was very short in hydroponics as compared to field experiments. Secondly, root architecture was completely different in both types of cultures. Further experiments are in process in order to acquire information on the mechanisms involved in the lead phytoaccumulation by various *Pelargonium* cultivars.

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Annex II: Arshad et al. 2008. 4th European Bioremediation Conference, Chania, Crete, Greece. 3-6 Sep, 2008

4th European BioRemediation Conference

ID 120

OPTIMISATION OF *IN VITRO* PLANT REGENERATION FOR LEAD HYPERACCUMULATOR SCENTED *PELARGONIUM* CULTIVARS

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ABSTRACT

A pre-requisite to understanding gene function in plants is the availability of a genetic transformation protocol, itself dependent on an efficient regeneration system. This work reports for the first time, the optimisation of culture conditions for *in vitro* plant regeneration of two Pb hyperaccumulator scented *Pelargonium* cultivars (*P. capitatum* cultivars Attar of Roses and Atomic Snowflake), using leaf discs as starting material. Of the different hormone combinations tested, 2 mg L^{-1} each of BAP and NAA, added to MS basal medium proved the best, resulting in regeneration efficiencies of about 84% for Attar of Roses and 97% for Atomic Snowflake. Elongation and rooting of plantlets, from both cultivars, were achieved on media containing 1.5 mg L^{-1} of IAA. Regenerated plants were successfully transferred from *in vitro* to greenhouse conditions for both cultivars on organic soil while in hydroponics, the survival efficiencies were 78 and 60% for Attar of Roses and Atomic Snowflake respectively.

Key words: *Pelargonium*, regeneration, *In vitro* culture, Pb hyperaccumulator.

Abbreviations: MS- Murashige and Skoog; BAP- N^6-benzylaminopurine; NAA- α-naphthaleneacetic acid; IAA- Indole-3-acetic acid.

1. INTRODUCTION

High concentrations of lead in soil are deleterious to the environment. Decontamination methods are expensive and disruptive to soil biogeochemical properties. Phytoremediation, the use of plants to clean up soil and water, is being addressed as a cost effective, promising and environmentally friendly technology [1,2]. Approximately 400 plant species have been identified as metal hyperaccumulators [1] and susceptible for use in phytoremediation strategies. Investigation of hyperaccumulators having better characteristics in terms of accumulation, tolerance, high biomass and rapid growth is continuously in progress. Considerable effort has been devoted to understand the physiological mechanisms underlying metal uptake and transport and sequestration. Despite the progress made, the genetic and biochemical processes involved are still poorly understood [3], especially for lead.

Over the past two decades, genetic transformation has emerged as a powerful tool to explain gene function and for crop improvement, either by modulating existing traits or introducing new ones. This allows the exploration of many aspects of plant physiology and biochemistry through analysis of gene function and regulation that cannot be studied by any other experimental method. Genetic transformation systems are based on the introduction of foreign DNA into plant cells, followed by the regeneration of such cells into whole fertile plants. The success of any plant genetic transformation strategy depends upon the availability of an efficient *in vitro* regeneration system coupled with appropriate selection regime and a method for introducing DNA into plant cells [4]. The efficiency of the regeneration system is greatly influenced by different factors particularly, the plant organ to be used as donor explants, the basic culture medium, growth hormones and their altogether interactions [5].

Our long term objective aims at understanding plant Pb hyperaccumulation, and specifically the genes that are involved in the phytoextraction process. We field-tested six Pelargonium scented cultivars for their lead accumulation potential. Three of them (*P. capitatum* cultivars Attar of Roses, Atomic Snowflake and *P. domesticum* cultivar Clorinda) were indeed very effective in Pb-phytoextraction [1] and could be qualified as lead-hyperaccumulators. In preliminary studies, we screened more than 15 scented Pelargonium cultivars for their aptitude to *in vitro* culture. Two cultivars Attar of Roses and Atomic Snowflake were selected on the basis of both better performances in phytoextraction as well as in *in vitro* culture. The objective of this work was to develop an efficient regeneration method as a prerequisite to genetic transformation experiments. We report for the first time, the optimization of plant regeneration for the scented cultivars *P. capitatum* cvs Attar of Roses and Atomic Snowflake.

2. MATERIALS AND METHODS

Scented *P. capitatum* cultivars (Attar of Roses and Atomic Snowflake), propagated from cuttings of commercial plantlets (http://www.pepinieres-heurtebise.com/), were grown in pots containing organic soil, in the greenhouse, and regularly irrigated with tap water. For regeneration purposes, the two latest fully developed leaves were harvested and washed with tap water for 30 min. Leaves were then dipped in 95% ethanol for 30 sec, immersed during 20 minutes in a filtered 2.5% Calcium Hypochlorite suspension. Explants were then rinsed thrice with sterile double deionised water. Sterilized leaves were cut into 0.25 cm^2 pieces, and about 10-12 pieces were placed adaxial side down on regeneration medium. Petri dishes were maintained at 25°C in a culture room illuminated with fluorescent lamps providing 70 µmol m^{-2} s^{-1}. For experiments requiring darkness, Petri dishes were wrapped in aluminum foil paper. The relative humidity was maintained at 70% with a 14/10h photoperiod provided by 40-W fluorescent lamps.

