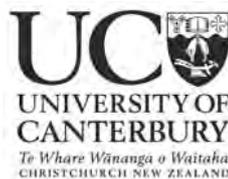


Investigations into Lead (Pb) Accumulation in
***Symphytum officinale* L.:**
A Phytoremediation Study

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ABSTRACT

Lead (Pb) is the number one heavy metal pollutant in the environment. The high cost and environmental concerns of conventional remediation technologies has led to an emerging alternative technology for heavy metal remediation: phytoremediation. This study was set out to advance Pb phytoremediation by investigating plant-associated factors (e.g. polyphenol levels, Pb-tannin chelation, and superoxide dismutase activity) and chemical-based factors (e.g. concentration of Pb, and the type and dosage of chelating agents in treatments) that may affect Pb accumulation.

Using a hydroponic system, sand-grown *Symphytum officinale* L. plants were exposed to nutrient solutions with or without lead nitrate ($\text{Pb}(\text{NO}_3)_2$) and ethylenediamine tetraacetic acid (EDTA). Using flame atomic absorption spectroscopy (to measure Pb content) and bovine serum albumin-protein precipitation (to measure polyphenol and tannin levels), a significant *in vivo* correlation between tannin level and Pb accumulation level was observed in roots of plants exposed to all Pb treatments. Higher tannin containing-lateral roots accumulated significantly more Pb than lower-tannin main roots. Transmission electron micrographs of unchelated Pb-treated plants supported these findings, whilst dialysis-based *in vitro* Pb-chelation studies with crude *S. officinale* root polyphenol extracts did not. The dialysis method was likely to be subject to fructan interference. A new, more accurate and simple method based on tannin immobilisation was consequently developed. Results using this method supported the hydroponic trends. This new method was also verified with purified tannic acid (from Sigma). Together, these findings demonstrate that *S. officinale* root tannins have the ability to chelate Pb. This may be a mechanism to cope with Pb stress (adaptive tolerance). Despite the typical signs of Pb stress at root level (e.g. root growth inhibition, and degraded cytoplasm), shoots showed no signs of stress under any Pb treatments. Most importantly, since this chelation-based tolerance mechanism also influences the accumulation levels, the phytochemical composition of plants should also be considered when screening plants for phytoremediation.

The level of Pb accumulated in the shoots depended on the concentration of $\text{Pb}(\text{NO}_3)_2$ and presence of chelating agents (EDTA or N-[2 acetamido] iminodiacetic acid (ADA)) in the nutrient solution. The highest level of Pb in shoots was between 0.05-0.06% (d.w. on average) using EDTA or ADA, well short of the 1% (d.w.) shoot accumulation target for Pb phytoextraction. The highest level of Pb in the roots (and of

all measurements) was with unchelated 500 μM $\text{Pb}(\text{NO}_3)_2$; on average 2% (d.w.) accumulated in root. Overall, since *S. officinale* accumulated Pb predominately in the roots, it is most suited for rhizofiltration and phytostabilisation. Whilst chelating agents enhanced Pb accumulation in shoots, root levels were unexpectedly reduced compared to unchelated Pb treatments. The level of Pb translocated did not completely account for this loss. Minor factors relating to EDTA desorption of roots, EDTA specificity, and charge repulsion of the PbEDTA complex may account for some of the loss, but the main cause remains unclear.

In vitro *S. officinale* cultures were developed and somaclonal variation (involving Pb pre-treatment of petioles) was used as a tool to further investigate, and attempt to improve its Pb phytoremediation potential. The shoots and roots of plants produced from petioles pre-treated with $\text{Pb}(\text{NO}_3)_2$ appeared more stressed than those without Pb pre-treatment. After re-treatment with Pb ($\text{Pb}(\text{NO}_3)_2$ or PbADA), plants developed from most Pb pre-treated petioles appeared to have reduced Pb accumulation and polyphenol levels, and increased superoxide dismutase activity in roots (although no statistically significant trends were found). Overall, plants produced from Pb pre-treated petioles in this study may have less phytoremediation potential.

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LIST OF ABBREVIATIONS

2,4 D	2,4-dichlorophenoxyacetic acid
AA	ascorbic acid
AAS	(flame) atomic absorption spectroscopy/spectrometer
ABC	ATP-binding cassette
ADA	N-[2 acetamido] iminodiacetic acid
ADS	antioxidative defence system
ATPases	adenosine 5'-triphosphatases
ATSDR	Agency for Toxic Substances and Disease Registry
BA	6-benzyladenine
BSA	bovine serum albumin
CAT	catalase
CEC	cation exchange capacity
CGM	callus growth media
CIM	callus induction media
d.w.	dry weight
DNA	deoxyribose nucleic acid
DPTA	diethylenetriamine pentaacetic acid
DTT	dithiothreitol (also known as Cleland's reagent)
DTZ	distal-transition zone
EDDHA	ethylenediaminedi (o-hydroxyphenylacetic) acid
EDDS	ethylenediamine dissuccinate
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol bis-2-aminoethyl ether-n,n',n'',n'-tetraacetic acid
EMS	ethyl methanesulfonate
ERMA	Environmental Risk Management Authority
EZ	elongation zone
f.w.	fresh weight
F/C	Folin-Ciocalteu
FAO/IAEA	Food and Agriculture Organization/International Atomic Energy Agency
GMOs	genetically modified organisms
HBH	4-hydroxybenzyl hydrazine
HC	Huang and Cunningham (nutrient solution)

HEDTA	hydroxyethyl ethylenediamine triacetic acid
IBA	indole-3-butyric acid
LR	lateral root
MR	main root
MS	Murashige and Skoog
MT(s)	metallothionine(s)
MZ	meristematic zone
Na ₂ EDTA	disodium ethylenediamine tetraacetic acid
NAA	α -naphthaleneacetic acid
NADPH	mono-hydrogen nicotinamide adenine dinucleotide phosphate
NBT	nitro blue tetrazolium
NR	nitrate reductase
NtCBP4 Δ C	truncated version Ni transporter gene (from tobacco) encoding of camodulin binding protein
o/n	overnight (shaking)
PC(s)	phytochelation(s)
PDR	physician's desk reference
POX	peroxidase
ppm	parts per million
PPM	plant preservative mixture
PVPP	polyvinyl polypyrrolidone
RIM	root induction media
ROS	reactive oxygen species
S.E.	standard error
SDS	sodium dodecyl sulfate
SMT	selenocysteine methyl transferase
SOD	superoxide dismutase
TA	tannic acid
TEA	triethanolamine
TEM	transmission electron microscope/microscopy
TI	tolerance index
u/w	ultrasonic water-bath
US(A)	United States (of America)
WPIM	whole plant induction media
YCF1	yeast protein from ATP-binding cassette transporter family

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CHAPTER 1

INTRODUCTION

Heavy metal pollution is a global environmental and health safety issue. The high cost and environmental concerns of conventional remediation technologies has fuelled the need for alternative remediation methods – phytoremediation, the use of plants to remove or detoxify pollutants, is one such emerging technology. The present study is directed towards advancing phytoremediation by understanding how plants take up, tolerate and accumulate heavy metals like lead (Pb). This section is devoted to reviewing: (i) heavy metals (particularly Pb), (ii) remediation methods, (iii) heavy metal uptake and tolerance mechanisms in plants and finally, (iv) the objectives of this study.

1 HEAVY METALS

1.1 Definitions

Heavy metals are a group of metals with an atomic density greater than 6g cm^{-3} (Alloway and Ayres, 1997). Although it is a widely recognised word, its definition is not useful in the biological field. Heavy metals are better described as ‘trace metals’ - metals found at low concentrations, usually less than one ppm or less, in a specified source, e.g. soil, plant tissue or ground water (Phillips, 1981). In this thesis, the term ‘heavy metals’ will be used, as it is the most widely recognised, but the description will be that of trace metals.

At low or background concentrations, heavy metals are not pollutants. They are naturally in the environment due to their presence in bedrocks. Some heavy metals in the environment, such as zinc and copper, are also essential micronutrients for living organisms. Therefore, the term ‘heavy metal pollution’ refers to when heavy metal levels are abnormally high relative to normal background levels; presence of the metal is insufficient evidence of pollution (Alloway and Ayres, 1997).

1.2 Sources of heavy metal pollution

There are two main sources of heavy metal pollution: nature and human activity. Naturally occurring pollution originates from excessive weathering of rocks with surface metal deposits and can equal or, in rare instances, exceed man-made pollution levels. Human activity is the main contributor to heavy metal pollution. The main man-made sources of heavy metal pollution are: (i) metal smelters and refineries, (ii) industrial wastes (e.g. electroplating) (iii) military operations, (iv) mining, (v) landfill run-offs, (vi) agricultural chemicals such as pesticides, herbicides and fertilisers, and (vii) automobile emissions (Saxena *et al.*, 1999).

In most developed countries, current heavy metal pollution cases are localised and are declining due to cleaner industrial practices and conversion to non-heavy metal-based products like unleaded fuel. However in Eastern Europe and developing regions of the world, namely India and China, heavy metal contamination of the environment is still widespread (Krämer, 2005). Globally, historically polluted environments (i.e. those from the 1800s industrial era) will also be a continuing problem because of the long-term persistence of heavy metals in the environment (Martin and Coughtrey, 1981).

2 LEAD

2.1 Lead pollution

Lead (Pb) is a very malleable, heavy and non-corrosive metal (Csuros, 1994). Past and present products containing Pb include solder, paints, plumbing, batteries, ammunition and petrol (Csuros, 1994; Alloway and Ayres, 1997). Lead pollution arises from the use of these products (particularly in the past) and the human activities listed above.

It is the focus of this study because of its toxic effect on living organisms, and widespread occurrence and persistence in the environment. Lead is ranked the number one heavy metal pollutant (and number two of all hazardous substances) by the Agency for Toxic Substances and Disease Registry (ATSDR, 2005). In the 1980s, global emission of Pb into soils was at least 36 times higher than the next two most toxic heavy metals, mercury (Hg) and cadmium (Cd) (Alloway and Ayres, 1997; ATSDR, 2005). In USA soils, Pb levels are between 1- 6,900 mg kg⁻¹; the regulatory limit in the USA is 600 mg kg⁻¹ (twice the regulatory limit in New Zealand) (Salt *et al.*, 1998). Furthermore

without intervention, Pb in these soils will continue to be present today as it persists in the environment for 150-5000 years (Kumar *et al.*, 1995).

2.2 Effect of lead on human health

The main entry routes of Pb into humans are through consumption of contaminated food and water. Inhalation of Pb particles from the air, soil and Pb-based products such as paints and batteries also occurs. Ingestion of Pb-based paint chips and swallowing soil or dust contaminated with Pb are the main sources of Pb poisoning in children. When ingested or inhaled, Pb is absorbed by the gastrointestinal tract and then accumulates in the blood stream and central nervous system. Once sufficient amounts of Pb accumulate in the body (0.3-0.8 ppm), it can cause permanent brain and nerve damage, kidney dysfunction, and disrupt haeme synthesis causing anaemia (Csuros, 1994; Yang *et al.*, 2000). Children are especially susceptible to its effects – about 50% of the ingested Pb is absorbed into a child's body, compared to between 10 to 15% in adults (Csuros, 1994).

2.3 Effect of lead on plant growth

Before hyperaccumulators were discovered in the late 1970s, the effect, accumulation and tolerance of heavy metals in plants were already under investigation because of their release into the environment from the industrial era (Koeppel, 1981). Presently, studying the effects of Pb on non-hyperaccumulating plants is important for two reasons: (i) plants are an entry path for Pb into the food chain and (ii) phytoremediation.

The most common symptoms of Pb toxicity are root and shoot growth inhibition (Huang *et al.*, 1974; Wierzbicka, 1987 and 1995; Vassil *et al.*, 1998; Huang and Cunningham 1996; Sobotik *et al.*, 1998; Geebelen *et al.*, 2002; Grčman *et al.*, 2003). These symptoms may occur because Pb binds strongly to a large number of bio-molecules like cell wall pectic acids, amino acids, enzymes, DNA and RNA (Xiong *et al.*, 2006; Kabata-Pendias, 2001) and in turn may cause:

- (i) Disruption to cell elongation and reducing cell wall elasticity (Lane *et al.*, 1978 Breckle, 1991; Kabata-Pendias, 2001).
- (ii) Disruption to microtubule alignment during cell division (Yang *et al.*, 2000).

- (iii) Accelerated aging/senescence of tissues as reflected by an increase in acid phosphatase, amylase and peroxidase activity (Lee *et al.*, 1976; Schützendübel *et al.*, 2001).
- (iv) Oxidative stress (Malecka *et al.*, 2001; Pilon-Smits, 2005)
- (v) Decrease in protein and carbohydrate content required for normal growth (Xiong *et al.*, 2006)
- (vi) Reduced photosynthesis and transpiration rates due to inhibition of the electron transport chain in mitochondria and chloroplasts (Bazzaz *et al.*, 1974; Carlson *et al.*, 1975; Koepe, 1981). A reduction in grana stacks, stroma and an absence of starch grains in chloroplasts has also been observed (Koepe, 1981). These events lead to leaf chlorosis.

Lead-induced toxicity symptoms may also be due to interference in ion uptake and translocation (Bazzaz *et al.*, 1974). Replacement of essential ions has also been reported (Pilon-Smits, 2005). Pb is also known to reduce uptake and accumulation of ions that are critical to the plant health such as calcium, phosphorous (Huang and Cunningham 1996) and nitrogen (particularly in nitrogen fixing bean-root nodules) (Huang *et al.*, 1974; Lee *et al.*, 1976; Xiong *et al.*, 2006). The reduction in nitrogen (N) is particularly problematic, as it is the most required element in plants, comprising 1.5-2% of plant dry matter. Nitrate (NO_3^-) is the most important form of N in plants. Once inside root cells, NO_3^- is reduced to NO_2^- by nitrate reductase (the key enzyme in nitrate assimilation, NR), and NO_2^- converted to $\text{NH}_4\text{-N}$. This product is then assimilated into an organic form such as glutamate and glutamine. These amino acids are involved in the synthesis of all other amino acids, nucleic acids, chlorophylls and hormones (Xiong *et al.*, 2006). Pb is known to reduce nitrogen levels in plants by inhibiting NR activity (Huang *et al.*, 1974; Bharti and Singh, 1993; Xiong *et al.*, 2006). Lead may directly inhibit its activity by binding to sulfhydryl groups on the enzyme or by reducing its production at a genetic level (by reducing the level of the NR gene inducer, nitrate)(Xiong *et al.*, 2006).

Whilst the above symptoms typically occur under *laboratory* conditions, there is a lack or actually no observable symptoms of Pb toxicity under natural conditions, even when plants contain 350 mg kg^{-1} Pb (d.w.) (Wierzbicka, 1995). Perhaps, one of the few reports of toxicity in the natural environment was evidence of decreased crop productivity by Pb and other heavy metals from a zinc smelter (Yang *et al.*, 2000). The

lack of observable effects may be due to: (i) irreversible binding of Pb to soil particles, leading to low Pb availability to plants (Koeppel, 1981; Eltrop *et al.*, 1991), (ii) binding of Pb to root tissue, leading to a lack of observable toxicity at shoot level, and (iii) plant species (e.g. hyperaccumulators vs. non-hyperaccumulator *Alyssum* species) and age (Koeppel, 1981), and (iv) not reaching the threshold Pb concentration for observable damage; this may be as low as 30-300 mg kg⁻¹ Pb (d.w.) (Zheljazkov and Fair, 1996) to 500 to 1000 mg kg⁻¹ Pb (d.w.) (Wierzbicka, 1995) (tissue unspecified in articles). Additionally, the bioavailable ambient concentrations of Pb are usually lower than the Pb treatment concentration used in laboratory studies. Higher Pb treatment concentrations in laboratory studies are often necessary to test, for example, if plants can effectively clean-up heavy metals from polluted water and within the time limitations of phytoremediation.

3 HEAVY METAL REMEDIATION TECHNOLOGIES

It was once thought that the passage of water through the soil exerted a purifying effect and that wastes dumped into the ground could be cleansed from the system (Suthersan, 1997). For organic pollutants, this view may contain some truth as some organic pollutants can be degraded into less harmful products by soil microbes. However, inorganic pollutants cannot be degraded. Inorganic pollutants such as heavy metals are persistent; Pb for example can persist in the environment for 150-5000 years (Kumar *et al.*, 1995). Remediation of such polluted environments therefore requires physical removal or conversion to non-toxic forms.

3.1 Conventional remediation technologies

Colloquially, conventional remediation technologies are generally termed as ‘pump and treat’ and ‘dig and dump’ techniques (Saxena *et al.*, 1999). They can be divided into either *in situ* or *ex situ* remediation. Some of the most commonly used technologies (summarised from Saxena *et al.*, 1999) include:

- (i) *Soil washing (in situ)*: The soil is washed with a chemical solution to separate metals bound to the soil. The subsequent liquid is then removed for treatment or disposal.

- (ii) *Chemical oxidation/reduction (in situ)*: The metals in the contaminated sites are chemically converted to less mobile or less hazardous forms.
- (iii) *Stabilisation (in situ)*: The metals are physically enclosed in a stabilised mass.
- (iv) *Excavation and off-site disposal (ex situ)*: The contaminated soil is removed and transported off-site for treatment or disposal. The area is then replaced with clean soil.

The environmental impact of such technologies can be very high. For example, soil-washing methods may render the soil infertile or spread the contaminant, and excavation methods can produce high waste volumes. Additionally, these remediation methods are often limited to small areas and depend on accessibility to the polluted site (Saxena *et al.*, 1999).

However, the most limiting factor is the high cost of these technologies. In the USA alone, heavy metal remediation has been estimated at US\$ 7.1 billion (Salt *et al.*, 1995a). Conventional excavation and disposal methods would cost US\$ 400,000 per acre of land with a remediation depth of 50 cm compared to US\$ 60,000-100,000 for phytoremediation (Salt *et al.*, 1995a). These costs are particularly uneconomical when used to remediate sites with pollution levels just above safety regulations.

3.2 Alternative remediation technologies (I) – Micro-organisms

Bioremediation uses microbes to remediate the subsurface of soils contaminated with hazardous chemicals. It is applied most effectively for organic pollutants (such as benzene, toluene and phenols) where, depending on the microbe and pollutant, it can result in the complete mineralisation of the pollutant. For example, benzene and phenols can be converted to products such as carbon dioxide and water (Suthersan, 1997; Davison, 2005).

For inorganic pollutants like heavy metals, this form of remediation is less effective. Microbes may immobilise pollutants by converting the elemental state of heavy metals, but ultimately they are not capable of removing them from the medium. One exception to this is the conversion of elemental Hg^{2+} (mercury) in the soil to volatile Hg^0 , which is released hazardously into the atmosphere (Suthersan, 1997; Davison, 2005).

3.3 Alternative remediation technologies (II) – Phytoremediation

3.3.1 Origin of phytoremediation concept

There are three types of metal-tolerant plants which are classified according to their tolerance and accumulation response on metal contaminated soils: (i) *excluders* - restrict metal uptake into roots except at extreme metal concentrations (ii) *indicator plants* - metal level accumulated in the shoot is relative to metal levels in soil and (iii) *hyperaccumulators* – concentrate metals in shoots, regardless of soil metal concentrations (Greger, 1999; Ghosh and Singh, 2005).

In the late 1970s, Brooks and Jaffre (Jaffre *et al.*, 1976) were one of the first to highlight the presence of plants that hyperaccumulate nickel (Robinson *et al.*, 2003). With the growing concerns of heavy metal pollution in the environment and inadequacies of conventional remediation technologies, this hyperaccumulator phenotype was used to introduce the idea of phytoextraction (Salt *et al.*, 1998) and eventually phytoremediation.

3.3.2 Phytoremediation: definition and classes

Phytoremediation is defined as the use of green plants to remove pollutants from the environment or render them harmless (Garbisu and Alkorta, 2001). The phytoextraction and rhizofiltration technologies are the most useful branches for heavy metal removal from soil and water respectively. The five classes of phytoremediation are outlined below.

- (i) *Phytoextraction*: Metal-accumulating plants are used to transport and concentrate metals from the soil into above ground shoots (Kumar *et al.*, 1995). There are two types of phytoextraction:
 - a. Chelate-assisted phytoextraction – high biomass crops are induced to accumulate high concentrations of heavy metals (> 1% d.w. of shoot) by application of chelating agents (e.g. EDTA) to soils after plants have grown to maximum biomass (Salt *et al.*, 1998).
 - b. Continuous phytoextraction – the natural ability of plants to accumulate and survive high metal concentrations over the entire growth cycle (i.e. hyperaccumulators) (Sas-Nowosielska *et al.*, 2004).

- (ii) *Rhizofiltration*: Use of plant roots to absorb, precipitate and concentrate toxic metals from polluted effluents (Dushenkov *et al.*, 1995).
- (iii) *Phytostabilisation*: Metal-tolerant plants are used to reduce the mobility of metals (via root uptake, precipitation or reduction), thereby reducing entry into waterways and airborne spread (Salt *et al.*, 1995a). Plant roots are also used to stabilise soil, thereby preventing erosion.
- (iv) *Phytodegradation*: Degradation of complex organic contaminants by root microbes (Saxena *et al.*, 1999).
- (v) *Phytovolatilisation*: This uses plant uptake and respiration mechanisms to remove volatile metals such as Hg from the soil. However, this is not desirable because this method relocates the pollutant from the soil into the air (Saxena *et al.*, 1999).

3.3.3 ‘Pros and cons’ of phytoremediation

Although phytoremediation is still at its initial stages of research and development, there are several potentially significant advantages of this approach over conventional remediation technologies. These include:

- (i) *Cost*: It is estimated to be least two to four fold less expensive than conventional remediation techniques (Cunningham and Ow, 1996).
- (ii) *Application scale*: Plants can be sown or translocated on a large scale.
- (iii) *Impact on the environment*: Since plants are able to concentrate the contaminant in their tissues, comparatively low waste volumes are produced. Furthermore, several plausible methods of reducing and recycling heavy metal contaminated plant tissues are available: pre-treatment steps e.g. composting can be used to reduce biomass, followed by incineration or ashing and liquid extraction (recycling) (Sas-Nowosielska *et al.*, 2004). In addition, plant roots will be able to prevent soil erosion and increase microbial communities.

However, phytoremediation also has a number of limitations and criticisms, as explained below:

- (i) *Remediation depth*: In order for the plant to absorb the contaminant, roots must be in the contamination zone. Most herbaceous plants are relatively shallow rooting, reaching only 0.5-1 m deep. Thus if the contamination is deeper than this, remediation can be limited (Pilon-Smits, 2005). Alternatively, in such cases deep-rooting (3 m deep) poplar or willow trees may be used instead (Suthersan, 1997; Pilon-Smits, 2005).
- (ii) *Bioavailability*: In the case of chelate-assisted phytoextraction, the use of chelating agents can cause metals to leach and spread into the groundwater below (Wenzel *et al.*, 2003). There is also uncertainty as to whether plants can remove enough of the bioavailable fraction to meet safety regulations (Pilon-Smits, 2005).
- (iii) *Time*: Phytoremediation can take up to ten years, which is more time consuming than conventional pump-and-treat methods (Pilon-Smits, 2005).
- (iv) *Multiple contaminants*: Some plants tolerate only certain types of heavy metals. Thus the presence of multiple types of heavy metals (e.g. Pb, Cd, Cr) and organic contaminants (e.g. PCB, TNT) may pose a challenge (Saxena *et al.*, 1999).
- (v) *Entry to the food chain*: There is also the possibility that wind dispersal of metal-laden dead leaves or animal feeding may be problematic. However, if the contaminated area has barriers and harvesting of contaminated plants is regulated, these problems may not be significant.

3.3.4 Ideal attributes of phytoextraction plants

For a plant to be considered as a hyperaccumulator of Pb, Ni, Cu, Cr, and Co, the plant *shoot* must accumulate more than 0.1% (d.w.) of these metals or 1% d.w. for Mn and Zn (Baker and Brooks, 1989). Of the over 400 plant species which have been identified as hyperaccumulators, 75% have been Ni hyperaccumulators (Clemens, 2001). Pb hyperaccumulators have been comparatively rare. To date 14 have been identified, although the exact number is controversial because some plants may have been mistakenly identified due to aerial contamination (Ghosh and Singh, 2005). Known Pb

hyperaccumulators include *Polycarphaeae synandra*, *Minuartia verna*, *Ameria martima*, *Thlaspi alpsetre* and *T. rotundifolium* (Huang *et al.*, 1997a).