Different regeneration media containing variety of combinations of auxins and cytokinins were tested to optimize culture medium (Table 1). BAP concentrations ranged from 0.5 to 3.0 mg L^{-1}. For NAA, concentrations varied between 0.05 to 2.0 mg L^{-1}. Explants were cultured on MS basal medium [6], MS vitamins, 30 g L^{-1} sucrose and specific hormone concentrations. The medium was adjusted to a pH of 5.8 ± 0.05 prior to autoclaving (20 min at 121°C, 1 bar) and solidified using 8 g L^{-1} Bacto Agar. Explants were regularly examined and the number of shoots developed per explant in culture was scored after 6 weeks. Regeneration efficiencies were calculated as the percentage of explants forming more than 10 shoots distributed over the whole explant, relative to the total number of explants. Regenerated shoots of *P. capitatum* cvs Attar of Roses and Atomic Snowflake were transferred into Vitrovent boxes (12-16 individual plants per box) containing 100 ml half strength MS medium (MS/2 Macro elements), 15 g L^{-1} of sucrose. Optimization of auxin levels required for best rooting and elongation was performed on media supplemented with three levels of IAA, 1.0, 1.5 and 2.0 mg L^{-1} containing 0.8% of agar as gelling agent,. Plants were sub-cultured every 4 weeks. Rooted plantlets were removed from Vitrovent boxes after 8-10 weeks and acclimatized either in organic soil or in hydroponics.in the green house at 22-26 °C and 60-70% relative humidity with a 14 h photoperiod (30–45 µmol m^{-2} s-1).

Nursing in organic soil: Initially plants were placed in a closed mini-green-house for one week to maintain high relative humidity. After one week, exposure to the green house conditions was introduced by gradually opening the apertures of the chamber every two days. After two weeks, plants were completely exposed to green house conditions. The survival efficiency was determined 4 weeks after the plants were introduced into the greenhouse. Plants transferred to soil were regularly irrigated with tap water and fortnightly with nutrient solution. The greenhouse transplants were grown to maturity until flowering in greenhouse conditions (21/19 °C day/night, relative humidity 70 %).

Nursing in hydroponics: Nutrient solution was as follows: 5000 μM KNO_3, 2000 μM $Ca(NO_3)_2$, 5000 μM KH_2PO_4, 1500 μM $MgSO_4$, 46 μM H_3BO_3, 9 μM $MnSO_4.H_2O$, 0.1 μM $MoNaO_4.2H_2O$, 0.9 μM $CuSO_4.5H_2O$, 15 μM $ZnSO_4.7H_2O$ and 90 μM Fe–EDTA. The same regime of acclimatization was applied as for nursing in organic soil to allow recovery after transfer from *in vitro* to greenhouse conditions.

3. RESULTS AND DISCUSSION

3.1 Optimization of shoot regeneration medium from leaf discs

In order to evaluate the efficiency in inducing shoot regeneration, two growth hormones, (BAP and NAA) were tested in different combinations resulting in five regeneration media. The composition and percentage shoot formation are given in Table 1. For Attar of Roses (Attar), there was no significant difference between three media RM1, RM3 and RM5 where BAP/ NAA ratios were 1, 1 and 3, respectively. Regeneration efficiency was the highest on RM3 medium containing 2 mg L^{-1} each of BAP and NAA with 84% of the explants producing shoots. For Atomic Snowflake (Atomic) cultivar, the regeneration medium containing 2 mg L^{-1} each of BAP and NAA (RM3) gave the best results inducing 97% of the explants to shoot formation followed by RM1 and RM5. There was no significant difference between RM1 and RM3 which had similar BAP/NAA ratio. According to Hassanein and Dorion [7], the combination of zeatin and BAP was necessary to achieve high regeneration frequency. Direct shoot regeneration, with 100% of explants inducing shoots and buds, was obtained from *in vitro*-leaf discs of *P. capitatum* cultivar Bois Joly on MS/2 medium supplemented with 0.5 mg L^{-1} NAA, 1.0 mg L^{-1} BAP and 1.0 mg L^{-1} zeatin in darkness. In their case, NAA proved to be most effective compared to IAA and 1.0 mg L^{-1} of BAP and, zeatin was better than 2.0 mg L^{-1}. Agarwal and Ranu [8] tested different media containing a range of BAP (0.22–1.1 mg L^{-1}) concentrations in combination with 0.35 mg L^{-1} of IAA by using leaves as explants for three cultivars of *Pelargonium x hortorum*. They observed the

responses in a narrow range of BAP (0.22-0.56 mg L^{-1}) and above 0.56 mg L^{-1} of BAP, the regeneration was completely suppressed. According to Saxena et al. [9], with *Pelargonium graveolens*, regeneration efficiency was low (24–76%) from leaf segments as compared to young explants, petioles. They obtained maximum number of shoots per leaf explant on the medium containing 5.0 mg L^{-1} of Kinetin and 1.0 mg L^{-1} of NAA. In contrast to this, the efficiency was 100% for *P. capitatum* cv Bois joly from leaf discs as explants [7].

Regeneration Medium (RM)	Growth regulators (mg L^{-1}) BAP	NAA	Ratio BAP/NAA	Regeneration efficiency (%) Attar	Atomic
RM1	1.0	1.0	1	73.4 ± 10.6	85.0 ± 13.2
RM2	1.0	0.5	2	25.0 ± 5.0	10.0 ± 5.0
RM3	2.0	2.0	1	83.9 ± 2.5	96.7 ± 5.8
RM4	2.0	0.05	40	1.7 ± 2.9	29.3 ± 23.4
RM5	3.0	1.0	3	80.2 ± 13.6	54.7 ± 6.3

Table 1: Effect of different growth hormones on regeneration of *P. capitatum* cultivars Attar of Roses and Atomic Snowflake.

It is well known that amenability to tissue culture is strongly species and genotype dependent. There are few studies that have related to optimization of regeneration conditions for scented Pelargonium cultivars. These include *Pelargonium sp.* 'Frensham' [10] and *Pelargonium graveolens* [9]. Our results confirm the strong genotype dependency for regeneration conditions as growth hormone requirements are different for the two *P.capitatum* scented cultivars. Our data show that, for *P. capitatum* cultivars Attar of Roses and Atomic Snowflake, the BAP/NAA ratio is important. Increasing the concentrations of BAP and NAA while maintaining the 1:1 ratio, regeneration efficiencies were increased. Higher concentration of BAP seemed promotive to the regeneration of Attar but 2 mg L^{-1} of BAP proved to be the best.

3.2 Gelling agent, shoot elongation and rooting medium

In view of increasing the efficiency of regeneration, physical conditions i.e. gelling agent and light/darkness were optimized. We tested Agar, Gelrite and Phytagel at concentrations suggested by the suppliers. There was no significant effect on the regeneration of both cultivars using those different gelling agents (data not shown). Results concerning the influence of IAA on rooting efficiency are shown in Fig 1. The rooting efficiency was below 50% for both cultivars when plantlets were transferred to Elongation and Rooting Medium (ERM) containing 1 mg L^{-1} of IAA. This increased to about 85% with 1.5 and 2 mg L^{-1} of IAA for both cultivars, with no significant differences between the two concentrations. Hassanein and Dorion [7] transferred the plantlets onto MS/2 medium gelled by Phytagel 0.3% (w/v) and supplemented with 1 mg L^{-1} IAA for rooting. All transferred plantlets rooted after 3–4 weeks. Boase et al. [11] tested the effect of NAA gradient (0, 50, 100, 150 and 200 µg L^{-1}) in rooting medium for *Pelargonium x domesticum* Dubonnet. The average root number increased with increasing concentrations of NAA and shorter roots with larger diameters were observed at higher concentrations (100, 150 and 200 µg L^{-1}). In the present study, the trends were similar in spite of the different rooting hormone i.e. IAA. Extremely dense rooting, enhanced root diameter and restricted growth were observed on the ERM containing 2.0 mg L^{-1} of IAA. In our case, the optimized IAA concentration was 1.5 mg L^{-1}. This provided the best rooting efficiency in addition to the better physical state of the rooted plantlets.

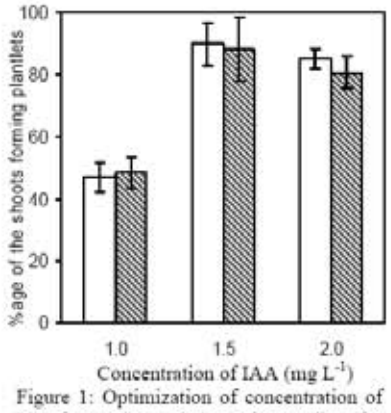

Figure 1: Optimization of concentration of IAA for rooting and elongation medium for *P. capitatum* cultivars Attar of Roses (□) and Atomic Snowflake (▨).

3.3 Acclimatization of *in vitro* plants

Rooted plants of both cultivars were transferred to organic soil and hydroponics. Fig 2 shows the acclimatization/survival efficiency, defined as the percentage of plants that survived the transition from *in vitro* to greenhouse conditions. All the plants were maintained in green house till flowering. On organic soil, efficiency was 100% for both cultivars as compared to hydroponics where it decreased to 78 and 60% for Attar and Atomic respectively. However, in spite of lower efficiencies of acclimatization in hydroponics, growth of plants was more vigorous and was quicker in hydroponics as compared to plants grown on organic soil. These

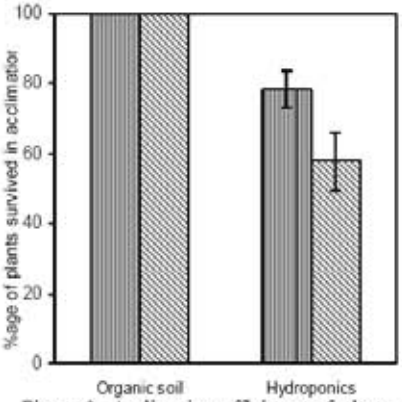

Figure 2: Acclimation efficiency of plants of *P. capitatum* cultivars Attar of Roses (▥) and Atomic Snowflake (▧) to organic soil and hydroponics.

efficiencies are higher than those reported by Agarwal and Ranu [8] where only 25-50% of the shoots/buds could be rooted and acclimatized from *in vitro* to green-house conditions.