Initial phytoextraction research began with hyperaccumulators, such as *Thlaspi caerulescences* and *Alyssum bertoloni* (Keller *et al.*, 2003). Whilst these plants are useful for studying metal tolerance and accumulation mechanisms, their slow growth rate and small biomass may limit their application in phytoremediation (Ebbs and Kochian, 1998). This is because the total amount of metals extracted (a measure of phytoremediation potential) is the product of biomass and tissue concentration (Kayser *et al.*, 2000).

The goal of phytoextraction is to reduce heavy metal levels in the soil to acceptable levels within three to ten years (Huang and Cunningham *et al.*, 1996). In order to achieve this goal, plants must be screened and selected for certain attributes. The ideal plant for phytoextraction would have: (i) a rapid growth rate, even under harsh conditions, (ii) a high shoot biomass (20 metric tones dry weight ha⁻¹ yr⁻¹) (Saxena *et al.*, 1999; Huang *et al.*, 1997b), and (iii) a capacity to accumulate high amounts of metals in shoots; in the case of Pb, 10,000 mg kg⁻¹ d.w. (1% d.w.) (Brooks, 1998). With regards to the latter point, this level of Pb in shoots is likely to be fatal to the plant, but it does not preclude it from harvesting (Wu *et al.*, 1999).

3.3.5 Plants used for lead phytoextraction

At this stage, no fully-grown (mature) plant has been found to meet all three attributes (above) on its own at a field scale – i.e. there is no evidence of a natural, high biomass Pb hyperaccumulator. However, in controlled bench and field-scale experiments, all three attributes have been apparently met, often by using seedlings or very young plants of high biomass plants and chelating agents like EDTA to artificially induce hyperaccumulation (see section 4.1.3).

In hydroponic phytoextraction studies, *Sesbania drummondii* (a high biomass leguminous shrub, which is found naturally on Pb contaminated sites) accumulated greater than 4% (d.w.) Pb in shoots (Sahi *et al.*, 2002). Similarly, *Brassica juncea* (L.) Czern.var. 426308 accumulated 3.5% (d.w.) Pb in plant shoots after 14 days in a sand/perlite mixture fed with 500 mg L⁻¹ Pb(NO₃)₂ (Kumar *et al.*, 1995). Both studies used seedlings and did not involve chelating agents or genetic modification. In hydroponic studies, the same *B. juncea* cultivar accumulated 1.1% Pb (d.w.) in shoots

after 14 days in Hoagland's nutrient solution containing 0.5 mM Pb(NO₃)₂ with 1 mM EDTA. This concentration was 400-fold higher than treatments without EDTA (Vassil *et al.*, 1998). Other plants investigated in bench-scale experiments include varieties of sunflower, scented geranium, and tree species such as poplar and willows (Saxena *et al.*, 1999).

Field-scale phytoextraction trials have also been conducted, although relatively few in comparison to bench-scale studies. Lesage *et al.*, (2005) showed that EDTA increased Pb accumulation in sunflower (*Helianthus annuus*) shoots, but only to a maximum of 0.007% (d.w.). The ability to extract Pb was hampered by the severe reduction in plant growth at higher EDTA concentrations (7-10 mmol kg⁻¹ soil). Using field-lysimeter studies, Wenzel *et al.*, (2003) demonstrated that canola (*Brassica napus* L.) could accumulate 0.385% (d.w.) Pb in shoots after 30 days in mildly contaminated soil and addition of EDTA in increments of 1g per kg of soil. The authors concluded that this is in contrast to field studies using Indian mustard (Blaylock *et al.*, 1997) and corn (Huang *et al.*, 1997b), where Pb levels in shoots were greater than 1% (d.w.) upon addition of chelating agents. However, this may in part be due to variations in the degree of soil contamination, plant species and age.

4 HEAVY METAL UPTAKE BY PLANTS

Heavy metal uptake by plants follows three key events: (i) bioavailability/mobilisation of metals, (ii) root uptake and xylem transport, and (iii) detoxification/tolerance (Saxena *et al.*, 1999; Clemens *et al.*, 2002). For enhancing the phytoremediation potential of plants, aspects from each of these events will be investigated in this study.

4.1 Factors influencing heavy metal bioavailability

One of the key factors that can influence the success of phytoremediation is **metal bioavailability** (Huang *et al.*, 1997b; Lasat, 2002). Metal bioavailability is the concentration of metals in the solution fraction of the contaminated soil that is available for root uptake. It is strongly affected by physical, biological and chemical factors*.

* This is a simplistic definition. Soils are a complex and dynamic matrix. The overall metal bioavailability also takes account of other factors such as the ability of the soil solid phase to re-supply metals to the soil solution once removed by plants.

Lead binds tightly to soil particles and exists in insoluble forms such as lead phosphate, carbonate and hydroxide. Thus, unlike Cd and Zn, the metal bioavailability of Pb is low (Alloway and Ayres, 1997; Lasat, 2002). However understanding and manipulating three factors, as detailed below, can improve Pb bioavailability and thus phytoremediation.

4.1.1 Physical and biological factors

Physical factors relate to the texture of soils. High organic matter content for example, would reduce metal bioavailability because metals are known to bind strongly to organic matter (Saxena *et al.*, 1999).

The biological factors include plant genotype and root-associated microbes. Dicots are thought to accumulate higher heavy metal concentrations than monocots because they have a higher number of negatively charged-metal attracting sites (cation exchange capability, CEC) in the cell wall than monocots (Saxena *et al.*, 1999). However, Huang and Cunningham (1996) suggested that monocots, such as corn, are superior in Pb translocation. They found that corn accumulated similar or more Pb in shoots than two of the best Pb-accumulating cultivars of *B. juncea* (a dicot). Root-associated microbes can influence metal uptake abilities of plants by modifying the pH around roots (Marschner, 1995).

4.1.2 Chemical factors (I): soil chemistry

Soil chemical factors that affect metal bioavailability include pH, cation exchange capability and redox potential. Of all the factors, pH is the most easily manipulated using soil acidifiers and ammonium containing fertilisers. The H⁺ ions have a higher affinity for negative sites on soil particles. Thus at low pH, metal bioavailability increases as more metals are released into the soil solution due to competition with H⁺ ions (Saxena *et al.*, 1999).

4.1.3 Chemical factors (II): chelating agents

Another chemical factor influencing metal bioavailability is the presence of chelating agents. Chelating agents increase metal bioavailability by removing metals from soil particle surfaces, forming metal-chelate complexes that are then taken up by plants

(Geebelen *et al.*, 2002). These compounds are naturally made by the roots (e.g. phytosiderophores such as mugenic acid, organic acids, and amino acids) or are synthetic and applied to the soil (e.g. ethylenediamine tetraacetic acid, EDTA) (Saxena *et al.*, 1999; Lasat, 2002). In phytoremediation, the latter is called chelate-assisted phytoextraction.

Translocation is considered as the other major hurdle in phytoextraction because there are no plants that can transport large quantities of Pb naturally to the shoots (Huang *et al.*, 1997b). This is important for soil phytoextraction because Pb accumulation is targeted at the harvestable above ground shoots of high biomass plants. EDTA has been used to address this hurdle. In addition to increasing bioavailability, EDTA has been reported to increase shoot accumulation of Pb in mesquite (*Prosopis* spp.) (Aldrich *et al.*, 2004), mustard plants varieties (Blaylock *et al.*, 1997; Salt *et al.*, 1998; Vassil *et al.*, 1998), corn (*Zea mays* L.cv. Fiesta) and pea (*Pisum sativum*) from less than 500 mg kg⁻¹ to more than 10,000 mg kg⁻¹ (>1% d.w. Pb in shoots) (Huang *et al.*, 1997b). Vassil *et al.*, (1998) suggested that the mechanism of this translocation may involve the destabilisation of the plasma membrane (the primary physiological barrier in the root that controls ion uptake and movement to shoots) via EDTA removal of Zn²⁺ and Ca²⁺ plasma membrane stabilising ions. Increased translocation due to transpiration was not supported as these authors found that PbEDTA reduced water loss in shoots.

Although EDTA is not Pb specific, it is the most effective chelating agent of those tested (EDTA> HEDTA>DTPA>EGTA>EDDHA) (Huang *et al.*, 1997b). However, its use may be limited as EDTA can cause substantial leaching and spread of heavy metals (McGrath and Zhao, 2003). However, this may be alleviated with agronomic management. For example, incremental addition of EDTA as opposed to a single high dose may be employed to reduce leaching (Barcosi *et al.*, 2003 and Wenzel *et al.*, 2003).

4.2 Heavy metal uptake, transport and accumulation

4.2.1 Metal uptake and transport

Although there are many gaps in the details of how plants uptake and transport metals, there is a consensus that four key steps in metal accumulation are involved.

- (i) *Mobilisation and uptake*: To increase the bioavailability of metals, the rhizosphere is acidified (by H^+ ion release from plasma membrane H^+ -ATPases) and metal chelators are released from the root (e.g. phytosiderophores, organic acids) (Clemens *et al.*, 2002). During uptake of metals, a high metal concentration gradient forms along the cell wall (a low affinity and unspecific ion-exchanger). Unless chelated, metals are thought to either remain in the negatively charged sites of the cell wall, or are transported apoplastically (between cells via cell walls and intracellular fluid) to the endodermis (Greger, 1999). There is also a fraction of metals that enters the root cytoplasm. This is thought to occur via:
- a. Passive transport: A high metal concentration in the cell wall promotes mass flow/diffusion of metals through the plasma membrane into the cell cytoplasm (Greger, 1999; Clemens *et al.*, 2002).
 - b. Active transport via plasma membrane transport proteins: These are required because metals cannot flow freely across the lipophilic plasma membrane at low concentrations (Lasat, 2002). Although, over 150 different cation transporters have been identified in *Arabidopsis thaliana*, presently little is known about the inorganic ion transporters (Salt *et al.*, 1998; Pilon-Smits, 2005). Inorganic transporters may include channel proteins (e.g. Ca^{2+} -channels) and H^+ -coupled carrier proteins (e.g. H^+ -ATPases) (Greger, 1999; Clemens *et al.*, 2002).
- (ii) *Compartmentalisation and chelation within roots*: Once inside the root cytoplasm, metals are chelated to organic acids (e.g. citrate, malate, histidine), thiol rich peptides (e.g. phytochelatins [PCs]), or cysteine rich metallothioneins (MTs) (Pilon-Smits, 2005). These are stored in root vacuoles or exported to the shoots (Salt *et al.*, 1998).
- (iii) *Xylem loading and transport to shoots*: To enter the xylem, metals must pass through the endodermis with the suberised Casparian strip. Since this is difficult, the younger parts of the root, where Casparian strips are not fully developed, are thought to be mostly responsible for metal uptake into the xylem (Greger, 1999). During this process (movement towards the xylem), metals are sequestered inside the root cells (cytoplasm) and are

symplastically transported to the stele. The transport of these ions into the xylem is a tightly controlled process mediated by membrane transport proteins, which are yet to be identified (Greger, 1999; Clemens *et al.*, 2002; Ghosh and Singh, 2005). Once in the xylem, metals are chelated (e.g. with citrate, histidine, or as hydrated ions) to avoid adhesion to the high CEC capability of the xylem cell wall (Salt *et al.*, 1998). Transport to the shoots is driven by transpiration (Pilon-Smits, 2005).

- (iv) *Sequestration and storage in leaf cells*: Metals from the xylem reach the apoplast of leaves (Clemens *et al.*, 2002). From here, metals are transported into the leaf cell via membrane transporter proteins such as ABC-type transporters in the vacuole and P-type ATPase pumps (Greger, 1999; Pilon-Smits, 2005). Once inside the leaf cells, their levels are also regulated by accumulation in the vacuole, cell walls, or at a tissue level (in the epidermis and trichomes). Within cells they are typically bound to chelators such as GSH, PCs, organic acids, and MTs.

4.2.2 Movement route and accumulation of lead in plant tissues

The accumulation site (root, shoot and cellular location) and movement route (apoplastic or symplastic route) of Pb once absorbed by the root is still controversially debated. However, it appears to be influenced by whether or not Pb is chelated.

For unchelated Pb-treated plants, most of the unchelated Pb is trapped in the root tissue, in root cell walls; very little is transported to the above ground shoot tissue. For example, Kumar *et al.*, (1995) found that *B. juncea* L. (Cern.) accumulated 10,300 mg kg⁻¹ Pb in the shoot (d.w.) compared to 103,300 mg kg⁻¹ Pb in the root (d.w.). The location of Pb in the root cell wall may be due to direct binding upon entry into the root tissue (Wierzbicka, 1987) or as the result of Pb from the cytoplasm being transported to the cell wall (via vesicles) (Malone *et al.*, 1974). Thus due to its deposition sites, unchelated Pb is considered to travel the apoplastic route in the root tissue (Blaylock *et al.*, 1997; Wierzbicka, 1995).

In chelated Pb studies, the root tissue absorbs the entire complex (PbEDTA) as most of the Pb in xylem fluid of *B. juncea* was found to be co-ordinated with EDTA (Vassil *et al.*, 1998). Once absorbed by the roots, the majority of this Pb is then

translocated and accumulated in the shoot tissue. Transmission electron micrographs of *Chamaecytisus proliferus* treated with PbEDTA showed that Pb was found in the shoot cell mitochondria, chloroplast and plasmodesmata. A small amount was also found in these organelles of root cells (Jarvis and Leung, 2001). Due to the location of Pb in the cytoplasm, chelated Pb is considered to travel the symplastic route (i.e. through cells via the plasmodesmata) from root to the shoot.

5 HEAVY METAL STRESS AND TOLERANCE IN PLANTS

In the previous section, understanding the factors that influence Pb bioavailability can improve phytoremediation. Another approach is to understand the mechanisms behind the plants' ability to tolerate Pb in the tissue as some notable, but not all, cases of heavy metal tolerance translate into greater metal accumulation ability. Examples include transgenic *A. thaliana* overexpressing a tobacco channel protein *NtCBP4ΔC* (Cherian and Oliveira 2005) or a vacuolar yeast transport gene *YCF1* (Song *et al.*, 2003), natural hyperaccumulators such as *Thlaspi caerulescens* (Yang *et al.*, 2004), and *B. juncea* after application of incremental doses (as opposed to one high dose) of chelating agents (Barcosi *et al.*, 2003). Moreover, some of the ways of how plants tolerate heavy metals (e.g. chelation) are also how they accumulate them. In this respect, tolerance is linked to accumulation.

This research will emphasise on phytoextraction, rhizofiltration and phytostabilisation technologies. In this introduction section, 'tolerance' will be reviewed in the context of how indicator and hyperaccumulator plants survive and avoid, (respectively) the effects of heavy metal stress. There are two types of plant tolerance mechanisms involved: **Mechanism 1 and Mechanism 2**. An indepth review on particular parts of these mechanisms will be presented as they will feature in the key objectives of this thesis: (i) Mechanism 1 – complex polyphenols and their role in chelating metal-ions (sections 7 and 8) and (ii) Mechanism 2 – superoxide dismutase (SOD), the first line of antioxidant enzymes in the *antioxidative defence system* and their role in decreasing metal-induced ROS levels (namely superoxide anions) and polyphenol polymerisation.

5.1 Positive effects of reactive oxygen species (ROS)

Reactive oxygen species (ROS) is a term used to describe a group of products produced from the one electron reduction of molecular oxygen (O_2) (Figure 1). The most common ROS molecules are superoxide anion/ radicals ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot OH$) (Wojtaszek 1997).

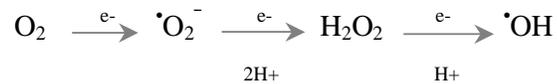


Figure 1. ROS production.

ROS, produced during plant development and primary defence (i.e. non-stress or moderate stress conditions), have a positive role in the biological system by acting as protectants, signal molecules and/or enzyme substrates for these processes (Greene, 2002; Wojtaszek 1997; Schöpfer, 1996). These include:

- (i) Photosynthesis and the respiratory electron transport chain (in the chloroplast and mitochondria), which involves oxidation of transition metals such as Fe^{2+} or Cu^+ (Fenton reactions).
- (ii) Cytoplasm/membrane bound/exocellular enzymes (e.g. NADPH oxidase, cell-wall peroxidase) involved in redox reactions during developmental stages such as seed maturation and cell wall/lignin biosynthesis.
- (iii) Exocellular enzymes in response to moderate stress conditions such as mild wounding (plant defence via oxidative burst reactions).

5.2 Negative effects of ROS and the antioxidative defence system

Whilst having a positive role in plant metabolism, ROS can also be damaging or fatal to the plant cell as they can easily react with bio-molecules (such as lipids, proteins and nucleic acids) (Schinkel, 2001).

To be able to utilise ROS for crucial plant development/defence and simultaneously avoid the damaging effects ROS, plant cells have an antioxidative defence system to tightly control the levels of these reactive molecules. This defence system comprises of several antioxidant enzymes (superoxide dismutase [SOD], catalase [CAT] and peroxidases [POX]) and a network of low molecular weight

antioxidant compounds (simple phenolic compounds, α -tocopherols) or reductants for antioxidant enzymes (ascorbate in ascorbate peroxidase and glutathione in glutathione peroxidase). ROS, such as $\cdot\text{O}_2^-$, are detoxified to H_2O_2 by superoxide dismutase (SOD) or by low molecular weight antioxidant compounds (above). The H_2O_2 is then converted into H_2O and O_2 by CAT or POX (that use either ascorbate or glutathione as reductants). In addition, a group of enzymes (dehydroascorbate reductase or monodehydroascorbate reductase and glutathione reductase) are involved in the regeneration of these reductants (Schinkel, 2001; Blokhina *et al.*, 2003).

5.3 Lead and oxidative stress

Elevated levels of lead ions (Pb^{2+}) can enhance ROS formation by binding to SH-groups of enzymes (such as those in the electron transport chain), substituting metal co-factors of membrane enzymes (causing electron leakage from the plasma membrane) or by binding directly to nucleic acids (Malecka *et al.*, 2001; Geebelen *et al.*, 2002). When ROS generation under such heavy metal stress cannot be controlled, the antioxidative defence system fails, resulting in lipid peroxidation, protein denaturation, DNA mutations and eventually cell death – a condition known as oxidative stress (Schinkel, 2001; Blokhina *et al.*, 2003). Some of these effects were reviewed in section 2.3 above.

6 HEAVY METAL TOLERANCE MECHANISMS

Plants can avoid or survive oxidative stress by: (i) controlling the concentration of metal-ion (i.e free metal ion) in the cell or tissue by isolation or chelation (Mechanism 1), or (ii) detoxifying ROS (produced by elevated levels of metals) using the antioxidative defence system (Mechanism 2).

Mechanisms 1 and 2 have been seen as two separate pathways - one focusing on prevention, the other focusing on repair. However, an increasing body of evidence suggests that the two are interlinked at certain metabolic points. In cadmium-exposed *B. juncea*, glutathione has not only been shown to be an antioxidant of Mechanism 2 but also a precursor to the phytochelatin chelator of Mechanism 1 (Navari-Izzo and Quartacci, 2001). After exposure to cadmium, nickel tolerant *Alyssum argenteum* showed higher antioxidant enzyme activity than nickel non-tolerant *A. maritimum*,

suggesting that the antioxidative defence system (Mechanism 2) plays a role in Cd tolerance (Schickler, 1999). In waterlilies, the role of the antioxidative defence system (specifically peroxidases) in heavy metal tolerance was suggested. The experimental results from this research suggest that increased peroxidase levels (Mechanism 2) induced polymerisation of phenolics, and that this polymerisation served to trap/chelate metal ions in the salt glands (Mechanism 1) (Lavid *et al.*, 2001b).

7 MECHANISM 1 (PART I): CONTROLLING METAL-ION LEVELS

Plants exhibiting metal tolerance or survival under stress are known to control and/or have the ability to reduce the effects of high free metal-ion concentrations. These can be categorised into two methods (not completely separate of each other): (i) to avoid uptake and build-up of toxic metals in the cytosol and (ii) detoxify metals that has entered the cytosol. The first method is achieved by tissue or cellular *isolation* and the second method involves metal-ion *chelation* to ligands.

7.1 Tissue isolation

The main heavy metal tolerance mechanism at whole plant level is the isolation of heavy metals to external plant structures, such as trichomes. Using electron microprobe techniques, Blamey *et al.* (1986) showed that Mn accumulated in and around leaf trichomes of sunflowers (*Helianthus annuus* L.). Using radioactively labelled Cd, Salt *et al.*, (1995b) showed that Cd preferentially accumulated in the leaf trichomes of Indian mustard plants (*B. juncea* L.). Isolation of metals to the root (and thus restriction to the shoot tissue) is also another possible mechanism (Thurman, 1981; Krämer *et al.*, 1997)

7.2 Cellular isolation

To control free metal-ion levels and protect cellular sites, metals are isolated by entrapment in the apoplast (cell wall and intracellular space), or are isolated in vacuoles or vesicles. Heavy metal entrapment in the cell wall has been observed in a variety of plant tissues including epidermal tissues of *Silene vulgaris* spp. *humulis* (Bringezu *et al.*, 1999), roots of *Allium cepa* (Wierzbicka, 1995) and protallium of *Lygodium japonicum* (Konno *et al.*, 2005). Heavy metals can also be detoxified by isolation in

vesicles and vacuoles (Clemens, 2001; Hall, 2002; Wierzbicka, 1995). In transmission electron micrographs of onion root cells, Pb deposits were found in vesicles fusing with the plasma membrane (Wierzbicka, 1998). In Zn-tolerant ecotypes of *Silene vulgaris*, Zn transport into vesicles was 2.5 times higher than sensitive ecotypes (Hall, 2002).

7.3 Detoxification by ligand chelation

Chelation of metal-ions to ligands is the third method of heavy metal tolerance in **Mechanism 1**. Chelation is thought to reduce metal toxicity and their ability to bind and disrupt cellular processes.

Both inorganic and organic ligands have been implicated in heavy metal tolerance, though the emphasis has been on the latter. The main inorganic compounds include phosphates (Cotter-Howells *et al.*, 1999) and silicates (Lichtenberger and Neumann, 1997; Neumann and zur Nieden, 2001). In terms of organic compounds (phytochemicals), PCs, MTs, organic acids and amino acids (reviewed by Clemens *et al.*, 2002 and Neumann *et al.*, 1995; Prasad, 1999), cell wall proteins (Lichtenberger and Neumann 1997; Bringezu *et al.*, 1999), cell wall pectins (Thurman, 1981), and polyphenols (Lavid *et al.*, 2001b) have been implicated in the tolerance of specific metals. Chelation of metals by these ligands is a result of the orientation and presence of N, O, or S atoms present in these compounds. Phytochelatins (PCs), which are cysteine rich polypeptides, has been extensively studied. In *A. thaliana* mutants, Cd tolerance was correlated with the amount of PCs (Howden *et al.*, 1995). Using *B. juncea*, it was shown that Cd accumulation resulted in a rapid induction of PC synthesis (Haag-Kerwer *et al.*, 1999). Phytochelatins have been shown to bind to Cu and Cd in *Silene vulgaris* cell cultures but not Zn or Pb (Leopold *et al.*, 1999). However, Leopold *et al.*, (1999) highlighted that the role of PCs is controversial. They also showed that in the case of Cu, PC-Cu complexation was only an initial and temporary response and that Cu was later found to complex with other unidentified compounds. Furthermore using *A. thaliana* mutants, Lee *et al.*, (2003) demonstrated that high levels of PCs lead to Cd hypersensitivity. Organic acids, such as citrate and malate, have also been implicated in heavy metal tolerance through the metal binding abilities of their carboxyl groups (Clemens, 2001). The carboxyl group (COO⁻) of cell wall pectins has been implicated in metal tolerance because of their ability to chelate metals. In Cu-tolerant *Agrostis tenuis*

(Thurman, 1981) and *Lygodium japonicum* fern (Konno *et al.*, 2005) it has been suggested that cell wall pectins, may be responsible for heavy metal tolerance as this fraction contained the highest concentration of metals. However, Thurman (1981) also cast doubt on the role of pectins. The pectin fraction of the Zn-tolerant *Agrostis stolonifera* was shown to accumulate five times more Zn than other cell wall fractions. He queried why this plant was also not Cu-tolerant given that Cu (according to Gurd and Wilcox, 1956) binds to carboxyl groups of pectins with a higher affinity than Zn. He also highlighted that the CEC of Zn-tolerant and non-tolerant roots of a number of plants were similar, which is not expected if pectic acids contribute to Zn tolerance.