4. CONCLUSION

An efficient regeneration system for green-house plants of *P. capitatum* cultivars Attar of Roses and Atomic Snowflake has been developed using leaves as starting explants on MS medium containing BAP and NAA each at 2 mg L^{-1}. The regenerated shoots were successfully rooted and acclimatized to green-house. This regeneration scheme is now being assessed for its suitability to genetic transformation.

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Annex III: Arshad et al. 2007. Info Chimie 482 : 62-65.

PHYTOREMÉDIATION

Des géraniums odorants pour décontaminer les sols

Baptisé « Pelargonium », ce projet du laboratoire Ecolab, installé à l'Ensat à Toulouse, vise à utiliser des géraniums pour débarrasser des sols, en environnement industriel, de leurs métaux lourds.

M. Arshad, J. Silvestre, J. Kallerhoff et C. Dumat [1]
Ecolab UMR 5245 INPT-CNRS-UPS [2].

En raison de leurs nombreuses utilisations industrielles actuelles et passées, et de leur persistance dans l'environnement, les métaux constituent désormais à l'échelle de notre planète un problème majeure en terme de qualité des sols (Chen et al., 2006), mais aussi d'autres compartiments : eaux superficielles et souterraines, végétaux cultivés (projet BAPPET, Pollutec 2007), etc. Les métaux et métalloïdes présents dans un sol constituent en effet un risque sanitaire potentiel au dessus d'une concentration seuil qui est fonction de nombreux paramètres : (i) abiotiques (nature et spéciation chimique de l'élément, pH, température…) et (ii) biotique (caractéristiques physiologiques et comportement de la cible) (Dumat et al., 2001 et 2006a; Ferrand et al., 2006).



De nombreuses techniques ont donc été développées afin de réduire la quantité totale et/ou la fraction disponible des métaux dans les sols pollués (He et al., 2005). Les techniques les plus couramment utilisées comme l'excavation du sol, « nettoyé » ensuite par des solvants, ont l'avantage de réduire très rapidement et efficacement les concentrations totales en métaux. Mais ces techniques sont coûteuses et peu respectueuses de l'environnement bio-géo-chimique du sol, qui suite à ce type de traitement devient parfois un simple matériau de remblai (Ensley, 2000). Certaines plantes sont capables d'adsorber (phénomène de surface) et d'absorber les métaux au niveau de leurs racines, puis de les transloquer vers leurs parties aériennes. La récolte répétée de ces parties aériennes réduit progressivement la concentration des métaux dans le sol.

Le terme phytoremédiation regroupe l'ensemble des techniques qui utilisent des plantes afin de réduire la concentration ou la disponibilité des métaux d'un sol contaminé. La phytoremédiation pourrait représenter une option pertinente dans le cadre de la gestion des sites industriels en activité ou en cessation d'activité (Tanhan et al., 2007; Alkorta et al., 2004). En effet, comme cela est schématisé sur le schéma ci-contre, certaines plantes judicieusement sélectionnées sont susceptibles de réduire rapidement (quelques semaines ou mois) et à moindre coût les transferts des métaux dans l'air (poussières de sol contaminé), les eaux et le sol (horizons plus profonds et nappes phréatiques éventuelles ou parcelles voisines) : le risque sanitaire lié aux métaux disponibles présents dans le sol est donc en conséquence réduit également (Deng et al., 2006). La phyto-extraction des métaux du sol, entraînant une réduction significative de leur concentration totale, est certes relativement lente (en compa-

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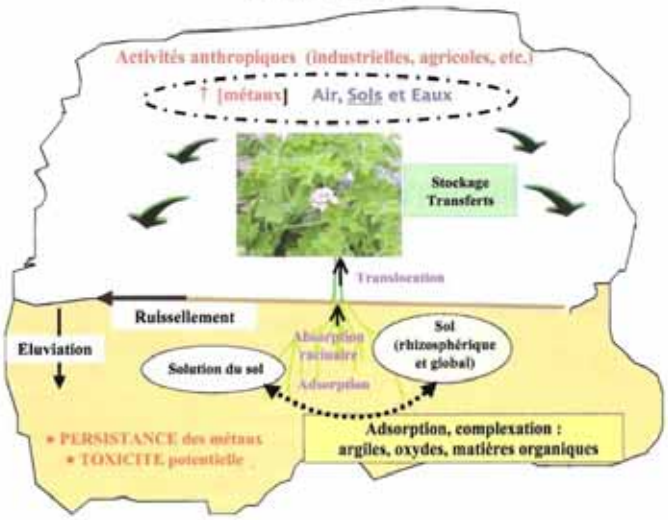
(2) http://www.ecolab.ups-tlse.fr/ Ensat PO box 107, avenue de l'Agrobiopole, 31326 Auzeville-Tolosane, Castanet-Tolosan cedex, France

raison avec des techniques physico-chimiques), mais elle est effective, et peut constituer une première étape intéressante dans le cas de nombreux sites pour lesquels une décision de remédiation est en attente.
Selon He et al. (2005), la phytoremédiation est une technique prometteuse mais encore peu étudiée en conditions réelles de plein champ, ce qui limite son développement. De plus, des recherches sur de nouvelles plantes sont nécessaires (Tanhan et al., 2007). Dans ce contexte, des chercheurs du laboratoire Ecolab (équipes des docteurs J. Sanchez et E. Pinelli) ont mis en place en étroite collaboration avec la STCM (http://w3.stc-metaux.com), entreprise de recyclage de batteries au plomb, volontaire en matière de management environnemental, des expériences sur site industriel afin de tester sur plusieurs années, et deux sols aux caractéristiques contrastées, les capacités d'extraction du plomb de six cultivars de géraniums odorants (Pelargonium) et de déterminer les mécanismes en jeu dans le transfert sol-plante et l'hyperaccumulation des métaux.

Premiers résultats, perspectives et applications

Trois cultivars de Pelargonium ont démontré un intérêt certain pour la phytoremédiation des sols contaminés. Cultivés en plein champ et en conditions d'utilisation courantes sur sites industriels en activité, à la fois sur sol acide très fortement contaminé en plomb (environ 40 000 mgPb.kg-1 sol sec) et sur sol calcaire moyennement contaminé (environ 2 000 mgPb.kg-1) ces trois cultivars présentent : (i) une forte concentration en plomb dans leurs parties aériennes (6900 mgPb.kg-1 MS) et sont des hyper-accumulateurs selon les critères de Baker et al. (2000) ; (ii) une biomasse élevée (45 tonnes par hectare et par an) en comparaison avec d'autres plantes (Schnoor, 1997) ; (iii) et aucun signe de toxicité même à très fortes concentrations (plus de ►

LE DEVENIR DES MÉTAUX DANS LE SYSTÈME SOL-PLANTE



Le transfert des métaux présents dans un sol contaminé, vers les plantes utilisées en phytoremédiation, 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).**
Ce transfert est difficile à modéliser de façon simple en raison des nombreuses interactions qui se produisent dans le système sol-plante, entre les constituants des sols et les métaux et entre la plante et le sol (Dumat, 2006b, HDR de l'INPT).

PHYTOREMÉDIATION **Des géraniums odorants**

> Pour une décontamination et un embellissement des sites industriels.

► 10 ppm) en Pb, Ni et Cd en solution (Dan et al., 2000). En utilisant nos données de terrain, les durées nécessaires : (i) pour extraire la totalité du plomb présent dans les premiers centimètres des deux sols, ou (ii) pour réduire la fraction estimée de plomb labile, bio-disponible et donc induisant directement un risque sanitaire et environnemental ; ont été calculées pour différents scénarios (type de sol, climat, cultivar, apport de chélates ou non). Quelques décennies permettraient d'extraire le Pb présent sur ces deux sites industriels. Ces durées pourraient être réduites à quelques années en optimisant les conditions de fertilisation et arrosage (KrishnaRaj et al., 2000) par augmentation de la biomasse, et/ou en ajoutant un chélate type EDTA qui favorise la solubilité du plomb (Huang et al., 1997).

De plus les géraniums odorants testés en phytoremédiation ont plusieurs avantages significatifs en comparaison avec les techniques physico-chimiques classiques de remédiation : (i) une réduction de la fraction labile des métaux en quelques semaines, (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, (iv) un coût bien moindre, (v) le respect de la biodiversité et du fonctionnement biogéochimique du sol, et (vi) un possible fractionnement intégré du végétal qui pourrait dégager des bénéfices. Ce dernier point est à l'étude en collaboration avec l'Ensiacet (laboratoire LCA et Critt Agroressources).

Les débouchés industriels envisageables concernent donc la remédiation des sols contaminés par les métaux. La réduction des risques de contamination par les métaux de l'environnement des sites industriels (par la réduction des transferts) et l'amélioration de l'impact visuel des sites industriels sont visés par ce projet. Les chercheurs impliqués dans ce projet remercient la STCM (P. Pradère) pour sa collaboration scientifique et son soutient financier. Ce projet est également soutenu par le programme national EC2CO-Cytrix 2007-2008 pour l'étude du devenir du plomb dans le système sol-plante et la SFERE (http://w3.sfere.fr). ■

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Annex IV: Arshad et al. 2008. 4th European Bioremediation Conference, Chania, Crete, Greece. 3-6 Sep, 2008. *Poster*

OPTIMISATION OF *IN VITRO* PLANT REGENERATION FOR LEAD HYPERACCUMULATOR SCENTED *PELARGONIUM* CULTIVARS

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Introduction

Scented *Pelargonium* cultivars

Ornamentals

Source of aromatic oils
Cosmetics
Perfumes
Aromatherapy



Hyperaccumulators of lead/heavy metals

Phytoextraction (*Arshad et al. 2008)

Our approach

Screening of Pb Hyperaccumulators (Scented *Pelargonium*), field cultures. → Regeneration method → Genetic transformation protocol → Understanding gene function in lead phytoextraction and hyperaccumulation.