The possible role of polyphenols has also been implicated (see section 8). Since polyphenols is the focus of this thesis, the next section is devoted to these compounds.

8 MECHANISM 1 (PART II): METAL-POLYPHENOL CHELATION

8.1 Polyphenols: class and structure

The basic structure of polyphenols consists of a 6-C aromatic ring with one or more hydroxyl group bound to it (Waterman and Mole, 1994). In a majority of scientific literature the term ‘polyphenol’ is interchangeable with the term ‘phenolic compound’ or ‘phenols’. However, a distinction between them is sometimes made (based on molecular weight), leading to confusion of the polyphenol definition. The term ‘phenolic acid’ however, is not interchangeable as it refers to one class of polyphenols. In this thesis, the term ‘polyphenols’ will be used to encompass all types of phenol-based compounds.

8.1.1 Classes of polyphenols

There is a wide range of polyphenols, ranging from simple structures with one aromatic ring to highly complex polymeric substances such as tannins and lignins. There are eight classes of polyphenols: (1) simple phenolics compounds, (2) tannins, (3) coumarins and their glycosides, (5) nathoquinones, (6) flavones and related flavonoid glycosides, (7) anthocyanidins and anthocyanins, (8) lignans and lignin. All polyphenols are formed via the shikimic acid pathway, also known as the phenylpropanoid pathway

(Evans, 1996). The thesis will focus on the extractable polyphenol levels and specific polyphenols called tannins.

8.1.2 Tannins

Tannins are the fourth most abundant phytochemical made by vascular plants, after cellulose, hemicellulose and lignin (Kraus *et al.*, 2003). Together with lignins, tannins are the most widespread and abundant polyphenols in plants (Scalbert *et al.*, 1999). One of the most satisfactory definitions of tannins is that of Horvath (Brown, 2001):

“Any phenolic compound of sufficiently high molecular weight and containing a sufficient number of hydroxyl or other suitable groups (i.e. carboxyls) to form effectively strong complexes with proteins and other macromolecules under the particular environmental condition being studied”.

This definition emphasises the character of tannins that sets it apart from all other polyphenols: their ability to bind and precipitate proteins (Hagerman, 1998).

The two groups of tannins are hydrolysable tannins and condensed tannins. Condensed tannins are more widespread than hydrolysable tannins. Hydrolysable tannins are as their name suggests, easily hydrolysed by acids or enzymes such as tannase (Evans, 1996). They are formed from several molecules of phenolic acids, commonly gallic acid or ellagic acids, which are united by ester linkages to a central glucose molecule. Unlike hydrolysable tannins, condensed tannins (proanthocyanidins) are not readily hydrolysable and do not contain a sugar moiety. They are related to flavonoid pigments and have a polymeric flavon-3-ol structure with A, B and C rings (Evans, 1996; Kraus *et al.*, 2003) (Figure 2).

8.2 General roles of plant polyphenols

The biological roles of polyphenols in plants are diverse. They are thought to help plants: (i) attract pollinators and seed dispersal agents, (ii) regulate growth and development, (iii) protect against herbivores, pathogens and environmental stress, (v) provide structural support at cellular and tissue level and (vi) serve as antioxidants (Chalker-Scott and Krahmer, 1989; Jackson *et al.*, 1996; Lozovaya *et al.*, 1999; Blokhina *et al.*, 2003).

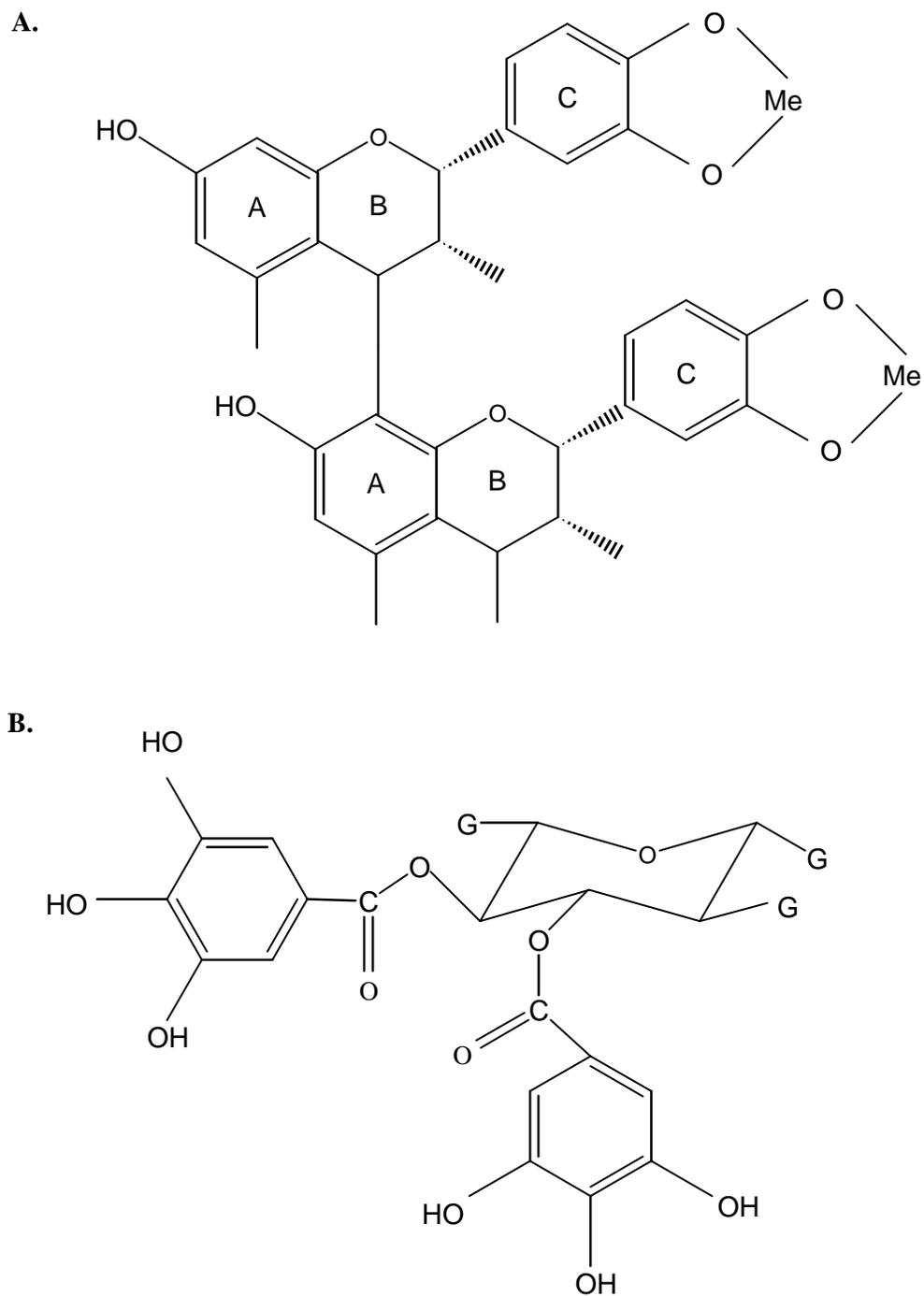


Figure 2. Example of condensed (A) and hydrolysable tannins (B). On the condensed tannin shown above, an example of metal (Me) chelation to two consecutive hydroxyl (-OH) groups (catechol residues) is illustrated.

Tannins are known to be involved in all these roles, except for role (i), which is fulfilled by the anthocyanidin class of polyphenols. As growth regulators, tannins have been shown to regulate certain plant tissues by inhibiting enzyme activities or acting as barriers to growth (Chalker-Scott and Krahmer, 1989). In terms of defence, the high concentration of tannins in protective tissues, such as bark, acts as ready-made anti-microbial agents and barriers to microorganisms for underlying living tissue (Scalbert, 1991). In a study on the bacterium *Erwinia chrysanthumi*, which causes rot in a wide range of plants, tannins inhibited the bacterium's growth by causing iron deprivation through iron-tannin complexation (Scalbert *et al.*, 1999). If underlying plant tissues are wounded a protective cover can also be produced by oxidative cross-linking of polyphenols (polymerisation). This process is also well known to influence the structure and extensibility of cell walls (McCallum, 1989). The high tannin content of plants also deters herbivores from foraging because of the astringent taste produced when plant tannins chelate to herbivore salivary proteins (Scalbert *et al.*, 1999; Fickel *et al.*, 1999). Polyphenols are also excellent antioxidants because they possess the ideal structure for free radical scavenging. Their antioxidative property is due to their ability to: (i) be hydrogen and electron donors, (ii) stabilise and delocalise the unpaired electron of ROS (in their radical form), (iii) chelate transition metal-ions (e.g. Cu and Fe) involved in ROS generation, (iv) alter lipid packing order and fluidity of membranes that could hinder the diffusion of free radicals, and (v) be involved in hydrogen peroxide scavenging cascade in plant cells (Blokhina *et al.*, 2003; Jung *et al.*, 2003).

8.3 Location of polyphenols in plants

8.3.1 Polyphenols at cellular level

Polyphenols are mostly detoxified by esterification with sugars moieties in the cell wall and in vacuoles (Parham and Kaustinen, 1977; Chalker-Scott and Krahmer, 1989). A disruption to the cell by pathogens or environmental stress releases these compounds from their ester linkages and location (Bussotti *et al.*, 1998; Chalker-Scott and Krahmer, 1989). They then become oxidised and quickly modify cell components such as enzymes and proteins in the plant or pathogen. In other cases they are synthesised and accumulate in the stressed tissue. The result is either a physical barrier or active antibiotic compounds (Chalker-Scott and Krahmer, 1989).

Polyphenols are present in the secondary and primary cell walls. In the secondary cell wall, condensed tannins and catechins are thought to be precursors of lignin biosynthesis due to their structural similarity to lignin precursors, and their ability to be proton acceptors during lignin synthesis reactions (Chalker-Scott and Krahmer, 1989; Lavid *et al.*, 2001b). Tannins in the primary cell wall, when complexed with pectin, cellulose or cell wall proteins, might form the initial matrix for lignin deposition (Chalker-Scott and Krahmer, 1989). The primary cell wall not only consists of cellulose and other sugars, but also phenolic acids (e.g. ferulic acids, *p*-coumaric acid) that are normally associated with the secondary cell wall) to control the extensibility of the cell wall (Chalker-Scott and Krahmer, 1989; Lozovaya *et al.*, 1999). Polyphenols in vacuoles of root and shoot tissue has been also reported (Mueller and Beckman, 1976; Scarlet *et al.*, 1989; Getachew *et al.*, 1998; Hutzler *et al.*, 1998).

Some phenolic compounds are also covalently linked to waxes (Hutzler *et al.*, 1998). An example is the waxy suberin layer, between the cell wall and plasma membrane, which may contain condensed tannins. It has been suggested that these polyphenols could stabilise the membrane-cell wall interface by providing a bridge between the two layers; hydrophilic polyphenols attachments to the cell wall and hydrophobic (suberin-wax fraction) attachments to the membrane (Chalker-Scott and Krahmer, 1989).

8.3.2 Polyphenols at tissue level

Polyphenols are found in all tissues: bud, leaf, stem, bark, trichome, seed and root (Chalker-Scott and Krahmer, 1989; Scalbert, 1991, Lavid *et al.*, 2001b).

The root tissue was the main focus in this study because this tissue of the experimental plant contained the highest level of polyphenols (see results section). There are few histochemical studies on the location of tannins in the root tissue. In cotton roots, catechins and gallocatechins have been reported in the hypodermis, endodermis, xylem, and root cap. In *Rheum* roots, tannins have been found in cork cells, particularly those close to the outside of the root (Chalker-Scott and Krahmer, 1989). In general, tannins are mostly found in the hypodermis. Such external distribution of polyphenols in the root tissue may serve as a chemical barrier to penetration and colonisation of roots by plant pathogens (Mace and Howell, 1974).

8.4 Heavy metal tolerance: chelation to polyphenols?

Polyphenols, especially tannins, are well known for their *in vitro* ability to chelate metals, such as iron (Fe) and Pb (Lavid *et al.*, 2001a; Evans, 1996). Virtually all tannins and most polyphenols have a catechol residue (two consecutive -OH groups on an aromatic ring) that makes them excellent metal-ion chelators.

These metal-chelating properties have formed the basis of a number of industrial products such as, Cu agents in wood preservation, writing inks, and foliar sprays (McDonald *et al.*, 1996; Laks and McKaig *et al.*, 1988). Polyphenol-metal chelation has also been an important factor in human and cattle nutritional studies (Bravo, 1998; Lavid *et al.*, 2001b). In cattle nutritional studies, it was found that high intake of tannin-rich foods inhibited Fe absorption, resulting in dietary deficiencies (Terrill *et al.*, 1992). Similarly, in humans the consumption of polyphenol-rich foods such as tea and coffee is known to decrease Fe absorption. In severe cases, this has led to Fe deficiency-related illnesses such as anaemia (Scalbert *et al.*, 1999).

Investigating the role of polyphenol-metal chelation in heavy metal tolerance may be important in developing successful phytoremediation. However, the biological importance of metal chelation by polyphenols (especially tannins) in plants towards heavy metal tolerance is not well known. Loponen *et al.*, (2001) suggested that polyphenols (e.g. catechin) in white birch leaves (*Betula pubescens*) may be involved in heavy metal tolerance as higher levels of these compounds were found in these plants at (and closer to) polluted sites (e.g. Ni-Cu smelter). An increase in amino acids precursors in these plants that make polyphenols (e.g. phenylalanine) (Moreno *et al.*, 2003), and soluble polyphenol levels (Schützendübel *et al.*, 2001; Tripathi and Tripathi 1999; Ruso *et al.*, 2001) has also been reported.

Studies in which tannins are thought to be involved in heavy metal accumulation and tolerance include: epidermal glands of *Nymphaea auroa* upon exposure to Cd, Pb and Cr (Lavid *et al.*, 2001a), root apices of *Lotus pendunculatus* Cav. during exposure to Al (Stoutjesdijk *et al.*, 2001), tannin cells of *Armeria maritima* with Cu exposure (Lichtenberger and Neumann, 1997). With respect to Pb, reports into the role of tannin-metal chelation in tolerance and phytoremediation are rare. The involvement of condensed tannins in Pb accumulation in *Athyrium yokoscense* (fern) gametophytes has also been suggested (Kamachi *et al.*, 2005). The role of polyphenols in chelated Pb

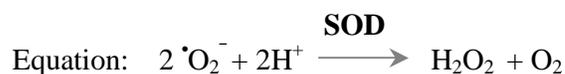
tolerance (as opposed to the above examples which all involve unchelated metals) is unknown.

9 MECHANISM 2: THE ANTIOXIDATIVE DEFENCE SYSTEM (ADS)

If the metal-ion concentration cannot be controlled with **Mechanism 1**, ROS formation will increase. The role of the antioxidative defence system is to remove excess ROS produced in order to avoid a build-up of ROS that will eventually cause oxidative stress. The role of this defence system in heavy metal tolerance is scant, especially in regard to the presence of chelating agents like EDTA. Of the ADS components that could be involved in heavy metal tolerance, the superoxide dismutase (SOD) enzyme will be the focus of this study.

9.1 Superoxide dismutase (SOD)

Superoxide dismutase (SOD, EC 1.15.11) is the first and sole enzyme of the antioxidative defence system which catalyses the destruction of the superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2) and elemental oxygen (O_2). There are three types of SOD, each differentiated by their metal ion co-factor and location in the cell: Fe-SOD (chloroplast), MnSOD (mitochondrion and peroxisomes) and Cu/ZnSOD (chloroplast and cell wall) (Del Rio *et al.*, 2003; Alscher *et al.*, 2002). SODs are found at these sites because they are where superoxide anions production and accumulation occurs (Greene, 2002).



9.2 Role of SOD in heavy metal tolerance

The role of the plants' antioxidative defence enzymes in heavy metal tolerance is a relatively new branch of study. Superoxide dismutase may contribute to Pb tolerance in two ways: (i) by detoxifying superoxide anions into H_2O_2 , and (ii) by producing a substrate (H_2O_2) which is involved in creating polymerised (also known as oxidised)

polyphenols that chelate metals. Overall, the trend in SOD activity on polyphenol levels is of interest in this study.

In most reported studies, the direction of SOD activity is variable (Gratao *et al.*, 2005). This is partly due to differences in experimental factors such as plant species, tissue type, metal type, metal concentration, and duration of metal exposure. However, most papers (using seedlings) report an increase in activity upon heavy metal exposure (Ali *et al.*, 2003; Ruley *et al.*, 2004; Reddy *et al.*, 2005). For example, in roots of peas (*Pisum sativum*) treated with 0.5-1 mM Pb(NO₃)₂, SOD activity increased by three-fold (Malecka *et al.*, 2001). After 15 days in the same concentrations of Pb, SOD activity increased by 87-100% in rice seedlings (*Oryza sativa*) (Verma and Dubey, 2003). However, onions (*Allium cepa*) exposed to wastewater containing 167.3 ppm Pb showed a steep decline in SOD (Fatima and Ahmad, 2005). The same decreasing trend (indicating oxidative stress) was also observed in peas exposed to Cd (Sandalio *et al.*, 2001).

10 TISSUE CULTURE AND PHYTOREMEDIATION

Tissue culture has been used as a tool to propagate and investigate tolerance mechanisms, such as those relating to the antioxidative defence system, in heavy metal tolerant plants. For example, shoot explants of *Acer psuedoplatanus* L. (sycamore) (Watmough and Dickinson, 1996) and *A. rubum* L. (red maple) (Watmough and Hutchinson, 1998) trees growing near metal smelters were cultured and callus tissues generated. Exposure of the callus tissues to Zn for example, showed that callus or cell lines generated from the most heavily contaminated Zn site, also exhibited the highest Zn tolerance (in terms of fresh weight and lethal dosage of metal). Similarly, in cell suspension cultures of *Sesbania drummondii*, tolerance was related to a positive correlation between Pb accumulation levels (0-500 mg L⁻¹ Pb(NO₃)₂) and induction of antioxidative enzyme activities (SOD and CAT) (Sharma *et al.*, 2005). Whilst the role of elevated antioxidative enzyme activity to heavy metal tolerance was demonstrated in this study, it is not true in all studies. Using heavy metal tolerant and sensitive clones of *Salix viminalis*, Landberg and Greger (2002) showed that there was no difference in antioxidative enzyme activities upon heavy metal exposure. However, they suggested

that *Salix* tolerance could be attributed to the elevated SOD level in tolerant clones prior to exposure to heavy metals. On average, SOD levels were 11% higher in shoots and 24% higher in roots of tolerant clones, suggesting that tolerant *Salix* species required less SOD production to achieve the same SOD level as the sensitive clones upon heavy metal exposure.

11 MUTANT STUDIES AND PHYTOREMEDIATION

There are four approaches to obtaining mutants for heavy metal tolerance and accumulation studies: (i) cultivar screening, (ii) somaclonal variation, (iii) genetic modification, and (iv) chemical mutagenesis. This section will review the last three approaches because they involve tissue culture; an example of cultivar screening is indicated in section 3.35 above.

11.1 Somaclonal variation approach

Somaclonal variations are mutations in plant tissue cultures that occur randomly, or are induced under *in vitro* selective pressures. The technique is often exploited to develop plants with desirable traits such as larger plant size, new flower and fruit colours, and resistance to stress conditions such as pathogens and salinity (Remotti, 1998). In this thesis, induced somaclonal variations in tissue culture will be used as a tool to investigate and attempt to improve the phytoremediation properties of our experimental plant growing under heavy metal (Pb) stress.

11.1.1 Types and advantages

There are three main types of somaclonal variations: (i) genetic variation - change in nucleotide sequence of DNA and chromosome number or structure (inheritable), (ii) epigenetic variation - change in gene expression (but not inheritable) and can be expressed without the presence of the stress condition, and (iii) physiological or adaptive variation - change in gene expression (not inheritable), but only present under stress conditions. The latter two forms of mutations occur frequently, whereas genetic variations are rare and reflect why only a few new cultivars developed via somaclonal mutations have been released to date (Tal, 1990).

The advantage of tissue culture-based somaclonal variation is in the flexibility and public acceptance of the technique when compared to genetic engineering. Tissue culture allows a wide range of plant materials to be examined; from whole plants to selected tissues such as bud, leaf or just root tissue, to undifferentiated cells in callus tissues and cell suspension cultures. It also allows a large population to be handled, selected and cloned. From a public acceptance stance, plants derived from somaclonal variations are more acceptable compared to transgenic plants (a genetically modified organism) as no genetic information has been deliberately inserted into the original plant genome; all variations rely on chance, which is not different from natural evolution (Predieri, 2001).

11.1.2 Induced somaclonal variation

The cell culture environment can be manipulated to enhance the rate of somaclonal mutations. Whole plants, tissues, or more commonly callus (undifferentiated cells) are exposed to the stress factor in a one-step high-dose approach (shock treatment) or via a more gradual incremental dosage approach. The surviving cells are then selected for regeneration with and without the stress factor to determine if the mutation is genetic or adaptive. Besides stress factor exposure, the normal nutrient media can also be altered. Mineral deficiencies in magnesium, calcium, phosphorous, nitrogen and sulphur, and the addition of the 2,4 D (a plant growth regulator) have been reported to induce chromosomal breakages and double mutation rates (Conner and Meredith, 1984a and b).

There are two debates within the approach of somaclonal mutation. Firstly, which plant tissue should be used for somaclonal variation: callus tissue or a differentiated tissue such as leaves. This is debated because mutations observed at callus level do not necessarily occur in differentiated tissues. Secondly, the duration of stress factor exposure: 'shock' treatment or gradual exposure approach. Genetic, heritable variations are said to increase with the one-step approach, but it is argued that this fast approach does not allow the cell or tissue to fully develop their tolerance capabilities (Tal, 1990; Remotti, 1998).

11.1.3 Developing heavy metal tolerant plants

In heavy metal tolerance studies, somaclonal variation has been rarely used to develop plants or tissues with heavy metal sensitivity or tolerance. *In vitro* Al tolerance has been developed in the plantlets of *Oryza sativa* and *Daucus carota* and Cd tolerance in the callus tissue of *Datura innoxia* and *Nicotiana tabacum* (Remotti, 1998). Gallego *et al.*, (2002) demonstrated an involvement of the antioxidative defence system in the adaptation of *Helianthus annuus* L. callus to heavy metals. In Cd- and Cr-adapted callus, all antioxidative enzymes except GR, (SOD, CAT, POX), were elevated relative to controls (non-heavy metal exposed callus). Somaclonal variation has also been applied to understanding salt tolerance mechanisms in crop plants such as *B. juncea* (Remotti, 1998) and *Lycopersicon esculentum* Mill. (Rus *et al.*, 2000).

11.2 Genetic modification approach

Another approach to develop metal-tolerant plant species is to use genetic modification. The goal of this approach is to develop fast growing, high-biomass plants with the metal accumulation traits of natural small biomass hyperaccumulators: ‘engineered phytoremediators’ (Ow, 1996). The advantage of this technique is the relatively short space of time and selective targeting of genes for improvement.

The possible areas of genetic manipulation include: (i) metallothioneins, phytochelatins and other natural metal chelators, (ii) metal transporters, (iii) alteration of metabolic pathways and (iv) alteration of oxidative stress mechanisms (v) alteration of root structure and shoot biomass (Kärenlampi *et al.*, 2000; Eapen and D’Souza 2005; Yang *et al.*, 2005).

Over the last few years, the genetic modification approach has gained significant momentum. Until recently, most metal or metalloid (semi-metals) tolerant genes used for engineering phytoremediators have been of microbial origin. Other genes used originated from non-hyperaccumulating plant genes, and mammalian metallothionein and phytochelatin synthase (Tong *et al.*, 2004; Eapen and D’Souza 2005). More recently, insertion of metal and metalloid tolerant genes from hyperaccumulating plants into non-hyperaccumulators has been reported (LeDuc *et al.*, 2004, see below).