Objective: Development of an efficient regeneration system as a prerequisite to genetic transformation experiments.

Materials and methods

Scented *P. capitatum* cultivars (Attar of Roses and Atomic Snowflake) → Two latest fully developed leaves as explants → Disinfection





Regeneration media : MS medium + hormones (Table 1) + agar (0.8%), pH 5.8 ± 0.05. → Shoot elongation and rooting: MS/2 macro elements + MS micro + MS vitamins + 1.5% sucrose + IAA + agar (0.8%). pH 5.8 ± 0.05. → Acclimatization

Regeneration Medium (RM)	Growth hormones BAP	NAA	Ratio BAP/NAA
RM1	1.0	1.0	1
RM2	1.0	0.5	2
RM3	2.0	2.0	1
RM4	2.0	0.05	40
RM5	3.0	1.0	3

Table 1. Hormonal combinations.

Results and discussions

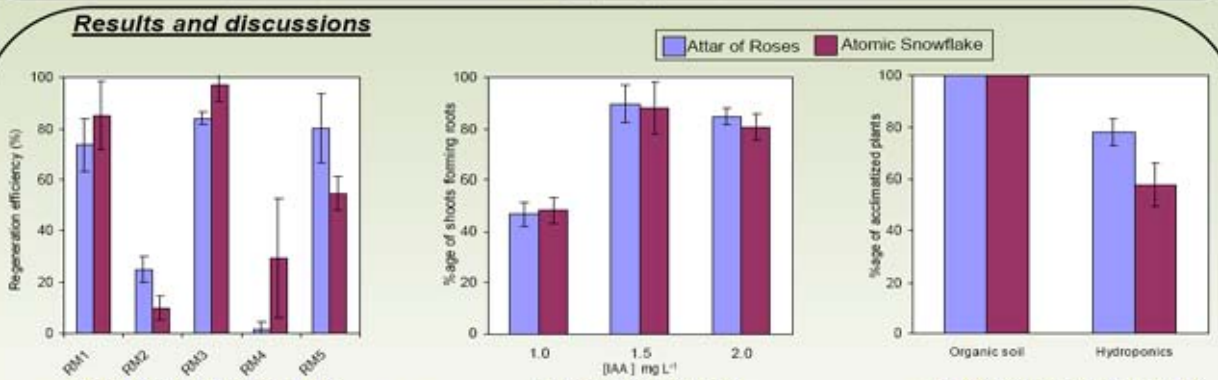


Fig 1. Regeneration efficiency : [% of explants forming shoots (≥10 shoots/explant)].

Fig 2. Rooting efficiency

Fig 3. Acclimatization efficiency

Regeneration efficiencies :
84% and **97%** regeneration efficiencies on **RM3** for **Attar** and **Atomic** respectively.

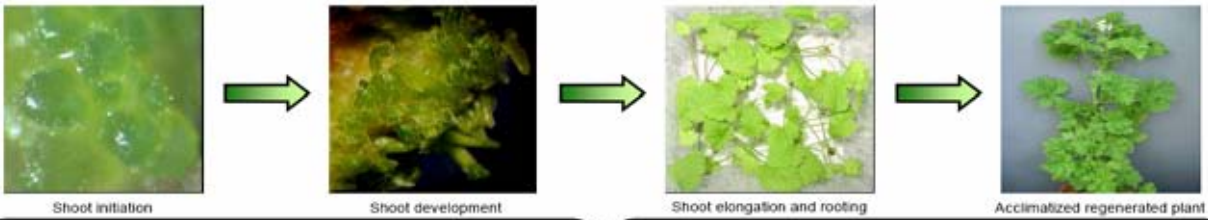
Regeneration media :
Attar cultivar gave high efficiencies on RM1, RM3 and RM5. However, Atomic cultivar only performed well on RM1 and RM3 (Fig 1).

BAP/NAA ratio :
Increasing the concentrations of BAP and NAA while maintaining the 1:1 ratio, increases in regeneration efficiencies.

IAA concentration :
1.5 mg L^{-1} for elongation and rooting medium (Fig 2).

Acclimatization efficiency : 100% on organic soil as compared to hydroponics (Fig 3).

Fig. 4: Different stages of regeneration.



Shoot initiation | Shoot development | Shoot elongation and rooting | Acclimatized regenerated plant

Conclusions

- Efficient regeneration protocol for Attar of Roses and Atomic Snowflake cultivars of scented *Pelargonium*.
- Best regeneration medium, RM3: MS + 2 mg L^{-1} each of BAP and NAA.
- High acclimatization efficiency to green-house conditions.