11.2.1 Transgenic plants

The development of engineered phytoextractors began with the phytovolatilisation form of phytoremediation. Mercuric-ion reductase (Mer protein), a bacterial protein which reduces Hg^{2+} to volatile Hg^0 , was inserted into *Arabidopsis*, tobacco and poplar plants. These transgenic plants were found to be more tolerant of organic mercury (Song *et al.*, 2003; Krämer, 2005). Other notable examples of transgenic plants modified with genes of microbial origin include those that detoxify arsenic (As) and selenium (Se) metalloids (reviewed in Eapen and D'Souza 2005).

The first high biomass engineered phytoextractor involved selenium (Se) (achieved at a laboratory scale). Although it is a metalloid and not a heavy metal, Se was the first contaminant where insertion and overexpression of a hyperaccumulator plant gene (*SMT* from *Astragalus bisulatus*) into a fast growing plant (*Brassica juncea*) resulted in increased tolerance, accumulation and volatilisation of selenate (the predominant form Se in soils) (LeDuc *et al.*, 2004). In field-scale studies, Se-transformed plants (inserted with *E. coli* genes which enhanced selenate assimilation and glutathione synthesis) have been shown to enhance Se phytoremediation (Bañuelos *et al.*, 2005). Most recently, Cd accumulation was enhanced when a metallothionein gene from *Silene vulgaris* L. was overexpressed in the high biomass *Nicotiana tabacum* L. (tobacco) (Gorinova *et al.*, 2006).

Recent developments in Hg and As transgenic plants have also included targeting the tolerant genes to specific cell sites and tissues or expressing a transgene only under certain conditions (Krämer, 2005; Pilon-Smits, 2005). In the case of Hg, targeting the Mer protein to the cell wall or endoplasmic reticulum (where non-volatile forms of mercury accumulate) enhanced the effectiveness of this transgene. Transgenic *A. thaliana* plants with the *ArsC* gene targeted downstream of the soybean promoter (which confers shoot-specific and light-induced expression), reduced root accumulation and increased As translocation (Krämer, 2005).

However, currently there are no transgenic plants adequate for commercial phytoextraction (Krämer, 2005). Furthermore, there is currently no fast growing, high biomass engineered phytoextractor for Pb. This may be because there is no known function of Pb in plants and thus there are very few genes conferring Pb tolerance and or accumulation. The exception to this is *Saccharomyces cerevisiae* (yeast) protein *YCF1*,

(a transporter protein which pumps Cd and Pb into vacuoles) and *TaPCSI* (which encodes PCs). Overexpression of *YCF1* protein in transgenic *A. thaliana* and yeast resulted in enhanced tolerance and accumulation to these heavy metals. Deletion of *YCF1* protein from yeast cells resulted in hypersensitivity to Pb (Song *et al.*, 2003). The overexpression of *TaPCSI* (involved in PC synthesis) in *Nicotiana glauca* also increased Pb accumulation and root growth under Pb treatment (Martinez *et al.*, 2006).

11.2.2 Risks associated with genetic engineering

The potential risks associated with using engineered phytoresponders include: (i) transgene flow, (ii) use of antibiotic resistance markers, (iii) cross contamination of human and animal foods, and (iv) effect on rhizosphere ecology. Of these risks, the most concerning is transgene flow because it may lead to the possible loss of genetic diversity and extinction of wild plants if genes flow from cultivated plants to wild relatives (Davison, 2005). To decrease the chances of spreading the transgene from engineered phytoresponders to natural plant populations, biological encapsulation has been proposed. This is where the gene(s) are inserted into the chloroplast genome instead of the nuclear genome (Krämer, 2005; Pilon-Smits, 2005). The use of conditional suicide genes may also be possible (Davison, 2005). For example, upon depletion of a certain pollutant or escape of the plant to non-contaminated soils, suicide genes could be activated. Another example is designing suicide genes that are activated upon an external environmental stimulus such as ethanol (Davison, 2005). However, this could be a concern if the suicide gene is transferred to non-target plants.

Currently in New Zealand, the Environmental Risk Management Authority (ERMA) strictly controls the use and release of genetically modified organisms (GMOs). At the time of this thesis, there was a moratorium (part of the Hazardous Substance and New Organism Act, 1996 and Biosecurity Act, 1993) on releasing GMOs in New Zealand (Ministry for the Environment – online, 2006). Consequently, since this situation led to an uncertain future for GM crops in New Zealand, the genetic modification approach was not the focus in this study.

11.3 Chemical mutagenesis approach and *Arabidopsis thaliana*

Whilst somaclonal mutations occur under heavy metal exposure, they are unlikely to be inheritable. For this reason, selection and analysis of chemically induced (EMS) *Arabidopsis thaliana* mutants is useful in understanding heavy metal tolerance mechanisms, especially in terms of Mechanism 1 and 2, or identifying specific inheritable genes involved in heavy metal accumulation and tolerance.

Since the 1960s, *A. thaliana* has been widely used as a model plant species to study basic plant processes and responses to conditions, such as nutrient deficiency and metal toxicity. It is used as a model plant system mainly because of its small well-characterised genome, short life cycle, large seed set and small seed size (Delhaize *et al.*, 1993). Its familial links with natural hyperaccumulators from the Brassicaceae family, such as *Thlaspi caerulescens*, has been cited as another reason for its use in metal toxicity studies.

Numerous *Arabidopsis* studies have increased the understanding of plant metabolism. These include the identification and analysis of mutants showing enhanced iron accumulation, defective phosphate translocation, and nitrate metabolism (Chen *et al.*, 1997). In metal toxicity studies isolation of mutants has enhanced the understanding of heavy metal toxicity, tolerance and accumulation. For example, callose was thought to be responsible for Al root inhibition, but isolation of mutants rejected this idea (Larsen *et al.*, 1996). Phytochelatins are thought to be involved in heavy metal tolerance and studies to test this have been made possible by isolating the PC synthase gene from Cd-sensitive mutants with deficient PC synthesis (Cobbett, 2003). In Pb-related studies, Chen *et al.*, 1997 initiated a research programme to identify *A. thaliana* Pb mutants to similarly enhance the understanding of Pb accumulation and tolerance in plants. It was envisaged that identification of genes controlling Pb accumulation and tolerance in *Arabidopsis* mutants, could be useful for engineering better phytoextraction plants. In the initial experiments over 500,000 seedlings were screened for Pb mutants, using root length indicators. Three mutants, APb2, APb7, APb8 were isolated. These mutants not only had elevated Pb shoot accumulation levels (three fold on a dry weight basis) relative to wild type plants, but also elevated accumulation levels of Cu, Mg, Zn and S. A possible mutation in the *man 1* gene controlling metal-ion uptake or levels in the mutant has been suggested as the basis for increased Pb accumulation and tolerance.

Isolating such genes may lead to genes for genetic engineering to enhance Pb phytoremediation.

12 SYMPHYTUM OFFICINALE L. (COMFREY)

Comfrey or knitbone (*Symphytum officinale* L.) is an erect, hairy perennial, native to Europe and temperate Asia, and naturalised in many countries such as New Zealand. It is a terrestrial plant that grows easily in damp, shady places, particularly near streams (Chevalier, 1996). *S. officinale* is an important animal feed in parts of the world such as Europe and Africa, and has been an important medicinal herb in healing fractures (Chevalier, 1996; Lawrence, 1976).

Symphytum officinale was selected for this project as an experimental plant for the following reasons. Firstly, it has a fast growth rate and high biomass (on average, some species of comfrey are able to produce 73 metric tones dry matter ha⁻¹ yr⁻¹) (Lawrence, 1976), three times that of corn which has been selected as a possible phytoremediation plant (Saxena *et al.*, 1999). Secondly, it has not been tested for phytoremediation. Finally, it contains the compounds under investigation: polyphenols and tannins. According to the PDR (1998) this plant contains 8-9% (d.w.) of tannins in shoots and 4-6% (d.w.) in roots. Additionally, *S. officinale* also has other features that could be beneficial to phytoremediation. These include: (i) a long and dense root system, (ii) regenerable shoots, well suited for continuous phytoextraction, (iii) abundance of trichomes which is a possible factor in metal tolerance (see above), (iv) its ability to withstand high salt environments, like near streams, may be advantageous for rhizofiltration, and finally (v) a high protein content (33% of dry matter) which could indicate an ability to produce protein-based metal chelators (e.g. PCs, MTs or transport proteins).

13 AIM AND OBJECTIVES

Due to its widespread distribution, persistence, and toxicity to human health (Csuros, 1994), lead (Pb) is often considered as the primary target for remediation studies, such as phytoremediation (Chen *et al.*, 1997).

Over the past decade, advances in phytoremediation have been a result of several research initiatives. These include the:

- (i) study of metal uptake, transport, tolerance and accumulation mechanisms
- (ii) study of metal solubility, and the concentration and type of chelating agents
- (iii) selection of plant varieties (ecotypes, cultivars, and transgenic plants)

In this study, three objectives in line with these initiatives were developed to advance Pb phytoremediation (using *S. officinale* as the experimental plant).

- (i) Objective 1: Association of plant polyphenols (particularly tannins) with the chelation-based heavy metal tolerance mechanism (tolerance mechanism 1) and Pb accumulation.
- (ii) Objective 2: Phytoremediation potential of *S. officinale* based on chemical-based variations to Pb concentrations and chelating agents.
- (iii) Objective 3: Use somaclonal variation to develop and screen for Pb mutants in *S. officinale* (sensitive or tolerant).

Recent studies have shown that plant polyphenols may be involved in heavy metal tolerance via its ability to chelate to metal ions (tolerance Mechanism 1) (Lavid *et al.*, 2001b). This is important for non-chelator based phytoremediation and for chelated-assisted phytoextraction where up to 40% of chelated Pb (PbEDTA) may dissociate into free Pb ions (Geebelen *et al.*, 2002). However, no studies to date have directly correlated the role of Pb-tannin chelation to Pb tolerance and accumulation in phytoremediation. Thus, the research began with the main objective of this project, objective 1. Central to this objective was to determine if there was a correlation between extractable polyphenol levels and Pb uptake/accumulation using *in vivo* and *in vitro* experiments. *In vivo* experiments firstly began with determining the distribution of polyphenols and tannins in *S. officinale* and the best possible experimental parameters for their analysis (e.g. assay type and extraction method). After hydroponic exposure to Pb(NO₃)₂ with or without EDTA, the extractable polyphenol and tannin levels of roots were analysed (because polyphenols were the highest in these tissues) All tissues (shoot and root) were used to determine Pb accumulation (and thus phytoremediation potential). Moreover, the large *in vivo* difference in polyphenol levels between the two root types of *S. officinale* (lateral and main roots), were utilised for Pb-polyphenol

correlation experiments. To further support the *in vivo* findings, transmission electron micrographs of roots with and without Pb treatment were examined to locate Pb accumulation at a cellular level and ultimately determine if they could be co-localised with known tannin sites in the cell. To consolidate the *in vivo* findings (i.e. to reduce the possibility that non-tannin factors were involved in Pb accumulation and tolerance) *in vitro* studies demonstrating that *S. officinale* tannins have the ability to chelate Pb ions under *in vitro* conditions were performed. However, the method employed for this purpose gave a completely contrary finding to the *in vivo* hydroponic experiments because the method was subject to interferences from other compounds in the extracts. Since there was no other established method, a new *in vitro* Pb-tannin chelation method had to be designed.

The second objective assessed the phytoextraction potential of *S. officinale* using increasing Pb and EDTA concentrations and testing a different chelating agent. In performing these experiments an unexpected trend in these experiments was observed. This led to an additional experimental question: “Why did the addition of EDTA to feeding solutions have a negative effect on accumulation of Pb in roots (which was not accounted for by the level translocated to the shoots)?” The effects of EDTA pre-treatment and alternative chelating agents (ADA) on polyphenol levels and Pb accumulation were subsequently assessed to elucidate an answer.

The final objective was to use somaclonal variation in tissue culture to develop *S. officinale* Pb mutants in order to further investigate and attempt to improve the Pb phytoremediation potential of this experimental plant. Firstly, *in vitro* tissue culture stocks of *S. officinale* were established. Secondly, plants from Pb-treated petioles were developed under *in vitro* conditions. Finally, these plants were re-treated with Pb (under non-sterile conditions). Changes to morphology, Pb accumulation, polyphenols levels, and SOD activity were assessed to determine if and how mutations may have occurred. Histochemical localisation of superoxide anions in any Pb-tolerant mutant was also planned. Since mutations in *S. officinale* may not be heritable, selection and similar assessment of genetically altered Pb-tolerant *A. thaliana* mutants was also to be performed (see Appendix K).

CHAPTER 2

MATERIALS and METHODS

Objective 1

*Association of polyphenols with chelation-based tolerance mechanism and Pb accumulation: Hydroponic studies with sand-grown *Symphytum officinale**

1 PLANT PROPAGATION

1.1 Root stock

Symphytum officinale root-stocks (approximately 10-years-old) were obtained from Millstream Gardens, Napier. All roots were stored at room temperature in the dark and kept moist.

1.2 Vegetative plant propagation

S. officinale plants were propagated using guidelines from Gardiner (1997), Seitz (1996) and Thompson (1992). Plants were established using root sections that were four cm long and one cm in diameter. The entire root section was placed vertically until the head was only 0.5 cm below the surface of the sand-based growth medium (consisting of approximately five grams of Nutricote™ 8-9 month slow-release fertiliser granules sprinkled on the top for every one litre of fine sand). All plants were grown in greenhouses for approximately 2-10 months depending on the experiment.

2 POLYPHENOL STUDIES

2.1 Histochemical studies

2.1.1 Detection of polyphenols

The presence of polyphenols was determined by adding 1-2 drops of stain (composed of 10% (v/v) sodium nitrate, 10% (v/v) acetic acid, 20% (v/v) urea) to each root cross section. After three minutes, two drops of 2M NaOH were added to the sections. Polyphenols produce colours from yellow to red (Reeve, 1951; Gahan, 1984).

Control samples were tissues covered with water or 2M NaOH instead of the stain.

2.1.2 Class of tannins

Root polyphenol extracts from methods section 2.3.2 were spot tested with 2% (w/v) FeCl₃ dissolved in 95% (v/v) ethanol to determine the class of tannins in *S. officinale* roots (Mace, 1963). Condensed tannins turn brown/green, or if they are hydrolysable tannins, black/blue. A change from brown/green to blue/black upon increasing drops of FeCl₃ indicates the presence of both tannins (Mace, 1963; Evans, 1996).

2.2 Factors affecting polyphenol analysis

Five parameters were tested using the combinations outlined in Table 1 and in methods section 2.3 below. Tissues were sampled from 3-4 month-old plants.

Table 1. Polyphenol parameters tested.

	Assay	Extraction method	Extraction solvent
Tannin recovery	F/C	u/w	70% (v/v) acetone
Extraction rounds	F/C	u/w	70% (v/v) acetone
Type of extraction solvent	F/C	u/w	70% (v/v) acetone 50% (v/v) methanol
Assay type	F/C or BSA	u/w	50% (v/v) methanol
Extraction method and time	F/C	20 minutes in u/w (room temperature) or overnight shaking (18-19 hours at 4°C)	50% (v/v) methanol

F/C = Folin-Ciocalteu, BSA = BSA protein precipitation assay, u/w = ultrasonic water bath

2.3 Polyphenol and tannin extraction

The following methods were performed using a combination of methods (some with modifications) from Lavid *et al.*, (2001b), FAO/IAEA manual (2002), and Waterman and Mole (1994). For mainly practical reasons, the use of dried tissue is the preferred method of sample preparation for polyphenol studies (Waterman and Mole, 1994; Brown, 2001), especially when the sample size is greater than three grams (d.w.).

2.3.1 Sample preparation

In a 4°C cold room, different plant parts (leaf blade, petiole, lateral root, main root) were cut into small pieces. Tissues were frozen and crushed into smaller pieces with liquid nitrogen before they were stored overnight at -80°C. Samples were freeze-dried (ThermoSavant, Super Modulyo 230) for at least two days, and then the dried samples were stored in capped glass vials enclosed in an airtight container with silica crystals.

In this study, the lateral roots were less than one mm wide in diameter and branching from a main root. The main roots were at least two mm wide in diameter from which lateral roots extended (Plate 1).

2.3.2 Extraction methods

To avoid condensation onto dried samples, vials were equilibrated to room temperature prior to opening. For method section 2.2 (testing extraction factors), freeze-dried tissues were ground to a fine powder (with a mortar and pestle). Approximately 0.2 g of tissue was extracted for polyphenols and the tissue extracted with 25 mL of 70% (v/v) aqueous acetone or 50% (v/v) aqueous methanol in an ultrasonic water bath (for 20 minutes at room temperature), or overnight on a shaker (for 18-19 hours at 4°C). Unless indicated in the methods or results, the extraction method was then standardised as follows. Dried tissues were ground with a ball mill (Retscher MM300) and extracted with 50% (v/v) methanol in an ultrasonic water bath (for 20 minutes at room temperature). Samples were centrifuged at 11,000g for 15 minutes, at 4°C. The supernatant volume was measured prior to each polyphenol/tannin assay.

2.4 Polyphenol quantification

In the preliminary experiments, pigments and fats from leaf and root extracts were removed by extraction with diethyl ether containing 1% (v/v) acetic acid prior to the



Plate 1. Lateral and main root of 2-month-old *Symphytum officinale*.

Folin-Ciocalteu or BSA assay. When necessary, extracts were diluted to obtain readings within the calibration curve. For the Folin-Ciocalteu and BSA assays, the standards were prepared with stock solutions of purified tannic acid (Sigma) at 0.1 mg mL^{-1} and 0.5 mg mL^{-1} respectively.

2.4.1 Folin-Ciocalteu assay for polyphenols

The following ingredients were added to 5-mL-capacity test tubes: 0.04 mL of polyphenol extract (from methods section 2.3.2), 0.36 mL of dH_2O , and 0.2 mL of 1 M Folin-Ciocalteu reagent (BDH). Samples were vortexed and stood for 3-4 minutes prior to addition of one mL of 7.91% (w/v) anhydrous sodium carbonate. Samples were vortexed again, stood for five minutes in a 45°C water bath (for polyphenol studies in results section 2) or 40 minutes at room temperature (in all other experiments). Absorbancy of all samples were read at 725 nm.

2.4.2 BSA-protein precipitation assay for polyphenols

The following ingredients were added to 3-mL-capacity test tubes: Increasing volumes of polyphenol extracts from methods section 2.3.2 (0-0.25 mL) adjusted to a total of 0.25 mL with 1% (w/v) SDS, 0.75 mL of 1% (w/v) SDS-7% (v/v) TEA and 0.25 mL of FeCl_3 reagent (0.01 M FeCl_3 in 0.1 M HCl). Vortexed samples were stood at room temperature for 30 minutes and then read at 510 nm.

2.4.3 Folin-Ciocalteu assay for tannins

The following ingredients were added to 1.8-mL-capacity-Eppendorf centrifuge tubes: 0.03 g of PVPP, 0.3 mL of dH_2O , and 0.3 mL of polyphenol extract from methods section 2.3.2. Tubes were then vortexed before and after standing for 15 minutes at 4°C , then centrifuged at 10,000g for 10 minutes at 4°C . The supernatant contains polyphenols *without* tannins. Polyphenols in this supernatant were measured as per methods section 2.4.1 except 0.12-0.15 mL of this supernatant was used. The tannin level is the loss in polyphenol levels after PVPP treatment. This is calculated by subtracting the polyphenol levels obtained in methods section 2.4.1 from those measured here (2.4.3).

2.4.4 BSA-protein precipitation assay for tannins

The following ingredients were added to 1.8-mL-capacity-Eppendorf centrifuge tubes: 0.5 mL of BSA (BDH) (1 mg mL^{-1} in 0.2 M acetate buffer, pH 4.8) and increasing volumes of polyphenol extract from methods section 2.3.2 (0-0.25 mL) that were adjusted to a total volume of 0.25 mL with 50% (v/v) methanol. Samples were vortexed and stood overnight at 4°C, then centrifuged at 12,000g for 15 minutes, at 4°C. The supernatant was discarded and the remaining BSA-tannin pellet gently washed with 0.2 M acetate buffer (pH 4.8). Pellets were dissolved in 0.25 mL of 1% (w/v) SDS and 0.75 mL of 1% (w/v) SDS (BDH)-7% (v/v) TEA (BDH) solution with vortexing. After 0.25 mL of FeCl_3 reagent (0.01 M FeCl_3 in 0.1 M HCl) was added, samples were stood at room temperature for 30 minutes and then absorbance read at 510 nm.

3 LEAD ACCUMULATION STUDIES (I)

3.1 Lead effects

Approximately 2-month-old plants (from methods section 1.2) were gently washed in tap water once to remove sand and then three times in dH_2O over a 10-minute period. Using a hydroponic system, plants were treated with four concentrations of $\text{Pb}(\text{NO}_3)_2$ salt solution (0, 100, 250, and 500 μM $\text{Pb}(\text{NO}_3)_2$), with dH_2O as a control. Three replicates were used for each level of $\text{Pb}(\text{NO}_3)_2$, and placed randomly on a table under growth room conditions (16 hour photoperiod at $26.5 \mu\text{mol m}^{-2} \text{s}^{-1}$, 22°C). Effects of the Pb treatments were observed over three weeks and solutions were changed every three days because no aeration unit was available at this stage.

3.2 Lead accumulation – hydroponic experiments

3.2.1 Lead treatment in hydroponic nutrient solution

For each replicate, approximately three uniform 10-month-old *S. officinale* L. plants, propagated from root cuttings, were washed to remove the growth medium. Plants were placed in a modified version of Huang and Cunningham (HC) low phosphate hydroponic nutrient solution (Appendix A1) for seven days, to adjust roots to the acidic (pH 4.5) solution. In this thesis, this solution is referred to as the **modified HC nutrient**

solution (pH 4.5). After seven days, solutions were replaced with one of three treatment solutions (pH 4.5):

- (i) modified HC nutrient solution (control)
- (ii) modified HC nutrient solution containing 250 μ M Pb(NO₃)₂
- (iii) modified HC nutrient solution containing 250 μ M Pb(NO₃)₂ and 125 μ M EDTA

All three treatment solutions were maintained at 0.5 L for seven days with the original solution. Nanopure dH₂O was used to make up all solutions for the hydroponic work. In Plate 2, the hydroponic lead treatment apparatus used in this study is shown.

3.2.2 Preparation of plant material for lead analysis

Lead (Pb) bound to the root surface was routinely desorbed with modified HC nutrient solution containing 1 mM EDTA (pH 4.5) for 30 minutes. Roots were then gently rinsed three times in dH₂O, dabbed dry on paper towels and separated into shoot (leaf and petiole), lateral root, and main root. In a 4°C cold room, tissues were cut into small pieces, and for those to be used for flame AAS, immediately dried in a fan-forced oven at 65°C for 24 hours. The dried tissues were then ground to a powder and re-dried overnight to remove any moisture obtained during grinding. Samples were transferred to capped glass vials, then placed in an airtight jar containing silica crystal and stored at -20°C.

Ten-mL-capacity porcelain crucibles (later replaced with 20-mL-capacity silica crucibles) with lids and 14-mL-capacity polypropylene tubes were soaked in 10% (v/v) HNO₃ for at least 24 hours, rinsed in dH₂O three times, and dried at room temperature prior to use. Between samples, crucibles were washed in detergent and rinsed in dH₂O before soaking in the above acid. Into each crucible, one gram dry weight of ground tissue was weighed and ashed at 495°C for at least 18 hours (time includes gradual increase of temperature to 495°C). After cooling, the ash in the crucible was dissolved in two mL of 10% HNO₃ (v/v), then diluted to 2% (v/v) HNO₃ with nanopure dH₂O, mixed, and transferred to 14-mL-capacity polypropylene tubes.

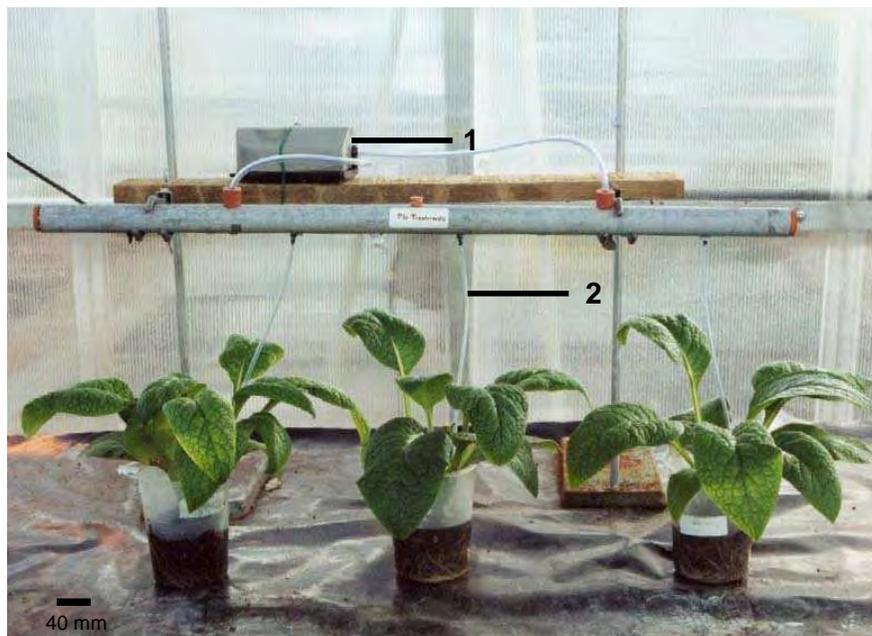


Plate 2. Hydroponic lead treatment apparatus showing 10-month-old *Symphytum officinale* (whole plants) after seven days of treatment.

Treatments (all pH 4.5), from left to right:

Modified HC nutrient solution containing $250 \mu\text{M Pb}(\text{NO}_3)_2$

Modified HC nutrient solution (control)

Modified HC nutrient solution containing $250 \mu\text{M Pb}(\text{NO}_3)_2$ and $125 \mu\text{M EDTA}$

Plants were supplied with continuous aeration via a portable air pump (1) and air supply tubes to the hydroponic solution (2).

HC = Huang and Cunningham.

3.2.3 Measurement of lead level in samples

All flame-atomic-absorption spectroscopy (flame AAS) analysis was conducted using a GBC Avanta Σ flame-atomic-absorption spectrometer with SDS-270 cletic auto-sampler. The instrument was calibrated with 0, 5, 10, 15 and 20 mg L⁻¹ Pb, made from 1000 mg L⁻¹ Pb (element) standard solution (nitrate-based analytical grade BDH) and diluted with 1% (v/v) HNO₃. The instrument was zeroed with 1% (v/v) HNO₃, and readings taken at 217 nm using a superlamp, with instrument settings set to a slit width of 1 nm, lamp current of 5 mA and, after optimisation with 15 or 20 mg L⁻¹ Pb, set to: gas flow rate 2, burner angle 0, working height 13 mm, working center -0.2 mm. For all tissue samples three replicate absorbance values were measured. The mean Pb concentration (in mg L⁻¹) was calculated automatically using the GBC Avanta version 1.33 computer program. When necessary, samples were diluted with 1% (v/v) HNO₃ to obtain readings within the calibration curve. Lead levels in tissue were calculated on the mg kg⁻¹ dry weight (d.w.) basis.

4 TRANSMISSION ELECTRON MICROSCOPY

All the TEM methods outlined below followed the methods from Jarvis (2001) and Ruzin (1999).

4.1 Preparation of ultra-thin sections

4.1.1 Fixation

Young lateral root and main root tissue sections of Pb-exposed and non-Pb exposed plants (methods section 3.2.1) were selected for observations. Tissue located about two mm above the root tip were cut into sections no thicker than one mm, and fixed for three hours in freshly made 3% (v/v) glutaldehyde in 0.075 M sodium phosphate buffer (pH 7.2) (under partial vacuum, at room temperature). The samples, in the same fixative, were then stored overnight at 4°C.

4.1.2 Post-fixation

After the fixative was removed, samples were washed in 0.075 M sodium phosphate buffer (pH 7.2) three times over a 30 minute period, then post-fixed in 1% (w/v) osmium tetroxide in 0.075 M phosphate buffer (pH 7.2) for three hours.

4.1.3 Dehydration

The samples were dehydrated in a series of acetone from 20, 40, 60 to 80% (v/v) for 10 minutes at each acetone concentration, followed by 100% acetone, three times, for 15 minutes each.

4.1.4 Infiltration

In step one of the infiltration, one part of Spurr's resin to two parts of 100% acetone was added to samples in vials, then placed on a slowly rotating wheel overnight.

In the second infiltration step, the resin was replaced with three parts of Spurr's resin to one part of 100% acetone and placed on a rotating wheel for at least three hours.

4.1.5 Embedding

Samples were transferred to shallow plastic caps (in a Petri dish) containing 100% Spurr's resin, covered in a glass petri dish, and heated in a fan forced oven set at 60°C overnight.

4.1.6 Sectioning

Specimens cut from resin blocks were glued to 'stubs' (empty pill capsules filled with hardened epoxy resin). These were set for one hour in a 60°C fan force oven. The stubs were then trimmed to remove excess resin and to obtain desired sections of the tissue. Once trimmed, the sections were mounted in an ultramicrotome (LKB 212B Ultratome) and approximately 90-100 nm ultra thin sections were cut and placed on copper TEM grids.

4.2 Microscopy

Ultra thin sections were viewed in a transmission electron microscope (Joel-1200 EX) at an accelerating voltage of 80 kV. Micrographs of cellular sites of interest were taken on Kodak Ester microscopy sheet film (8.3 x 10.2 cm).

5 IN VITRO LEAD CHELATION TO POLYPHENOLS

In this section, polyphenol extracts from lateral and main roots were used to determine if they could chelate Pb ions and to confirm the *in vivo* hydroponic trends.

5.1 Established protocol: Lavid's dialysis method

Following the method outlined in Lavid *et al.*, (2001b), approximately five grams fresh weight of lateral or main roots (from three 10-month-old plants, grown as per methods section 1.2 above) were ground to a powder with liquid nitrogen, then extracted for two hours with 50 mL of 50% (v/v) methanol (bubbled with N₂) at 4°C. Homogenates were centrifuged at 11,000g for 15 minutes at 4°C and the polyphenol supernatant used for Pb chelation studies. Excess polyphenol supernatant was assayed for polyphenols using the BSA-protein precipitation method. Dialysis tubes containing five mL of polyphenol extract were placed in 100 mL of 250 M Pb(NO₃)₂ solution (pH 5.3) for 48 hours with gentle shaking at 4°C. The entire content of the tube at t = 0 hours and t = 48 hours was collected, dried at room temperature, then dry ashed at 500°C for 18 hours and used for Pb analysis as described above.

To make a better comparison with between the Lavid dialysis method and the new Pb chelation protocol outlined below, the Lavid dialysis method was repeated with some modifications – plant material and polyphenols were used and analysed as per 5.2.1 to 5.2.2 and 50 mL of 250 mg L⁻¹ Pb(NO₃)₂ was used. Since fructans were suspected to interfere with this method, additional dialysis tubes containing five mL of 0, 0.15, 0.3% (w/v) purified fructan (chicory inulin) were also tested for Pb chelation. This was equivalent to approximately 0, 15, 30% (w/w) fructans – the level of fructans in comfrey root tissue if (although unlikely) completely extracted into the polyphenol extract. After 48 hours, the entire volume of each tube was measured, and two mL of this was digested with 30% (v/v) HNO₃ in a 60°C water bath overnight (18 hours). This is **the modified protocol of the Lavid dialysis method.**

5.2 Developing a new method: PVPP-immobilised tannin method

The general PVPP-immobilised tannin method is illustrated in Figure 3 and detailed in the sections below.

5.2.1 Plant material

Three-month-old *S. officinale* plants were grown from root cuttings in North-end sand supplemented with Nutricote™ 8-9-month slow-release fertiliser (as per methods section 1.2). All plants were grown under growth room conditions (16 hour photoperiod at $26.5 \text{ mol m}^{-2} \text{ s}^{-1}$, 22°C).

5.2.2 Polyphenol extraction and assay

Ball-mill ground freeze-dried root powder from 3-month-old *S. officinale* (methods section 5.2.1) was used for all polyphenol extractions. Polyphenols were extracted at a ratio of 0.2 g of tissue to 15 mL of 50% (v/v) methanol with an ultrasonic water bath for 30 minutes. Samples were centrifuged at 10,000g for 30 minutes at 4°C and supernatants were used for polyphenol assays. Polyphenol and tannins were quantified using the Folin-Ciocalteu assay (methods section 2.4.1 and 2.4.3).

5.2.3 Preparation of immobilised root tannins

Insoluble polyvinyl polypyrrolidone (PVPP) was used to bind and immobilise tannins from extracts. For each of the three replicates, the following ingredients were added in the following order:

- (i) For each Pb treatment level (50, 250, and $500 \text{ mg L}^{-1} \text{ Pb}(\text{NO}_3)_2$), 0.1 g of insoluble PVPP was weighed into five 14-mL-capacity PEG tubes.
- (ii) Polyphenol extracts or methanol was added to the tubes. Tube 1 contained five mL of 50% (v/v) methanol, tubes 2 and 3 contained five mL of lateral root polyphenol extract, and tubes 4-5 contained five mL of main root polyphenol extract.
- (iii) All five tubes were then shaken horizontally for 20 minutes and then centrifuged at 10,000g at 10°C for 15 minutes. This cool temperature was selected to keep any fructans soluble in the polyphenol extract; 4°C may result in fructan precipitation (Megazyme, 2004; Massardo *et al.*, 2002) and contamination of PVPP-tannin pellet after centrifugation. The liquid polyphenol extract was removed for the tannin assay and the remaining tannin bound-PVPP (PVPP-immobilised root tannin) pellet rinsed two times with nanopure dH_2O .

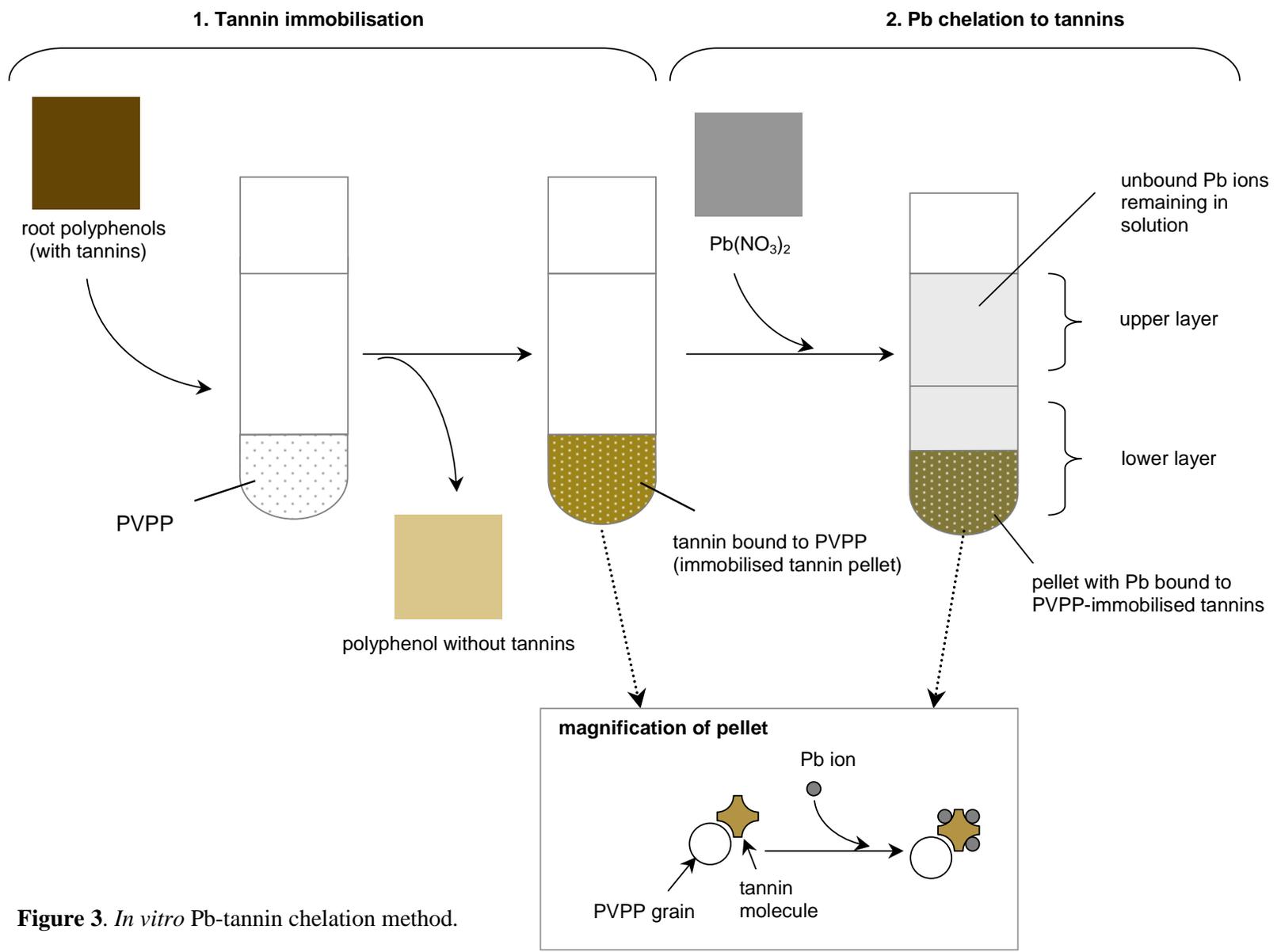


Figure 3. *In vitro* Pb-tannin chelation method.

5.2.4 Lead chelation to immobilised root tannins

Tube 1 (control 1): PVPP (50% (v/v) methanol) + $\text{Pb}(\text{NO}_3)_2$

Tube 2 (control 2): PVPP-immobilised lateral root tannins + HNO_3

Tube 3 (sample 1): PVPP-immobilised lateral root tannins + $\text{Pb}(\text{NO}_3)_2$

Tube 4 (control 3): PVPP-immobilised main root tannins + HNO_3

Tube 5 (sample 2): PVPP immobilised main root tannins + $\text{Pb}(\text{NO}_3)_2$

Tubes 1, 3 and 5 contained five mL of 50 mg L^{-1} $\text{Pb}(\text{NO}_3)_2$ solution (pH 4.5); tubes 2, 4 contained 2% (v/v) HNO_3 . All five tubes were shaken for four hours at room temperature under low light conditions, and then centrifuged for 15 minutes at 10,000g at 10°C . To determine if Pb was removed by the immobilised tannins, each five mL solution was quantitatively divided into two halves (about 2.5 mL each): the upper layer (UL, Pb solution only) and the lower layer (LL, Pb solution + PVPP pellet). Total Pb recovery from wet acid digestion was determined by comparing the Pb level in the upper layer of tube 1 with level of Pb in the prepared Pb solutions (i.e. prior to the addition to tubes). Each tube (containing the UL or LL) was digested with 30% (v/v) HNO_3 in a 60°C water bath overnight (18 hours). The experiment was repeated for 250 and 500 mg L^{-1} $\text{Pb}(\text{NO}_3)_2$ levels. The concentration of Pb was determined using a flame-atomic-absorption spectrometer (model GBC Avanta Σ) with analytical grade $\text{Pb}(\text{NO}_3)_2$ (BDH) as the standard.

Note that the Pb treatment level in the methods and results section refers to $X \text{ mg L}^{-1}$ lead nitrate ($\text{Pb}(\text{NO}_3)_2$) for Pb solution preparation purposes. In the results section, the actual mean elemental Pb values ($X \text{ mg L}^{-1}$) of these solutions are also indicated because this was the form and level of Pb measured in the upper and lower layers of the control (tube 1) by AAS.

5.3 Lead chelation to immobilised lateral root tannins

In this section, *S. officinale* polyphenols from only lateral roots were used in Pb chelation studies. This was to confirm that the Pb chelation trends observed in results section 6 were due to differences in polyphenol concentrations, as opposed to root-associated factors (e.g. root type or age). Purified tannic acid (Sigma) and purified fructan (chicory inulin) were also used to validate the design and need for a new *in vitro* Pb-tannin chelation protocol.

The same protocol was applied as methods section 5.2, but in a micro-scale version and with the following modifications: (i) extraction of polyphenols was at a ratio of 0.05 g root powder to five mL of 50% (v/v) methanol, (ii) tannins were immobilised using 0.02 g PVPP with one mL of polyphenol extract at increasing concentrations (0, 12.5, 25, 50, and 100% (v/v) of initial extract), (iii) one mL of 50 mg L⁻¹ Pb(NO₃)₂ (pH 4.5) was added to immobilised tannins and Pb chelation period reduced to two hours at room temperature, (iv) the level of Pb removed from solution was directly determined by AAS after diluting 0.5 mL of the top layer solution with two mL of 1% (v/v) HNO₃. No wet acid digestion was necessary to determine Pb levels in this solution as 100% of initial Pb solution was recovered without digestion (data not shown).

5.4 Lead chelation to immobilised purified tannic acid

The experiment was repeated exactly as methods section 5.3 except purified tannin and fructan (from Sigma and not *S. officinale*) were used. Tannin concentrations ranged from 0-0.4 mg mL⁻¹ to 0-10 mg mL⁻¹. Tannins were also spiked with 0-9 mg mL⁻¹ purified fructan (chicory inulin) – to reflect the level of fructans (expressed as the tannin:inulin ratio) expected in the polyphenol extracts.

Objective 2

*Phytoremediation potential of Symphytum officinale:
Hydroponic studies with sand-grown S. officinale*

Unless indicated, all plants used in this section were 3-4 month-old plants grown under growth room conditions (16 hour photoperiod at 26.5 mol m⁻² s⁻¹, 22°C) in sand-based growth media (methods section 5.2.1) to avoid whitefly problems in greenhouses.

6 LEAD ACCUMULATION STUDIES (II)

In some plants a threshold concentration of Pb(NO₃)₂ or chelating agent (e.g. EDTA) is required before Pb can be adsorbed by the roots and/or translocated to the shoots. Thus, in order to determine the phytoremediation potential of *S. officinale*, the effect of EDTA and Pb(NO₃)₂ concentrations were investigated.

6.1 Effect of EDTA level on lead accumulation

Roots of plants were washed to remove sand and held overnight in aerated tap water. Plants were then transferred to modified HC nutrient solution (pH 4.5) for one week and then replaced with 300 mL of one of four treatment solutions (pH 4.5):

- (i) modified HC nutrient solution (control)
- (ii) modified HC nutrient solution containing 250 μ M Pb(NO₃)₂ and 250 μ M EDTA (1:1 molar ratio of Pb to EDTA)
- (iii) modified HC nutrient solution containing 250 μ M Pb(NO₃)₂ and 500 μ M EDTA (1:2 molar ratio of Pb to EDTA)
- (iv) modified HC nutrient solution containing 250 μ M Pb(NO₃)₂ and 750 μ M EDTA (1:3 molar ratio of Pb to EDTA)

All solutions were aerated and topped up with the original solution daily. After seven days in treatment solution, plants were harvested and roots desorbed for 30 minutes in 300 mL of modified HC nutrient solution containing 1 mM EDTA (pH 4.5), then rinsed with nanopure dH₂O. Plants were divided into roots and shoots, frozen in liquid nitrogen and freeze-dried. All samples were ground to a fine powder with a ball mill (Retscher MM300) and Pb levels measured as per methods section, 3.2.2 and 3.2.3.

6.2 Effect of 500 μ M Pb(NO₃)₂ level on lead accumulation

The effect of Pb concentration on the level of Pb accumulation in tissues were conducted as per methods section 6.1 except the treatment solutions (all pH 4.5) were:

- (ii) modified HC nutrient solution (control)
- (iii) modified HC nutrient solution containing 500 μ M Pb(NO₃)₂
- (iv) modified HC nutrient solution containing 500 μ M Pb(NO₃)₂ and 500 μ M EDTA (1:1 molar ratio of Pb to EDTA)

7 LEAD UPTAKE STUDIES (III): EDTA STUDIES

Surprisingly, increased EDTA concentrations decreased Pb accumulation levels in roots (that could not be accounted for by translocation) of *S. officinale* (see results section 7 and 8). The following experiments were performed to further investigate this finding.

7.1 EDTA pre-treatment studies

7.1.1 Root segment test

For each replicate, 3-4 month-old lateral roots were divided into six 1.2 g f.w. portions and each portion placed in mesh-like ‘tea bags’. Three ‘tea bags’ were pre-treated with modified HC nutrient solutions (pH 4.5) containing 500 μ M EDTA and three without 500 μ M EDTA. After 18 hours, the root containing ‘tea bags’ were rinsed in dH₂O and one ‘tea bag’ from each half transferred to one of three solutions (all pH 4.5): (i) modified HC nutrient solution, (ii) modified HC nutrient solution with 500 μ M Pb(NO₃)₂ or, (iii) modified HC nutrient solution with 500 μ M Pb(NO₃)₂ and 500 μ M EDTA. After 24 hours, roots were harvested and washed in dH₂O. One portion of the roots (0.2 g f.w.) was removed and freeze dried for total polyphenol analysis whilst the remaining one gram was used for Pb analysis as per methods section 3.2.2 and 3.2.3. Pb levels in Pb treatments were corrected against controls. In total, the experiment took three days (18 hours for pre-treatment and 24 hours for treatment). A concurrent seven day test (72 hours for pre-treatment and 96 hours for treatment) was also performed, except only Pb data was collected as roots segments were entirely browned after day seven. All solutions were aerated.

7.1.2 Whole plant test

For each replicate, six sand-grown plants were used. The first set of three plants were treated with modified HC nutrient solution (pH 4.5) for three days whilst the remaining set of three plants were treated with modified HC nutrient solution plus 500 μ M EDTA (pH 4.5). On day four, one plant from each set was then treated for four days in one of three new solutions: (i) fresh modified HC nutrient solution (pH 4.5), (ii) modified HC nutrient solution containing 500 μ M Pb(NO₃)₂, and (iii) modified HC nutrient solution containing 500 μ M Pb(NO₃)₂ and 500 μ M EDTA. All solutions were constantly aerated and topped up to 200 mL with the matching solution every second day. Plants were harvested after four days. Roots (lateral and main roots combined) were washed three times with dH₂O, separated into shoot and roots, frozen in liquid nitrogen and freeze-dried. Polyphenol and Pb levels were measured as outlined in methods section 2.4.2, 3.2.2 and 3.2.3 respectively. Fe levels were similarly measured with analytical grade Fe(NO₃)₂ (BDH) as the standard.

7.2 Lead accumulation: alternative chelators (ADA)

The same hydroponic method outlined in methods section 6.2 was conducted, but with the following treatment solutions set at pH 7.0 (the optimal working pH for ADA):

- (i) modified HC nutrient solution (control)
- (ii) modified HC nutrient solution containing 500 μ M Pb(NO₃)₂ and 500 μ M EDTA (1:1 molar ratio of Pb to EDTA)
- (iii) modified HC nutrient solution containing 500 μ M Pb(NO₃)₂ and 500 μ M ADA (1:1 molar ratio of Pb to ADA)

Except for the root Pb desorption step with EDTA, Pb levels were measured as per methods section 3.2.2 and 3.2.3 respectively. Fe levels were also measured. Instead, roots were carefully washed three times in nanopure dH₂O to avoid mixing the two different chelating agents in treatments.

Objective 3

Pb mutant plant studies:

Symphytum officinale tissue culture

8 DEVELOPING *SYMPHYTUM OFFICINALE* IN TISSUE CULTURE

8.1 Explant selection

Symphytum officinale root division clumps with only one central crown were washed to remove soil and pruned to ten cm in length. Roots were planted in two litres pots containing North-end sand supplemented with about ten grams (two teaspoons) of 8-9 month Nutricote™ fertiliser. Plants were also propagated via singular root cuttings (methods section 1.2). All plants were grown under growth room conditions as described previously.