Perspectives

- Assessment of suitability of this system to genetic transformation – in process.
- Understanding gene function in phytoextraction and hyperaccumulation of lead.

Abbreviations: MS- Murashige and Skoog; BAP- N6-benzylaminopurine; NAA- α-naphthaleneacetic acid; IAA- Indole-3-acetic acid.

*Arshad, M., J. Silvestre, E. Pinelli, J. Kallerhoff, M. Kaemmerer, A. Tarigo, M. Shahid, M. Guiresse, P. Pradere and C. Dumat (2008) "A field study of lead phytoextraction by various scented *Pelargonium* cultivars", Chemosphere, 71, 2187-2192.

Annex V : Arshad et al. 2007. Pollutec, Nov, 27-30, 2007. Paris - Nord Villepinte, France.

Poster

Des Pélargoniums odorants pour la phyto-remédiation de sols contaminés par les métaux

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Contexte Scientifique et Objectifs

-Les métaux: problèmes majeurs en terme de qualité du sol, eaux superficielles et souterraines, végétaux cultivés.
-Un risque sanitaire potentiel.
-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 du projet:
➢Tester les capacités d'extraction du Pb de 6 cultivars de Pélargonium odorants sur 2 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

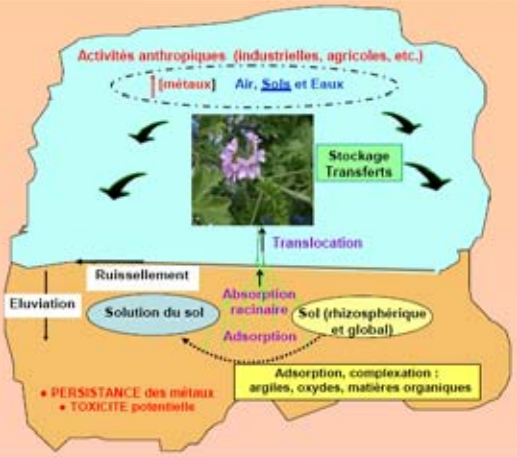
Le transfert des métaux présents dans un sol contaminé, vers les plantes utilisées en phyto-remédiation, va dépendre en particulier des caractéristiques :

(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 (Sol-B: 1830 mg Pb kg^{-1} de sol sec) et sol acide fortement contaminé (Sol-T: 39250 mg Pb kg^{-1} de sol sec).
➢Cultures en plein champ et en conditions d'utilisation courantes.

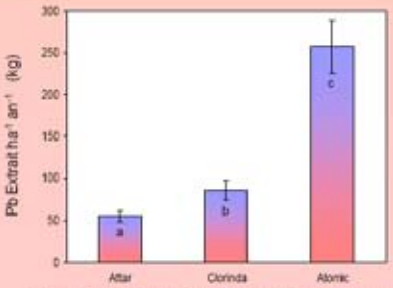


Séchage 80 °C pendant 48H → Broyage → Digestion des échantillons à l'acide (DigiPrep) → Filtration des aliquotes → Mesure [Pb] à l'ICP-AES





Résultats et applications



Quantité de Pb extraite par les cultivars Attar, Clorinda et Atomic, sur sol acide fortement contaminé.

⇒ *Arshad et al., INFO-CHIMIE, 2007*

- 3 cultivars ont un intérêt certain en 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) Diminution de la 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 Pélargoniums à l'étude qui pourrait dégager des bénéfices (Zheljazkov et al., 2006).

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Prix des Techniques Innovantes pour l'Environnement, Pollutec 2007, Paris - Nord Villepinte, France.