Petioles of 2-month-old *S. officinale* plants grown from root divisions (a clump of roots attached to a shoot crown) and cuttings (just a root section, see Plate 1) were collected as explants for tissue culture. The petioles selected were attached to open leaf blades of between 15 and 20 cm long.

8.2 Sterilisation regime

Following a combination of methods (Huizing, 1983; Hall, 1999, Gamborg and Phillips, 1995), two sets of tissue culture sterilisation regimes were tested:

Set I: 10, 20 and 100% (v/v) commercial bleach for 10 and 20 minutes

Set II: 5, 10 and 20% (v/v) commercial bleach for 10 and 20 minutes

For each sterilisation treatment, six petiole segments (two cm long) were rinsed in tap water for 15 minutes and then sterilised with one of the treatments regimes above (in a laminar air flow cabinet). Each treatment regime contained Tween 20 at a rate of two drops per 100 mL of bleach. Explants were rinsed three times with sterile distilled water, before two mm was cut off the petiole section ends and placed on MS B media (methods section 8.3.1). The petiole sterilisation regime for subsequent experiments involved the initial water-rinsing step and 10% (v/v) bleach with Tween 20 (as above) for 10 minutes.

8.3 Media development

General tissue culture media preparation instructions followed that of Sutter (1996) and Gamborg and Phillips (1995).

8.3.1 Callus induction media (CIM)

Petiole tissue from 2-month-old root cutting and division plants were sterilised using the protocol established above. Callus and shoot induction response from these petiole sections on six types of media were investigated to determine the best media for plant propagation *in vitro*. All media were based around the Murashige and Skoog medium (MS, Murashige and Skoog, 1962) supplemented with plant growth regulators (BA or IBA) (Appendix A2).

In these cultures, the petioles of plants grown from root divisions (older explant) and root cuttings (younger explant) were not cut longitudinally for several reasons: (i) to determine the necessity of this step, (ii) to compare the response to longitudinally cut sections, and (iii) to avoid browning of thinner petioles harvested from plants developed from root cuttings.

- (i) MS A: MS basal nutrients (pH 5.8)
- (ii) MS B: MS basal nutrients + 2% (w/v) sucrose

- (iii) MS C: MS B + BA (1 mg L⁻¹)
- (iv) MS D: MS B + BA (1 mg L⁻¹) + IBA (0.01 mg L⁻¹)
- (v) MS E: MS B + BA (1 mg L⁻¹) + IBA (0.1 mg L⁻¹)
- (vi) MS F(0): MS B + BA (1 mg L⁻¹) + IBA (1 mg L⁻¹)

In vitro generated shoots and callus formed on MS F(0) media (using petioles from 2-month-old root cutting plants) were used for subsequent root, shoot, and callus growth media (RIM, SIM, CGM) trials. MS F(0) liquid and agar media was also tested.

8.3.2 Callus growth media (CGM)

Six types of CGM were tested, all based around the Murashige and Skoog media (1962):

- (i) MS F(0): MS B + BA (1 mg L⁻¹) + IBA (1 mg L⁻¹)
- (ii) MS F(1): MS B + BA (2.5 mg L⁻¹) + IBA (2.5 mg L⁻¹)
- (iii) MS F(2): MS B + BA (3.5 mg L⁻¹) + IBA (3.5 mg L⁻¹)
- (iv) MS F(3): MS B + BA (5.5 mg L⁻¹) + IBA (5.5 mg L⁻¹)
- (v) MS G: MS B + kinetin (0.3 mg L⁻¹) + NAA (4 mg L⁻¹)
- (vi) MS J: MS B + BA (2 mg L⁻¹) + 2,4 D (0.5 mg L⁻¹)

8.3.3 Root and whole plant induction media (RIM, WPIM)

Four types of RIM were tested using *in vitro* shoot tissue (shoot blade with petiole). When crown tissues were of a sufficient quantity, six types of WPIM were tested using *in vitro* crown tissue. All *in vitro* tissues used in this section emerged from petiole explants on MS F(0). All media were based around the Murashige and Skoog (MS) media (1962). The best medium was determined by the speed and formation of roots and shoots from the shoot or crown tissue.

RIM media were:

- (i) MS A: MS basal nutrients (pH 4.5)
- (ii) MS B: MS basal nutrients + 2% (w/v) sucrose
- (iii) MS H: MS A + IBA (1 mg L⁻¹)
- (iv) MS L: MS B + IBA (1 mg L⁻¹)

WPIM media were:

- (i) MS L: MS B + IBA (1 mg L^{-1})
- (ii) MS M: MS B + IBA (0.1 mg L^{-1})
- (iii) MS N(0): MS B + IBA (1 mg L^{-1}) + BA (0.1 mg L^{-1})
- (iv) MS N(1): MS B + IBA (1 mg L^{-1}) + BA (0.2 mg L^{-1})
- (v) MS N(2): MS B + IBA (1 mg L^{-1}) + BA (0.4 mg L^{-1})
- (vi) MS E: MS B + IBA (0.1 mg L^{-1}) + BA (1 mg L^{-1})

8.3.4 Propagation of *Symphytum officinale* plant lines

The MS N(0) media formed excellent root and shoot tissue, thus it was also an excellent whole plant propagation media. Thus, every 4-5 weeks, whole plants were cut back to leave just the crown; the shoots were cut back to leave one cm of petiole attached to the crown, all roots and dead tissue surrounding the crown were removed. Depending on the size of the crown, most crown tissue were divided longitudinally in half and placed three mm into the MS N(0) media. MS N(0) liquid was also tested.

9 IN VITRO LEAD-TREATED PLANTS

A variety of Pb treatments were performed on whole plants and shoot cuttings (shoot blade with petiole) before the 'Pb pre-treated' petioles were harvested for shoot and whole plant generation (Figure 4). The chelating agent (EDTA and ADA) used were at the same concentration as $\text{Pb}(\text{NO}_3)_2$, and therefore are abbreviated as X M PbEDTA and PbADA. Each treatment was performed successively and according to the previous treatment results. Table 2 below outlines all the treatments.

9.1 Whole plants pre-treated with lead

Petioles from whole plants pre-treated with PbEDTA and PbADA (c.f. just shoots in subsequent experiments) were initially chosen for shoot generation to mimic 'normal' plant uptake of Pb. All pre-treatments were performed under sterile conditions.

Table 2. Lead pre-treatment of *in vitro* *Symphytum officinale* whole plants or shoot cuttings.

Treatment	Plant description	Treatment description (all solutions)
# 1	whole plant, 4-weeks-old	HC*, no sucrose, pH 4.5 (one week adaptation phase) HC \pm 500 M PbEDTA, no sucrose, pH 4.5 (two weeks)
# 2	whole plant, 4-weeks-old	HC, no sucrose, pH 7.0 (one week adaptation phase) HC \pm 500 M PbEDTA, pH 7.0, no sucrose (two weeks).
# 3	whole plant, 3-weeks-old	HC, 2% (w/v) sucrose, pH 7.0 (one week adaptation phase) HC \pm 500 M PbEDTA or PbADA, 2% (w/v) sucrose, pH 7.0 (two weeks)
# 4	shoot only, 4.5 weeks old	HC \pm 100, 250, 500 M Pb(NO ₃) ₂ , 2% (w/v) sucrose, pH 4.5 (one week)
# 5	shoot only, 3-weeks-old	HC \pm 500 M Pb(NO ₃) ₂ , PbADA, 2% (w/v) sucrose, pH 7.0 (one week)

*HC refers to modified Huang and Cunningham nutrient solution.

9.1.1 Pre-treatment # 1: 0, 500 M PbEDTA, pH 4.5

One plant from each of the four lines (each representing one replicate) were transferred from MS N(0) agar to 25 mL of modified HC nutrient solution (pH 4.5), after removing the MS N(0) agar medium from the roots. Plants were grown in the medium for seven days to allow the plant to adjust to the new pH and media prior to transfer to 25 mL of modified HC nutrient solution (pH 4.5) with or without 500 M PbEDTA. To maximise stress treatments, the medium did not contain sucrose and were treated with PbEDTA for two weeks. After two weeks, petiole tissues were taken about one cm above the height of the solution and rinsed with sterile distilled water prior to placement on MS F(0) media (for *de novo* shoot generation).

9.1.2 Pre-treatment # 2: 0, 500 M PbEDTA, pH 7.0

The pre-treatment was the same as 9.1.1 above, except pH 7.0 was used solution. An additional set, in which *in vitro* petioles were surface sterilised again prior to culture on MS F(0), was also performed. This was to due to contamination arising from pre-treatment #1.

9.1.3 Pre-treatment # 3: 0, 500 M PbEDTA and PbADA, pH 7.0

Similar to pre-treatment # 1, whole plants were pre-treated in modified HC nutrient solution with or without PbEDTA and PbADA (1:1 molar ratio), but with pH 7.0 solution and 2% (w/v) sucrose. The *in vitro* petioles were not re-sterilised.

9.2 Shoots from sand-grown plants pre-treated with lead

The *in vitro* petioles from tissue-cultured plants exposed to Pb failed to produce adequate *de novo* shoot tissue (see results section). Since one possibility was a loss in shoot regeneration ability, Pb-pre-treated petioles from 2-week-old sand-grown shoot cuttings were attempted as an alternative approach to developing Pb mutants. Shoots were treated with 35 mL of modified HC nutrient solution (pH 7.0) with or without 500

M PbADA. Solutions were constantly aerated for seven days. The leaf blade and the water-rinsed shoot base (i.e. petiole) were discarded. The remaining petioles were sterilised as per methods section 8.2, cut into one cm sections and placed on MS F(0) media with or without 0.05% (v/v) PPM (Plant Preservative Mixture, Bio-Strategy Distribution Ltd, Auckland, NZ). The addition of PPM, an anti-microbial agent, was tested as the petioles could be difficult to sterilise after exposure to PbADA in a non-sterile environment.

9.3 Petioles from *in vitro* shoots pre-treated with lead

9.3.1 Pre-treatment # 4: 0 to 500 M Pb(NO₃)₂, pH 4.5

In vitro shoots (4.5-week-old) were pre-treated with modified HC nutrient solution (pH 4.5) containing 0, 100, 250 or 500 M Pb(NO₃)₂ and 2% (w/v) sucrose. Shoots were dried overnight at 70°C, ashed, and Pb levels analysed as per methods section: 3.2.2 and 3.2.3.

9.3.2 Pre-treatment # 5: 0, 500 M Pb(NO₃)₂ and PbADA, pH 4.5

Approximately uniform *in vitro* shoots (shoot blade with petiole) were excised from 3-week-old whole plants grown on MS N(0) media. Shoots were cut to approximately the same petiole length and placed in a 30 mL culture vial containing three mL of one of the following treatment solutions:

Modified HC nutrient solution (pH 4.5) with 2% (w/v) sucrose plus:

- (i) neither $\text{Pb}(\text{NO}_3)_2$ nor ADA (control)
- (ii) 500 μM $\text{Pb}(\text{NO}_3)_2$
- (iii) 500 μM $\text{Pb}(\text{NO}_3)_2$ with 500 μM ADA
- (iv) 500 μM $\text{Pb}(\text{NO}_3)_2$ with 1000 μM ADA

After seven days, shoots were harvested and the base of the petiole (about one cm immersed in the treatment solution) rinsed, excised and discarded. After the blade was removed, the remaining petiole section was divided into two sections (between 0.5-1 cm in length) and placed on MS F(0) media for shoot /callus induction. Shoots were selected from four lines, each representing one replicate. To determine the Pb level in the petiole and blade tissue, a separate second set of shoots was treated for Pb analysis. Tissues pieces were dried overnight at 70°C, then ashed and analysed as per methods section 3.2.2 and 3.2.3.

9.4 Propagation of plants pre-treated with lead

After one or two months (see results section), *de novo* shoot/crown tissue from pre-treatments 2, 4, and 5 petioles were transferred to MS N(0) media for rooting and whole plant propagation. To obtain a minimum of four plants per pre-treatment (two stock plants, two for re-treatment), three to four subcultures (each subculture 4-5 weeks long) were required. This was because only a maximum of two shoots were large enough for culture to MS N(0) and the shoots/crown from the first two subcultures were not large enough to be divided into two plants.

9.5 Re-treatment regime

Plants were subcultured at least three times to obtain sufficient plant numbers. Lead pre-treated plants were fully grown (adequate shoot and root growth) in MS N(0) media within four weeks of subculture. Two plants from each pre-treatment, (0, PbEDTA, unchelated Pb or PbADA) were used for re-treatment tests; one plant was used as a control and fed with modified HC nutrient solution (pH 7.0, control), while the second plant was treated with modified HC nutrient solution (pH 7.0) containing either $\text{Pb}(\text{NO}_3)_2$ or PbADA.

Under non-sterile conditions, whole plants were transferred to a semi-enclosed container (i.e. covered with a perforated plastic bag) with water to minimise transplant

stress (e.g. water loss) and to soften the agar around the roots for easy removal. After the agar was removed, all plants were grown in the semi-enclosed container for one week in 50 mL of modified HC nutrient solution (pH 7.0). After this, the modified HC nutrient solution was replaced with modified HC nutrient solution with or without Pb treatment solutions (pH 7.0) as detailed below. All solutions did not contain sucrose. Solutions were constantly aerated and topped up as necessary with the original solution.

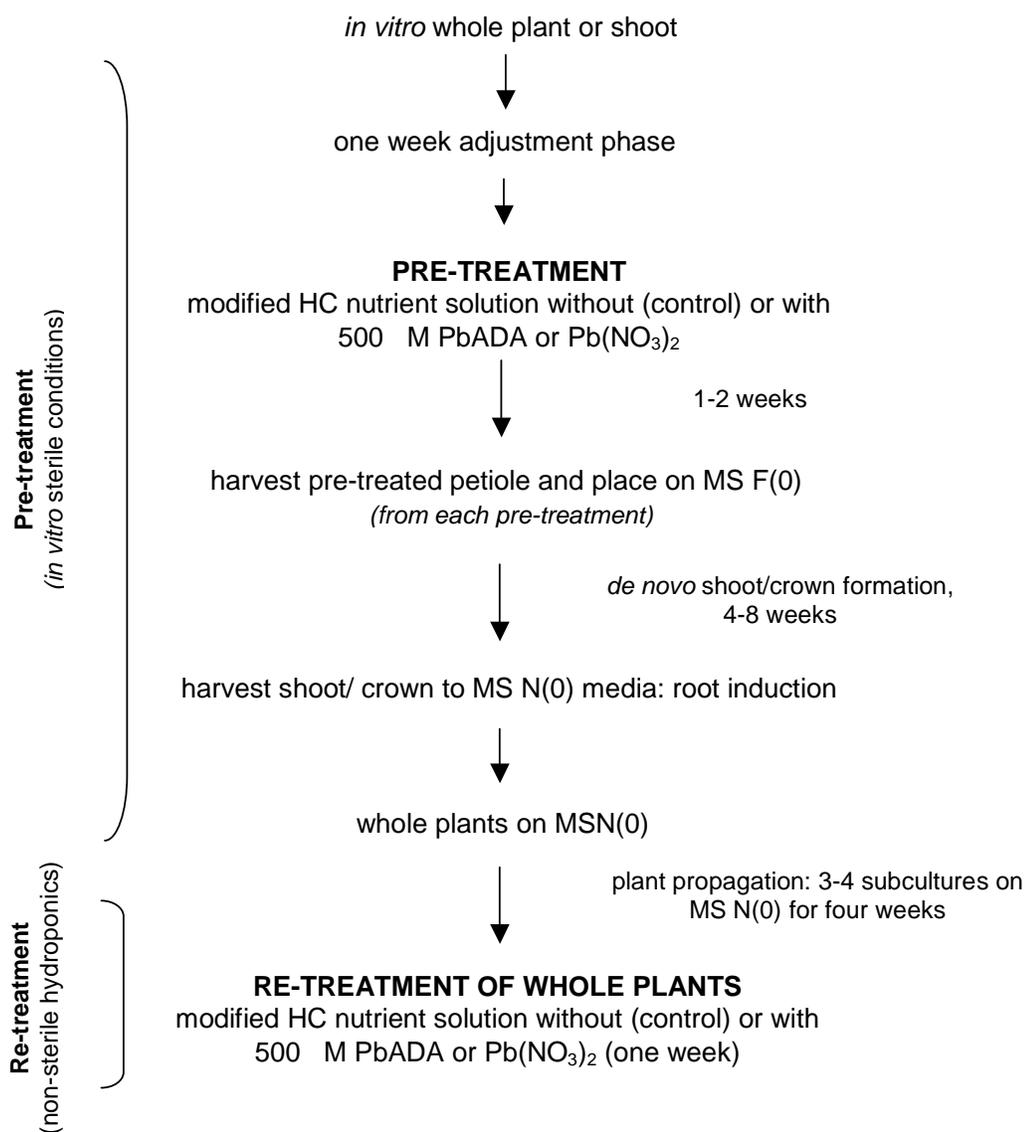


Figure 4. Overall Pb pre-treatment and re-treatment protocol for *in vitro* *Symphytum officinale* tissues and plants (for successful lines). HC = Huang and Cunningham.

10 INDICATOR TESTS FOR POTENTIAL MUTANTS

After the plants were re-treated with Pb solutions in hydroponics (methods section 10.5) three tests were performed on the plants to determine if and how mutants formed:

- (i) Pb level in shoots and roots (d.w.)
- (ii) Polyphenol level in roots (f.w.)
- (iii) Superoxide dismutase (SOD) activity in roots (per mg protein or f.w.)

The Pb and polyphenol analysis were performed as in objective 1 except polyphenols were extracted at a rate of 0.1 g (f.w.) tissue to one mL of 50% (v/v) methanol. Both histochemical and quantitative assays developed for superoxide dismutase (SOD) activity and superoxide anions localisation are outlined below.

10.1 Histochemical localisation of superoxide anions

A histochemical test for superoxide anions was developed because it would be a useful *in vivo* visual tool should Pb mutants be developed or identified. The superoxide anion ($\cdot\text{O}_2^-$) is one type of free radical that is increased under heavy metal stress. NBT (nitro blue tetrazolium) is a positive purple stain for the superoxide anion. Under heavy metal stress, increased antioxidative defence system reduces the level of this free radical and the degree of purple staining. Therefore this staining allows: (i) the degree of stress to be visualised and (ii) the generation and/or diffusion sites of superoxide anion to be localised *in vivo*. It is also an indirect indicator of possible SOD enzyme levels; specific indication of SOD levels is not possible with NBT as there may be other non-enzyme antioxidants involved in superoxide anion removal.

10.1.1 Leaf discs

Leaf discs (0.5 cm in diameter bored from young leaf tissues) were vacuum infiltrated, up to 24 hours in the dark at room temperature in one mL of water-based treatment solutions (due to the presence of Pb). This was to aid penetration of the treatment solution and subsequent NBT stain solution. After treatment, leaf discs were washed three times with distilled water. Except for controls (no-NBT), all discs were placed under vacuum in one mL of 0.1-0.2% (w/v) NBT (in potassium phosphate buffer, pH 7.8) initially for five minutes. This was extended to 1.5 hours when uneven staining was observed. Discs were rinsed in potassium phosphate buffer (pH 7.8) and chlorophyll

removed with boiling in 70% (v/v) ethanol for 5-10 minutes (as heating for up to one hour in 96% (v/v) ethanol at 40°C was ineffective at chlorophyll removal).

10.1.2 Petiole sections

Petiole sections (one mm cross sections) were tested in a similar manner except: (i) sections were incubated in 0.1 M potassium phosphate buffer (pH 7.8) for one hour before exposure to treatment solution to reduce wound-related superoxide production, and (ii) the vacuum infiltration and chlorophyll removal steps were omitted. This was because boiling in ethanol turned the petiole section a light crimson colour and severely reduced the initial contrast between NBT purple staining and light green petioles.

The treatments for 10.1.1 and this section were dH₂O, 500 μM and 1000 μM of Pb(NO₃)₂, H₂O₂, EDTA, and PbEDTA (at equal concentrations).

10.1.3 Roots of intact whole plants

For each treatment set, cloned *in vitro* propagated plants were exposed to one of seven water-based treatment solutions for 24 hours in a growth room (16 hour photoperiod at 26.5 μmol m⁻² s⁻¹, 22°C):

- (i) dH₂O, pH 3.5
- (ii) dH₂O, pH 6.0
- (iii) 5 mM H₂O₂
- (iv) 250 μM EDTA
- (v) 250 μM Pb(NO₃)₂
- (vi) 250 μM Pb(NO₃)₂ and 250 μM EDTA
- (vii) 250 μM Pb(NO₃)₂ and 500 μM EDTA

All plant roots were then rinsed three times with dH₂O, stained with two mL of 0.1% (w/v) NBT (dissolved in 0.1 M potassium phosphate buffer, pH 7.8) for one hour in the dark and rinsed three times again with the same buffer.

10.2 Superoxide dismutase (SOD) activity

10.2.1 Extraction

Fresh tissue (blade, petiole, or roots) were homogenised in ice cold 0.1 M potassium phosphate buffer (pH 7.8) containing 1% (w/v) PVPP*. All tissues were homogenised at a ratio of 0.2 g (f.w.) to two mL of this extraction buffer (with a small amount of fine sand) for two minutes on ice. Tissue weights and supernatant volumes were recorded for quantification of SOD activity.

*Before determining this as the best SOD extraction buffer, the potassium phosphate buffer containing one or more of the following reagents were tested: (i) 0.625 mM Na₂EDTA (to ensure the EDTA level would exceed the Pb level in tissue), (ii) 0-0.25% (v/v) Triton X-100, (iii) 10% (v/v) glycerol, (iv) 0.2 mM ADA, (v) 2 mM DTT. These tests are recommended when tissues contain membrane-bound enzymes and heavy metals (Bollag *et al.*, 1996).

10.2.2 Superoxide dismutase assay

The SOD assay does not follow typical enzyme assays based on the Beer-Lambert law. The nitro blue tetrazolium (NBT) assay for SOD is an inhibition-based photochemical assay. In this assay, superoxide ions (generated from the redox reaction between photon-excited-riboflavin and methionine) converts NBT (originally a clear yellow solution) to formazan. Formazan is a blue-purple coloured product that is measured at 560 nm. Being a scavenger of superoxide anions, SOD reduces the concentration of superoxide anions (the NBT substrate) and thus the level of formazan produced. The extent of loss in formazan production is a measure of the SOD activity in the sample (Beyer and Fridovich, 1987).

Following Malecka *et al.*, (2001), the one mL reaction mixture contained enzyme extract volumes up to 0.2 mL, 0.05 M potassium phosphate buffer (pH 7.8), 13 mM methionine, 63 M NBT and 13 M riboflavin. All samples were illuminated in growth room lights ($26.5 \text{ mol m}^{-2} \text{ s}^{-1}$, 22°C) for 15 minutes (or for seven minutes with buffer containing Triton X-100), and absorbance measured at 560 nm. Two controls, boiled SOD enzyme and buffer-only samples were performed, but only the former was factored into the SOD activity calculation (Asada *et al.*, 1974). The latter control

served to compare and check SOD assays between experiments. An inhibition curve was made against the fresh weight of tissue in the volume of extract. SOD activity was expressed as units per mg protein, where one unit was defined as the amount of tissue (mg f.w.) in the extract that caused 50% inhibition of NBT photo-reduction.

Prior to analysis of SOD activity in plant tissues, absorbance readings of potassium phosphate buffer solutions (all pH 7.8) spiked with four separately tested reagents (500 μ M PbEDTA, 1000 μ M PbEDTA, 26 mg L⁻¹ FeEDTA, or 260 mg L⁻¹ FeEDTA) were measured at the wavelength for SOD analysis (560 nm).

10.2.3 Total protein assay

Total protein was measured using the micro-assay version (1.1 mL assay volume) of the Bradford Assay (Bradford, 1976). Bradford reagent (0.1 mL) was added to the enzyme extract (1 mL), vortexed and stood for five minutes before the absorbance was measured at 595 nm. A range (0-25 μ g mL⁻¹) of bovine serum albumin (BSA, from BDH) solutions was used as the standard.

11 STATISTICAL ANALYSIS

To ensure homogeneity of variances, the F (max) values of all data were calculated to determine if the data required transformation prior to ANOVA analysis. When the experimental F (max) values were greater than the corresponding tabular value, data were log₁₀/log₁₀+1 transformed and F (max) re-tested prior to ANOVA. All data presented in this thesis (unless indicated) passed the F (max) test. One way ANOVA on data were significant at the 95% or 99% level if P values were less than or equal to 0.05 or 0.01 respectively. Statistically significant treatments were determined using the Tukey test and indicated as different letters in graphs or tables.

CHAPTER 3

RESULTS AND DISCUSSION

Objective 1

*Association of polyphenols with chelation-based tolerance mechanism and Pb accumulation: Hydroponic studies with sand-grown *Symphytum officinale**

1 PLANT PROPAGATION

Vegetative root cutting was the fastest and most successful propagation method for *Symphytum officinale*. Within one month, at least 90% of the cuttings started to develop shoots and roots. Hydroponic studies used only approximately uniform plants.

2 POLYPHENOL STUDIES

In this thesis, **polyphenols** refers to both simple and complex phenolic compounds. Tannin is one class of complex polyphenols that was investigated.

2.1 Histochemical detection of polyphenols

Free hand horizontal cross-sections of fresh roots and petioles stained red with nitrous acid, indicating the presence of catechol-type polyphenols in these tissues. The heaviest red staining was observed in the epidermal and casparian strip region of the root. In the petiole cross-sections, unicellular trichomes, head of glandular trichomes, epidermis and vascular bundle also stained red, though lighter in intensity than roots. No red staining was observed in controls (preliminary observations and no photos shown).

It was difficult to be certain of the location of polyphenols in these tissues because the red stain appeared to smear throughout the tissue – most probably because polyphenol-containing vacuoles were ruptured during free hand sectioning. However, the results are consistent with previous histochemical observations on *S. officinale* by

De Padua and Pantastico (1976). In their studies, tannins were found to be more abundant in roots than shoots.

2.2 Class of tannins

The lateral and main root contained condensed tannins as both root polyphenol extracts turned olive-green upon addition of iron chloride. No green colour was observed in controls. The lateral root extracts were a darker green than main roots, reflecting a higher level of tannins in the lateral root. No blue/ black colour, which is indicative of hydrolysable tannins, was observed upon further addition of iron chloride (Plate 3). This supports the fact that condensed tannins are found most commonly in angiosperms (e.g. *S. officinale*), gymnosperms and ferns (Harborne and Baxter, 1993).

2.3 Factors affecting polyphenol analysis

A single recommended protocol for quantifying polyphenols in plants does not exist because polyphenol and tannin levels can vary significantly depending on the plant species and how the tissue is handled from sample preparation to assay (Silanikove *et al.*, 1996; Waterman and Mole, 1994).

In the present study, factors concerning polyphenol extraction and analysis were conducted to determine the most suitable method for this project. These factors were:

- tannin recovery after freeze-drying
- number of extraction rounds
- type of extraction solvent
- extraction method and time
- type of quantitative assay

It is important to note that the objective of this experiment was to assess only *trends* relating to Pb accumulation and polyphenol levels, ‘total’ polyphenol levels were not necessary. Thus, the polyphenol level measured in *S. officinale* represents (and was limited to) the ‘extractable’ polyphenol fraction from the first round of extraction, not the total polyphenol content. Extractable polyphenols will be referred to with the shortened phrase ‘**polyphenol level**’. Total polyphenol analysis would involve at least two extraction rounds and boiling the residue in 1% (w/v) SDS and 5% (w/v) mercaptoethanol for 75-90 minutes. This is required to extract all the fiber and protein

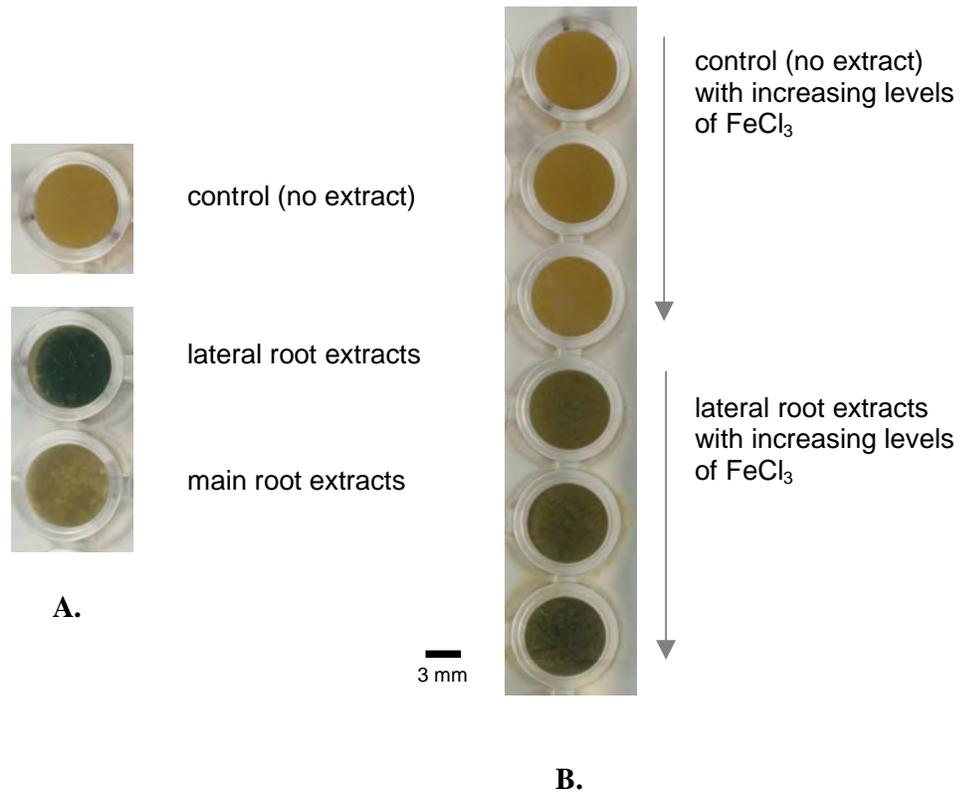
Plate 3. Class of tannins in roots of *Symphytum officinale* (determined by the FeCl₃ spot test on polyphenol extracts from roots).

A. Same amount of iron chloride applied to each root extract.

Olive-green colour is indicative of condensed tannins and relative levels of tannin in roots.

B. Lateral root polyphenol with increasing levels of iron chloride.

Remains olive-green, thus is indicative of only condensed tannins in extracts.



bound condensed tannins (which can make up at least 30% of the total polyphenols in some plants) (Jackson *et al.*, 1996).

2.3.1 Tannin recovery after freeze-drying

As recommended by Waterman and Mole (1994), and the FAO/IAEA laboratory manual (2002), all plant tissues were freeze-dried due to the large sample sizes involved in this section. To determine tannin recovery after freeze-drying, lateral root tissues were spiked with 0.1 mg mL^{-1} tannic acid. No tannin was lost due to this drying process as 100% of the tannic acid spike was recovered (Appendix B).

2.3.2 Number of extraction rounds

Two rounds of extraction were performed on four types of *S. officinale* tissues: leaf blade, petiole, lateral root, and main root. In all tissues, the first round of extraction (R1) released more than 80% of the total (R1 plus R2) polyphenols ($P \leq 0.05$) (Figure 5). Thus, only one extraction round was performed in subsequent experiments.

2.3.3 Extraction solvent

In this section, polyphenols were more effectively extracted from root tissues using 70% (v/v) acetone than 50% (v/v) methanol because only acetone resulted in significantly different polyphenol levels ($P \leq 0.05$) between the lateral and main root. Although not statistically significant ($P \geq 0.05$), this difference appears to be because acetone is more effective in extracting lateral root (not main root) polyphenols than methanol (Figure 6A). These trends were also true for the tannin levels measured (Figure 6B). Why only the lateral root? One reason could be the differences in biochemical composition between the lateral root and main root extracts. For example, acetone is known to prevent polyphenol-protein precipitation (Brown, 2001). If lateral roots (although not tested) have more protein-precipitable tannins than main roots, this could explain why acetone was more effective in extracting polyphenols from only the lateral root. However, the precise reason is unknown.

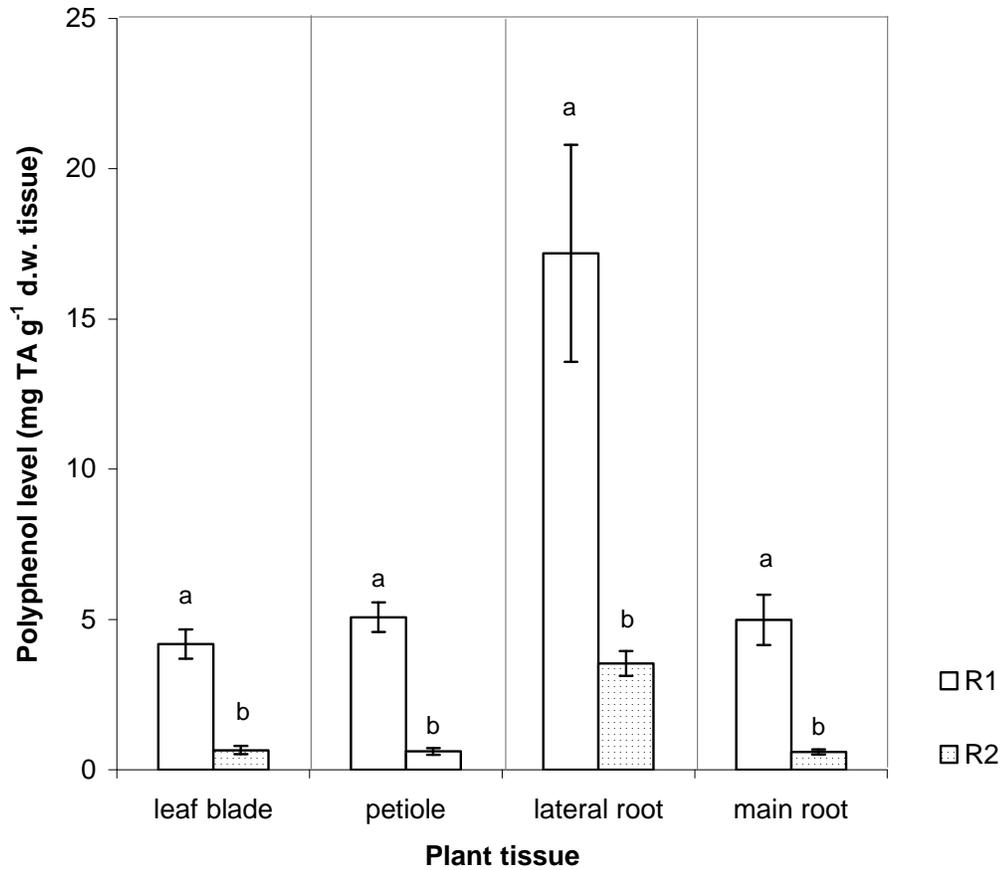


Figure 5. Polyphenol level after first (R1) and second (R2) round of extraction. Values are means \pm S.E. of three replicates. R1 and R2 within each tissue type (from 3-4 month-old *Symphytum officinale* plants) were compared; values not sharing the same letter *within* the tissue type are significantly different ($P \leq 0.05$). TA = tannic acid.

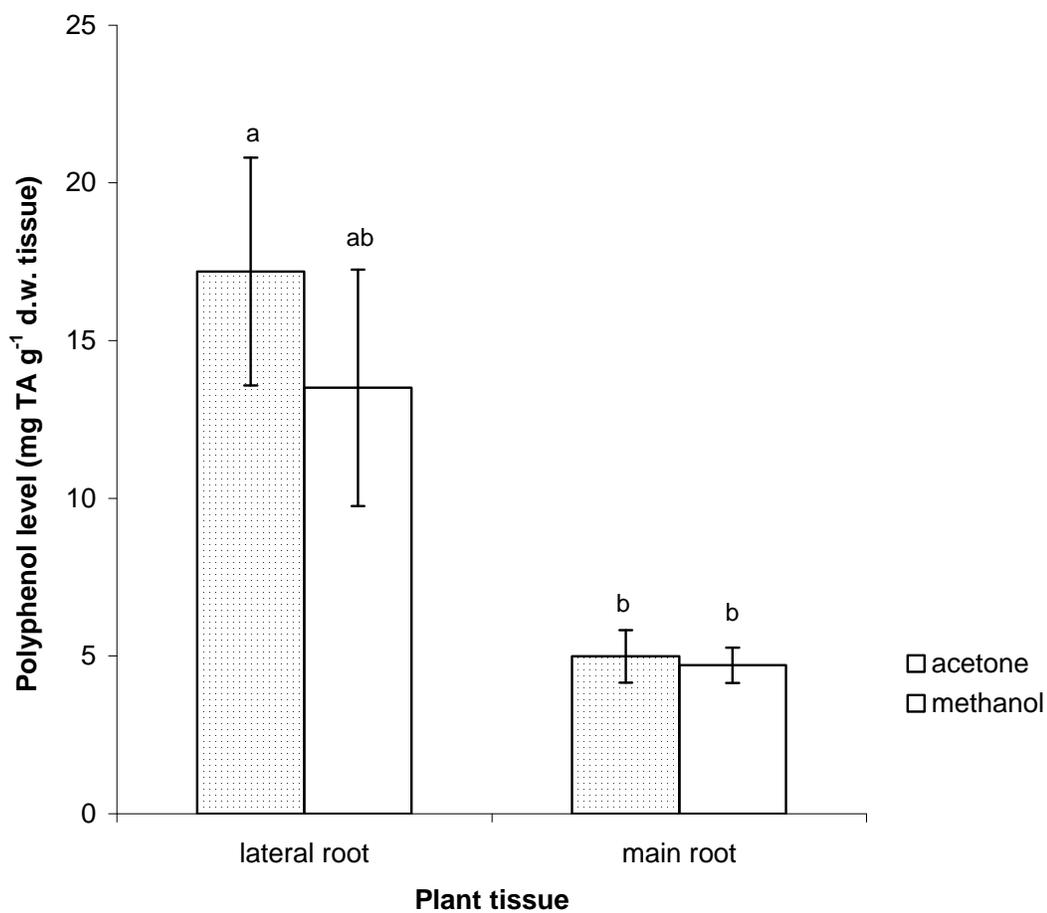


Figure 6A. Effect of 70% (v/v) acetone and 50% (v/v) methanol solvents on polyphenol levels extracted from 3-4 month-old *Symphytum officinale* roots. Values are means \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). TA = tannic acid.

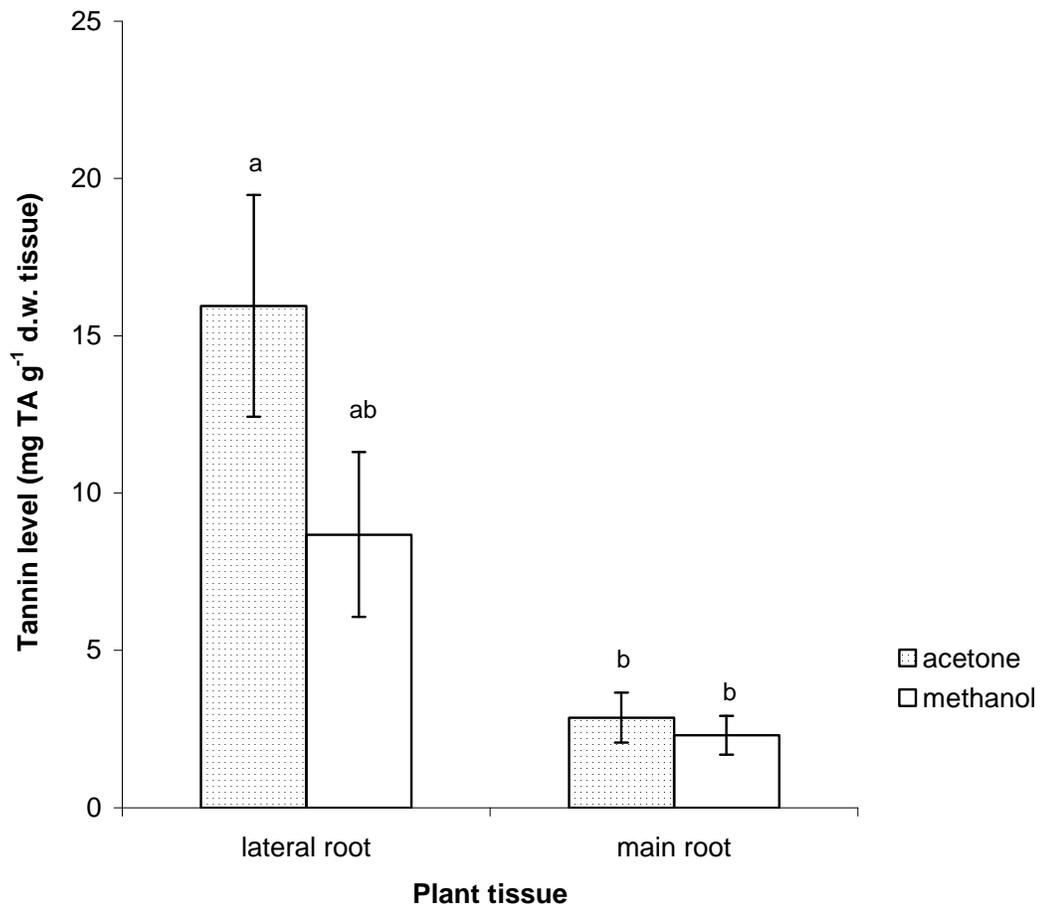


Figure 6B. Effect of 70% (v/v) acetone and 50% (v/v) methanol solvents on tannin levels extracted from 3-4 month-old *Symphytum officinale* roots. Values are means \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). TA = tannic acid.

In subsequent experiments only 50% (v/v) methanol was used for polyphenol extraction because: (i) in order to compare the Folin-Ciocalteu assay to the BSA-protein precipitation assay, an extra step was required to remove acetone (otherwise it would inhibit the protein-tannin precipitation reaction of the assay, and (ii) in subsequent experiments, longer methanol-based extraction (overnight) resulted in significant differences in polyphenol/tannin levels between these root types.

After 50% (v/v) methanol was selected, additives were added to this solvent to determine if the polyphenol and tannin yield could be further improved. In numerous publications, the 0.1% (w/v) ascorbic acid additive was added to methanol (without pH adjustment) for overnight extractions to reduce the loss of polyphenols by oxidation and increase condensed tannin yields (Dalzell and Kerven, 1998; Terrill *et al.*, 1992). To avoid tannin precipitation by Pb in polyphenol extracts of Pb-treated tissue, 1.5 mM EDTA was also added as an additive. However, it was found that both these additives inhibited root-tannin extraction when compared to plain 50% (v/v) methanol. Five minutes into extraction and at the end of the extraction period, the brown intensity of both the extracts (indicative of polyphenol levels) was much lower. The BSA-protein precipitation assay for tannin levels supported this observation (Appendix C). The acidity of these additives may have inhibited effective tannin extraction. The pH of 50% (v/v) methanol was about 5.4, compared to pH 4.2 for 50% (v/v) methanol plus 0.1% (w/v) ascorbic acid, and pH 3.6 for 50% (v/v) methanol plus 0.1% (w/v) ascorbic acid and 1.5 mM EDTA. This is consistent with beer production methods in which tannin extraction is avoided by maintaining pH well below 6.0 (Scandrett, 1997), but the precise reason for this effect is unknown. Ascorbic acid was also found to interfere with the Folin-Ciocalteu assay (see 2.3.4 below).

2.3.4 Type of quantitative assay

Two types of assays were performed on root polyphenol extracts: radial diffusion (Hagerman, 1998) and colorimetric assays (FAO/IAEA laboratory manual, 2002; Hagerman and Butler, 1978). Radial diffusion did not detect tannins from the extracts as a cloudy ring (indicative of tannins bound to the BSA-protein in the radial diffusion medium) was not observed (preliminary observations). However, both the Folin-Ciocalteu and BSA-protein precipitation colorimetric assays successfully detected polyphenols and tannins in the root extracts. In the case of the Folin-Ciocalteu assay,

this was only after one of the reagents – PVP (soluble) – was changed to PVPP (insoluble). Both reagents complex tannins in the polyphenol extract (FAO/IAEA, 2002; Silanikove *et al.*, 1996), but only insoluble PVPP was capable of removing tannins from the polyphenol extract; the molecular weight of soluble PVP was not large enough to precipitate tannins from the root extract.

Although absolute levels of tannins in the root extracts were different, the BSA-protein precipitation and Folin-Ciocalteu assays gave the same trend – lateral roots contained on average 3.7 times and 4.6 times more tannins than the main roots using respective assays ($P \leq 0.05$) (Figure 7). The BSA-protein precipitation assay was used in preference to the Folin-Ciocalteu assay since it was later found that the Folin-Ciocalteu assay could be subject to interferences from compounds such as ascorbic acid, which could be used in later experiments as chelator, antioxidant*, or in polyphenol extractions (Appendix D). (In *in vitro* experiments to follow, the Folin-Ciocalteu assay was used because such interference compounds were not part of the experiment and because the assay steps were incorporated into the experimental design).

2.3.5 Extraction method and time

In this section, the main root was tested because the thick and more fibrous nature of this tissue (compared to other tissues) could be affected more by the method and duration of extraction. For practicality, the overnight extraction period of 18-19 hours was chosen because it did not appear to result in any significant tannin loss compared to the shorter two-hour extraction period (Appendix E). Three times more polyphenols were extracted from the main root tissue using overnight shaking (for 18-19 hours at 4 °C) compared with the ultrasonic water-bath (20 minutes) method (Figure 8A).

Although not statistically different, the trend was also the same for tannins (Figure 8B). This is likely due to the particle size of the root powder; being manually ground, the root powder may not be fine enough (< 0.2 mm) for effective ultrasonic water-bath extractions. A ball-mill, as opposed to manual grinding, would be required to grind the tissue to the required particle size for ultrasonic extractions; this instrument was available and implemented in later experiments (section 5 of results onwards).

* ascorbic acid (vitamin C) in the treatment medium was considered as it has been shown to play a role in detoxifying ozone in the apoplast (Luwe *et al.*, 1993; Burkey and Eason, 2002) and during salt stress when added to the external medium (Shalata and Neumann, 2001).

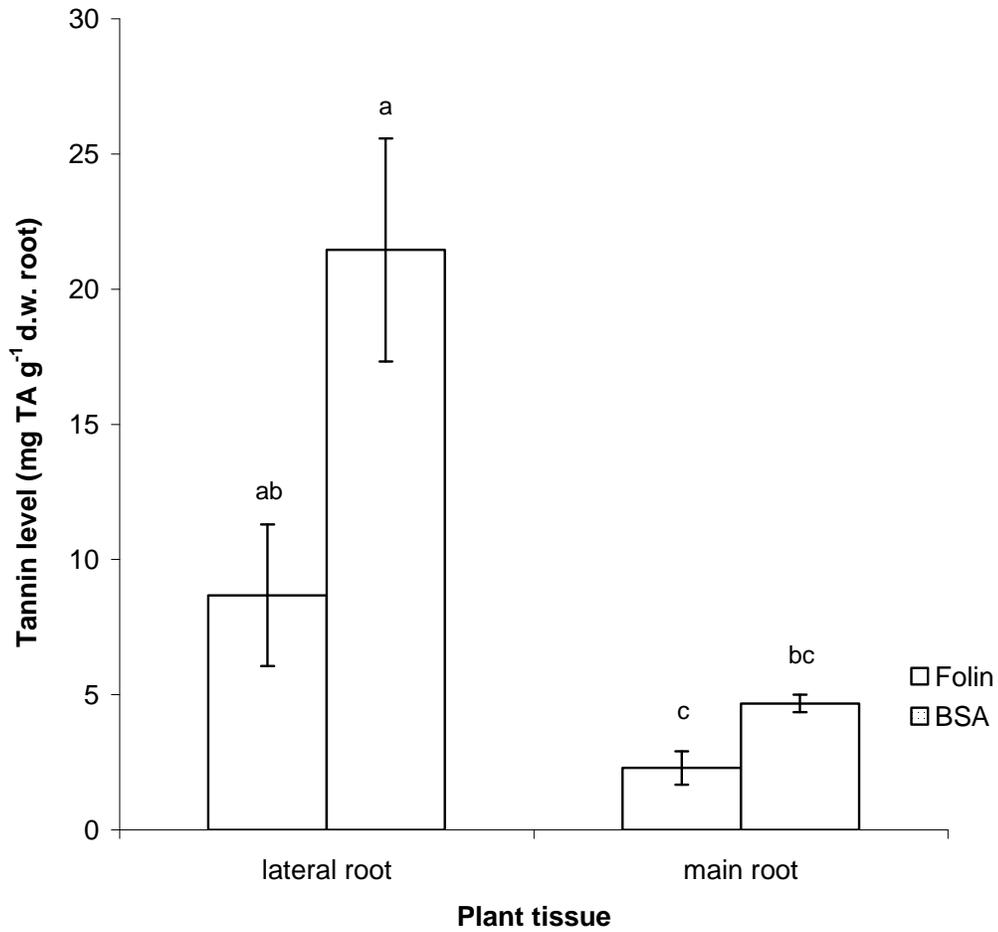


Figure 7. Comparison of two assays for determination of tannin levels extracted from root tissue of 3-4 month-old *Symphytum officinale*. Values are means \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). TA = tannic acid.