ABSTRACT

Metal removal from contaminated soils using plants can provide an environment friendly solution. However, its successful application on a large scale is still limited due to unavailability of plants with desired set of characteristics i.e. hyperaccumulation, high biomass and rapid growth. The objective of this work was to assess the potential of scented *Pelargonium* cultivars for lead (Pb) extraction under field conditions, plant induced rhizosphere changes, soil factors influencing availability of Pb and to develop an efficient genetic transformation protocol for the selected cultivars. Of the six scented *Pelargonium* cultivars field-tested, three cultivars (Attar of Roses, Clorinda and Atomic Snowflake) accumulated more than 1000 mg Pb kg^{-1} DW, with high biomass reaching up to 45 tons ha^{-1} y^{-1} dry matter. During assays in controlled conditions, Attar of roses (Pb hyperaccumulator) significantly acidified its rhizosphere and increased Dissolved Organic Carbon (DOC) concentration as compared to Concolor Lace (non-accumulator), probably due to enhanced exudation in response to the metal stress. Lead concentrations in both cultivars were best correlated with $CaCl_2$ extracted Pb. Extended X-ray Absorption Fine Structure (EXAFS) and Environmental Scanning Electron Microscopy─Energy Dispersive x-ray Spectroscopy (ESEM-EDS) demonstrated that Pb was mainly complexed to organic acids within plant tissues whereas the dominant form in soil was $PbSO_4$. Parallel to the soil-plant Pb transfer assays, a genetic transformation protocol was optimized in view of better understanding biochemical processes involved in lead hyperaccumulation and gene function, in the future. The best regeneration scheme was based on the pre-culture of explants on 10 µM TDZ (Thidiazuron) in addition to 1 mg L^{-1} each of N^6-benzylaminopurine (BAP) and α-naphthaleneacetic acid (NAA), followed by removal of TDZ from the culture medium. Kanamycin and hygromycin proved to be efficient selectable markers for genetic transformation. Two *Agrobacterium* strains, C58 and EHA105 harboring binary vectors carrying the selectable marker genes *hpt* and *nptII* were chosen for transformation experiments. They also contained the *uidA* gene coding sequence as reporter gene. After infecting with C58, 4 and 107 rooted plants on hygromycin-containing medium were obtained for Attar and Atomic cultivars, respectively. The four Attar plants and 82 Atomic plants expressed Gus in leaves, petioles, stems and roots as expected with a sequence driven by the 35S constitutive promoter. Polymerase Chain Reaction (PCR) screening was performed on Gus positive plants and 2 and 20 plants of Attar and Atomic were screened as PCR positive, respectively. After infection with EHA105, 23 and 133 rooted plants were obtained on kanamycin selection medium but none of these expressed Gus. Southern hybridization patterns will enable to correlate gene copy numbers to expression levels in these different events. The optimized protocols could be used for understanding molecular mechanisms of Pb accumulation and improvement in phytoextraction technique.

Key words: Phytoremediation, scented *Pelargonium*, Pb, hyperaccumulators, phytoavailability, speciation, DOC, pH, regeneration, genetic transformation, *Agrobacterium.*

RESUME

L'utilisation des plantes pour décontaminer les sols pollués par les métaux est une solution respectueuse de l'environnement. Mais le développement de cette technique à grande échelle est encore limité en raison de l'indisponibilité de plantes avec les caractéristiques souhaitées (hyperaccumulation, biomasse élevée et croissance rapide). Les objectifs de ce travail étaient d'évaluer le potentiel de plusieurs cultivars de *Pélargonium* odorants pour l'extraction du Pb au champ, étudier la disponibilité du plomb en relation avec l'activité rhizosphérique et développer un protocole de transformation génétique. Parmi les six cultivars de *Pélargonium* odorants testés au champ, trois : Attar of Roses, Clorinda et Atomic Snowflake ont accumulé plus de 1000 mg kg^{-1} Pb, avec une forte biomasse. Pendant les expérimentations en conditions contrôlées, Attar of roses (le cultivar hyperaccumulateur) acidifie sa rhizosphère et augmente la concentration en COD significativement plus par rapport Concolor Lace (le cultivar non hyperaccumulateur), sans doute en réponse à la pollution métallique. Les concentrations en plomb dans les deux cultivars sont corrélées avec l'extraction au $CaCl_2$. Les analyses par EXAFS et ESEM-EDS ont montré que le plomb présent dans les racines était principalement sous forme de complexes organiques alors que les sulfates de plomb prédominent dans le sol. Parallèlement à ces essais, un protocole de transformation génétique a été mis au point en vue de mieux comprendre les processus biochimiques impliqués dans l'hyperaccumulation et la fonction des gènes, Le système de régénération optimisé se base sur la pré-culture d'explants sur un milieu contenant 10 µM TDZ + 1 mg L^{-1} de chacun de BAP et NAA suivie par l'enlèvement de TDZ du milieu de culture. La kanamycine et l'hygromycine se sont avérés être de bons marqueurs sélectifs pour le *Pélargonium*. Deux souches d'Agrobacterium, C58 et EHA105 contenant des vecteurs binaires avec des gènes marqueurs *hpt* et *nptII* ont été choisis pour des expériences de transformation. Ils ont également le gène codant *uidA* séquence du gène rapporteur. Après l'infection avec C58, 4 et 107 plantes enracinées sur hygromycine ont été obtenues pour Attar of Roses et Atomic Snowflake, respectivement. Parmi ces plantes enracinées, les quatre plantes d'Attar et 82 d'Atomic Snowflake ont exprimé le Gus dans les feuilles, pétioles, les tiges et les racines comme prévu avec une séquence sous contrôle du promoteur constitutif CaMV ^{35}S. De 20 plantes qui expriment le Gus, 7 plantes se sont avérées être positives après criblage par PCR. Après infection par EHA105, 23 et 133 plantes enracinées ont été obtenues après sélection sur kanamycine, mais aucune n'a démontré d'activité GUS. Seule des expériences d'empreintes par Southern blotting permettront de corréler le nombre d'insertions et niveau de l'expression dans ces différents événements de transformation.

Mots-clefs: Phytoremediation, *Pélargonium* odorants, Pb, hyperaccumulateur, phyto-disponibilité, spéciation, COD, pH, régénération, transformation génétique, *Agrobacterium*.