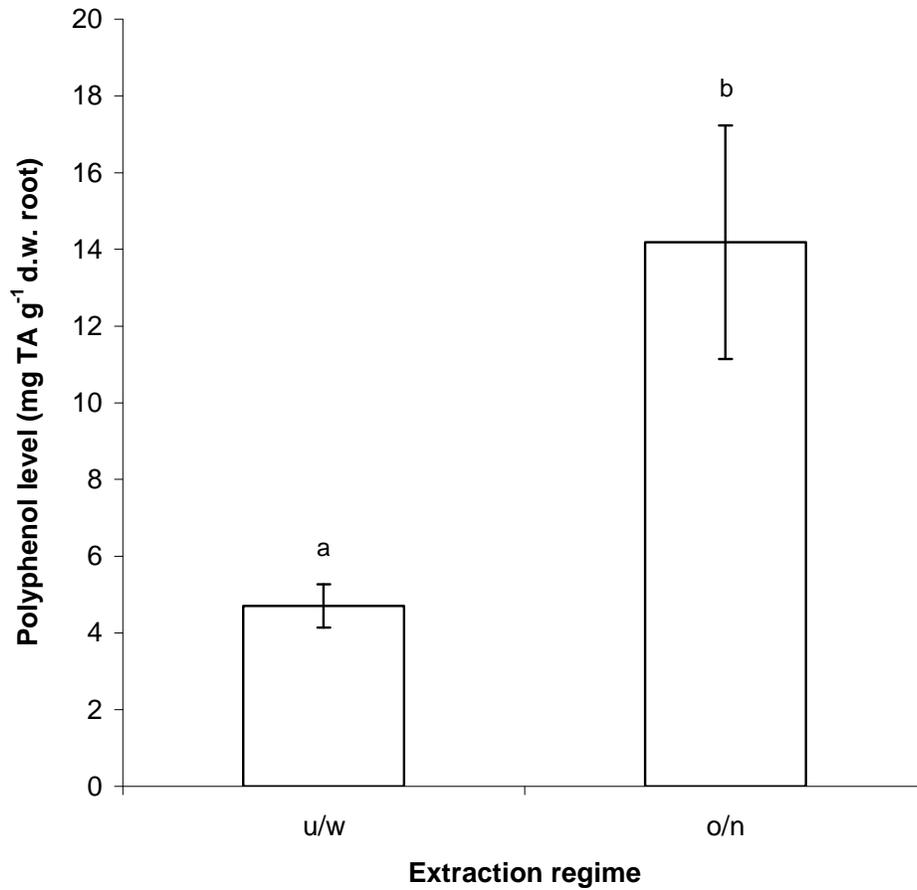


Figure 8A. Effect of extraction method on polyphenol levels extracted from 3-4 month-old *Symphytum officinale* main root tissue. Ultrasonic water-bath (u/w) vs. overnight shaking (o/n). Values are means \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). TA = tannic acid.

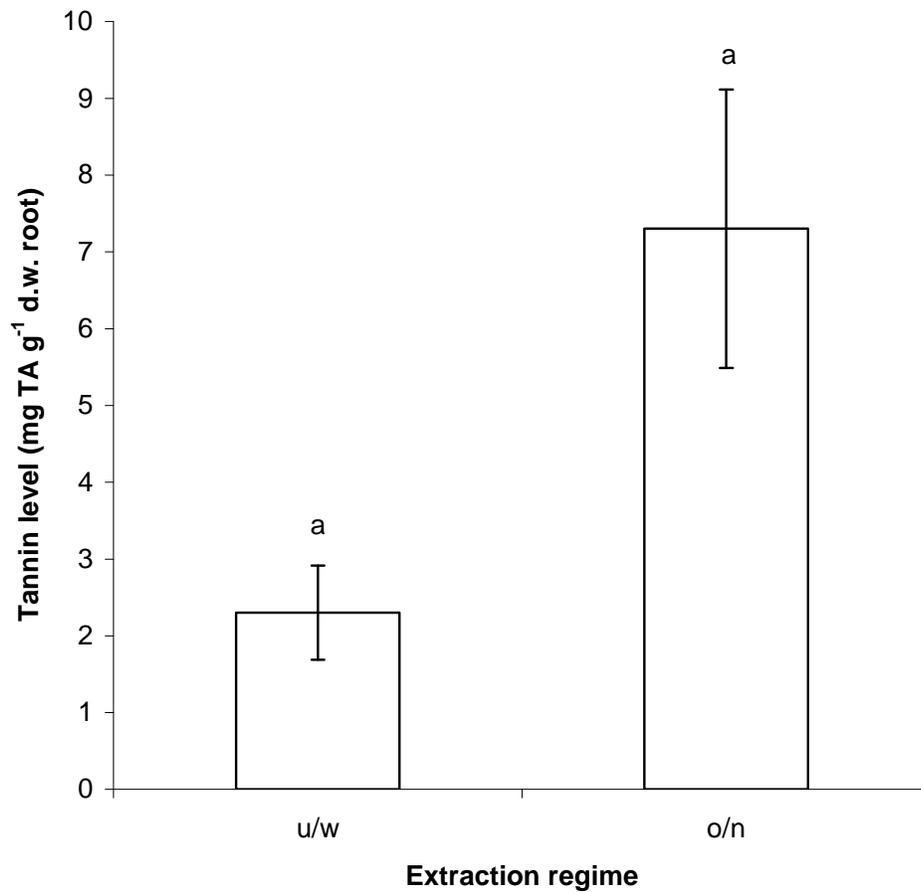


Figure 8B. Effect of extraction method on tannin levels extracted from 3-4 month-old *Symphytum officinale* main root tissue. Values are means \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). Ultrasonic water-bath (u/w) vs. overnight shaking (o/n). TA = tannic acid.

2.3.6 Overall methods chosen

Based on the five factors analysed above, the routine protocol for determination of polyphenol and tannin levels in plants from hydroponic experiments included the following features:

1. freeze-drying of plant materials
2. one extraction round
3. 50% (v/v) methanol as extraction solvent
4. BSA-protein precipitation assay
5. overnight shaking at 4°C (hand-milled tissues)

2.4 Distribution of polyphenol and tannins in *Symphytum officinale*

There are conflicting reports on the distribution of tannins in *S. officinale*. According to the PDR (1998), shoots contained more tannins than roots (8-9% d.w. in shoots compared to 4-6% d.w. in the roots) where as De Padua and Pantastico (1975) and others report that these compounds are more dominant in roots. The results presented here support the latter findings. The distribution of polyphenols in *S. officinale* was found to be in the following decreasing order (average % of total extractable plant polyphenols in methanol-based extraction solvents): lateral root (49%) > petiole (20%) = main root (17%) = blade (13%) (Figure 9A). The distribution of tannins in these tissues was also the same (Figure 9B).

Of the polyphenols measured in the lateral root, an average of 64% were tannins; similarly in the main roots 48% of the polyphenols measured were tannins. On a dry weight basis, polyphenols in the lateral roots were an average of 1.3% and in main roots 0.4%; tannins were 0.8% and 0.2% in these respective tissues. These values are smaller than those reported previously, possibly because of the age, extraction and growth regime of the plants. Nonetheless, there were sufficient differences in tannin levels between tissues and only trends were of interest in the studies to follow.

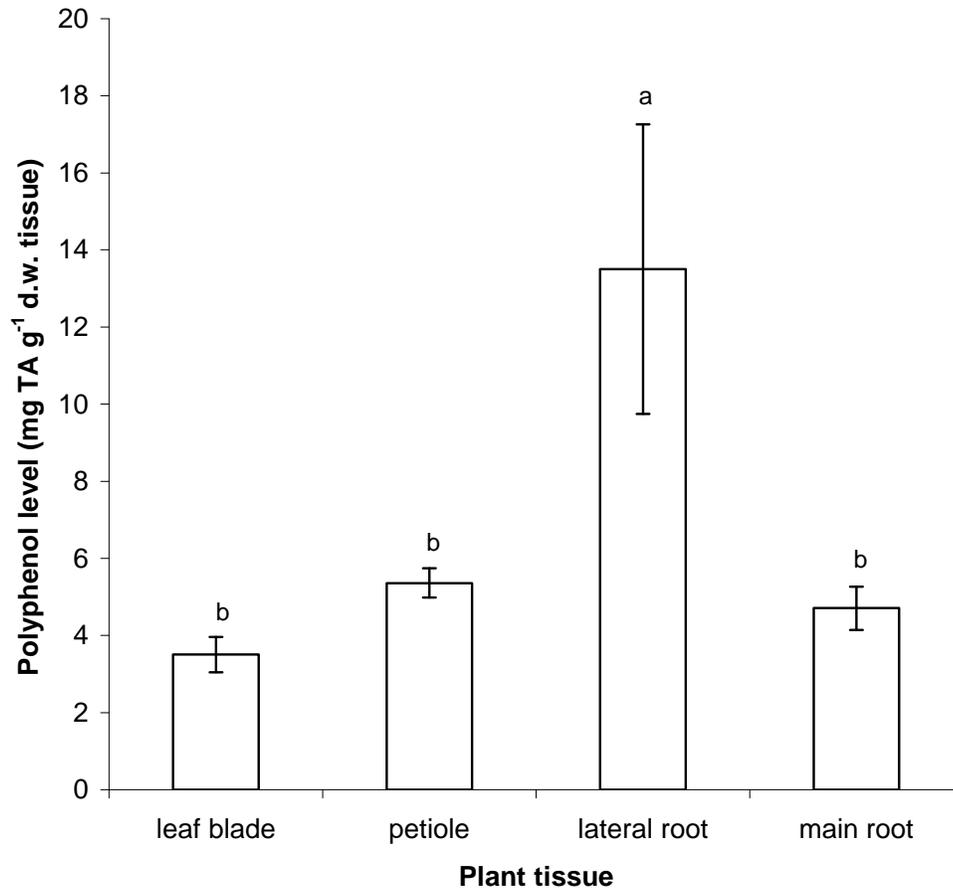


Figure 9A. Distribution of polyphenols in 3-4 month-old *Symphytum officinale*. Values are means \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). TA = tannic acid.

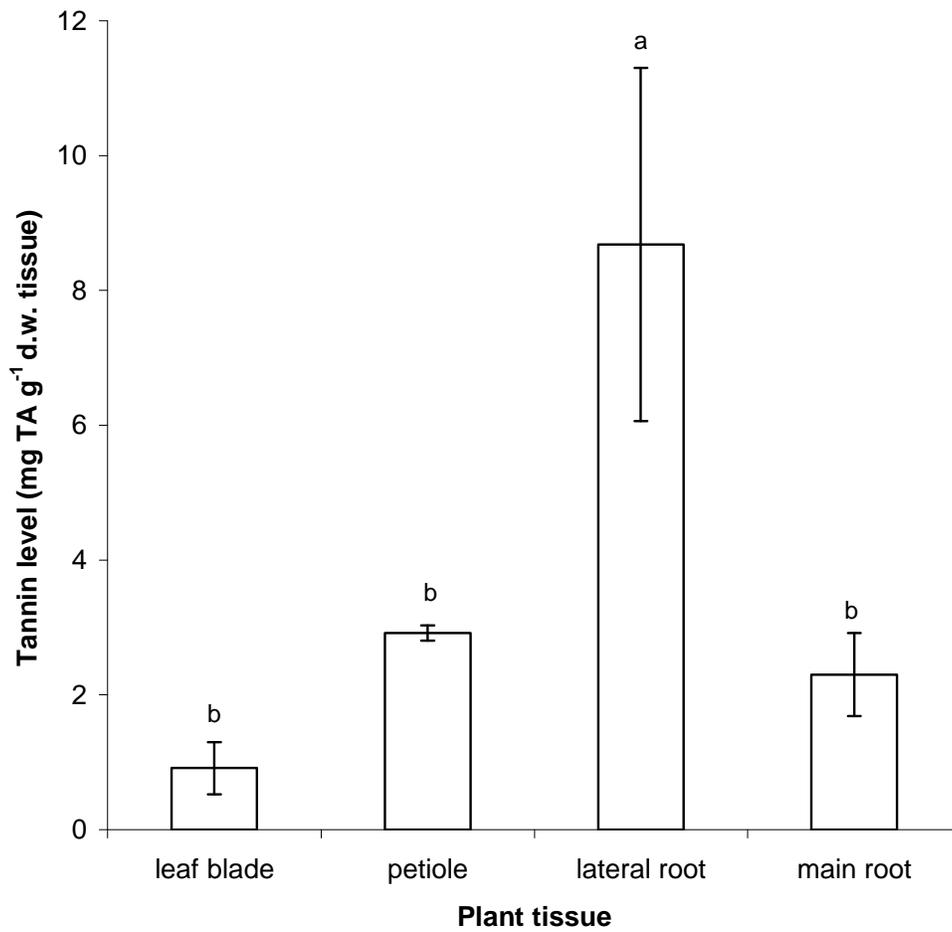


Figure 9B. Distribution of tannins in 3-4 month-old *Symphytum officinale*. Values are means \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). TA = tannic acid.

3 LEAD ACCUMULATION STUDIES (I)

3.1 Ashing temperature

To determine the effect of the ashing temperature on Pb recovery, one g (d.w.) of *S. officinale* root tissue was spiked with 12 mg L⁻¹ Pb (element) (from analytical grade Pb, BDH standard) and ashed at three different temperatures (480°C, 495-500°C, 550°C). Control tissue was spiked with 1% (v/v) HNO₃.

The 480°C and 495-500°C ashing temperatures were the best temperatures for Pb recovery as least 85% of the 12 mg L⁻¹ Pb spike solution was recovered; there was no difference between the two temperatures (Appendix F). However, of these two temperatures, the 495-500°C ashing temperature was selected for subsequent Pb analysis as it resulted in the most complete ashing (as determined by the 'whiteness' of the ash). Only 75% of the Pb was recovered at the 550°C ashing temperature (as used in previous publications such as Dushenkov *et al.*, 1995); the loss of Pb is presumably due to Pb volatilisation above 500°C.

3.2 Lead effect on plants: visual observations

Root growth and health of 2-month-old *S. officinale* plants decreased with increasing Pb(NO₃)₂ concentrations (Table 3). After one week, root growth of plants exposed to 100 M Pb(NO₃)₂ did not appear to be affected when compared to the control; new roots up to five cm long were observed in both control (no Pb(NO₃)₂) and 100 M Pb(NO₃)₂ solutions. From 250 M to 1000 M Pb(NO₃)₂, new root growth was visibly inhibited; at 500 to 1000 M roots appeared severely stunted and thick compared to other Pb concentrations. This root growth inhibition is a typical effect of Pb plant growth (Sobotik *et al.*, 1998). With increasing Pb concentrations, the colour of the roots appeared to change from white/yellow to brown (indicative of cell death and/or polyphenol polymerisation), particularly when comparing the control to 250-1000 M Pb(NO₃)₂. These effects were maintained over the three-week duration of this experiment (Plate 4).

In all treatments, shoot growth appeared to be relatively green and healthy after one week. By the end of week two some yellowing of leaves, particularly around the edges, could be seen in all plants (no chlorophyll measurements were taken). However,

Table 3. Effect of $\text{Pb}(\text{NO}_3)_2$ on new root growth after one week.

	0 M $\text{Pb}(\text{NO}_3)_2$	100 M $\text{Pb}(\text{NO}_3)_2$	250 M $\text{Pb}(\text{NO}_3)_2$	500 M $\text{Pb}(\text{NO}_3)_2$	1000 M $\text{Pb}(\text{NO}_3)_2$
New root growth length	> 5 cm	> 5 cm	up to 2 cm	up to 1.5 cm	< 0.5 cm

**Plate 4.** Effect of increasing $\text{Pb}(\text{NO}_3)_2$ concentrations on 2-month-old *Symphytum officinale* root growth after three weeks.

one or two new shoots still formed after week two in all $\text{Pb}(\text{NO}_3)_2$ treatments. By week three, significant leaf yellowing was observed for all plants, probably due to a lack of nutrients from the $\text{Pb}(\text{NO}_3)_2$ salt solution. Throughout the three-week duration of this experiment, there were no observable differences in shoot health between any of the Pb treatments and the control. This is consistent with AAS data from Figure 10A, which show that very little Pb is transported to the shoots.

Interesting in this study, this suggests that root growth inhibition had little consequence on shoot growth and health. Presumably, root-shoot ‘communication’ went on as usual, with or without Pb. The age of the plant and Pb treatment concentration may have some bearing on this observation. Previous studies showing shoot stress (Vassil *et al.*, 1998; Huang and Cunningham 1996; Geebelen *et al.*, 2002) used seedlings or very young plants. Sobotik *et al.*, (1998) showed that partial root inhibition was only observed at Pb concentrations starting at 0.01 M, while at 0.1 M shoot and root growth were severely inhibited.

4 ROLE OF POLYPHENOLS IN LEAD ACCUMULATION

The possible role of polyphenols in the metal-chelation based tolerance mechanism and Pb accumulation in *S. officinale* was investigated at root level for two reasons. Firstly, the above experiment established that lateral root tissues contained the highest level of polyphenols such as tannins. Secondly, there was an *in vivo* difference in polyphenol and tannin levels between the lateral and main root tissues; lateral roots contained about three times more polyphenols and four times more tannins than main root tissue (on a dry weight basis). This difference forms the basis of the following *in vivo* studies (Pb, polyphenol and TEM analysis of tissues after Pb treatment). The basic question here was: “*does the root with more polyphenols/tannins accumulate more Pb?*”

4.1 Hydroponic conditions

To ensure that all plants used in the experiments were uniformly treated, the initial hydroponic Pb experiments (result section 4.2) were re-started because the plants required spraying to control an unexpected infestation of whiteflies.

Another hurdle was the unexpected precipitation of Pb in the nutrient solution. This would not only reduce Pb bioavailability but also may result in contamination of

the root tissue. Acid washing of all glassware, to remove any phosphate contaminants, did not solve the problem. For Pb concentrations up to 100 μM , adjusting the pH of the nutrient solution prior to Pb addition removed Pb precipitation. However above this concentration, precipitation was still observed – this was due to the phosphate levels of the nutrient solution. When the phosphate level of the HC nutrient solution was lowered by 50% (to 5 μM KH_2PO_4 ; see Appendix A1), precipitation was almost eliminated. The plants grown in this HC nutrient solution (referred to as *modified* HC nutrient solution) did not exhibit any phosphate deficiencies.

4.2 Pb uptake and accumulation

Each level of treatment (modified HC nutrient solution containing 0 μM $\text{Pb}(\text{NO}_3)_2$ (control), 250 μM $\text{Pb}(\text{NO}_3)_2$ (unchelated Pb form), and 250 μM $\text{Pb}(\text{NO}_3)_2$ with 125 μM EDTA (chelated Pb form) was replicated three times.

After seven days in nutrient solution containing 250 μM $\text{Pb}(\text{NO}_3)_2$ with and without 125 μM EDTA, new root growth was significantly reduced compared to the control plant. Of the two Pb treatments, the degree of root growth inhibition was slightly less with EDTA, which was consistent with the slightly lower Pb levels in the roots. At the shoot level there were no observable Pb effects (Plate 2, methods section). This is consistent with AAS data, which show that very little Pb is in the shoots (Figure 10A).

Under the conditions of this experiment, *S. officinale* appears to accumulate Pb primarily in its roots. The distribution of Pb (for both unchelated and chelated Pb forms) in *S. officinale* plant tissues, from highest to lowest was: lateral root > main root > shoot (Figure 10A and B). For both unchelated and chelated Pb-exposed plants, most of the Pb accumulated in the root tissue. In both forms of Pb-exposed plants, only a small but significant ($P \leq 0.05$) amount of Pb was translocated from the root to shoot tissue. For chelated Pb-treated plants, the Pb shoot level was on average 12 times higher compared to unchelated Pb-treated plants. This is consistent with the function of EDTA, which is to increase Pb translocation (Figure 10A).

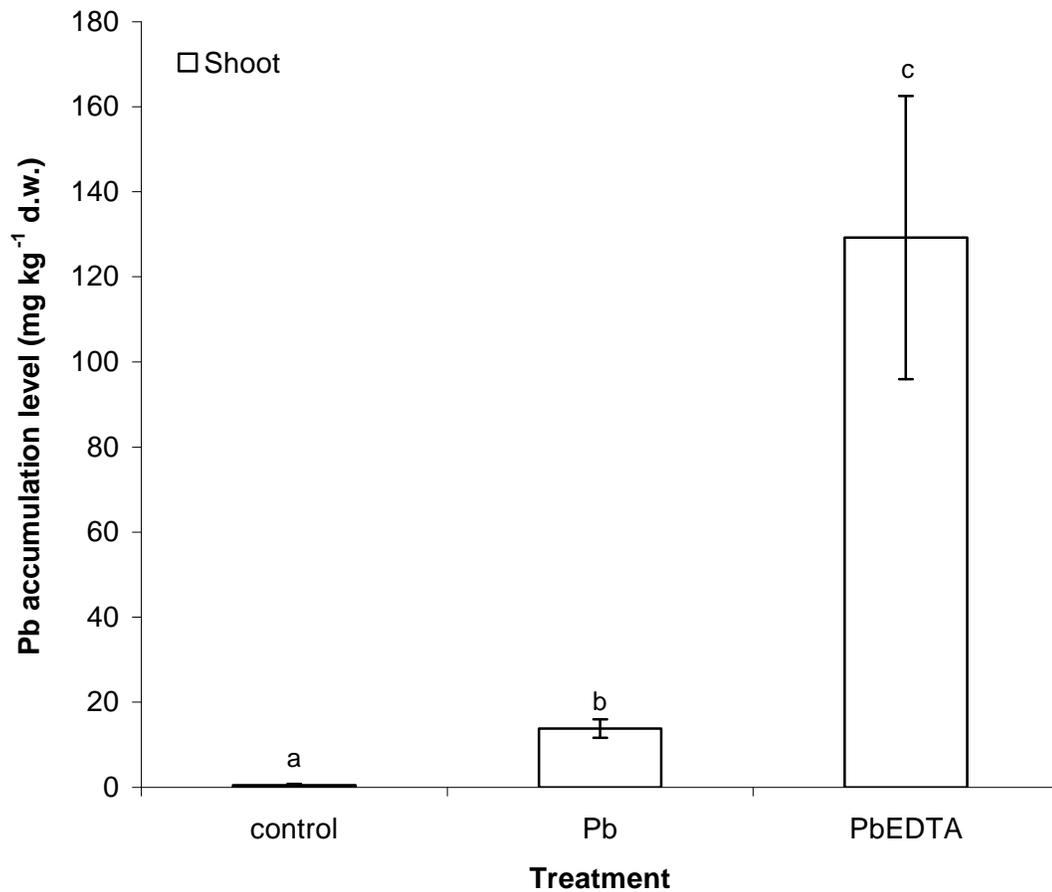


Figure 10A. Distribution and accumulation of Pb in 10-month-old *Symphytum officinale* shoots after whole plants were treated for seven days in modified HC nutrient solution (pH 4.5) containing 0 (control), or 250 μ M Pb(NO₃)₂ with or without 125 μ M EDTA solution. Values are means \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.01$). HC = Huang and Cunningham.

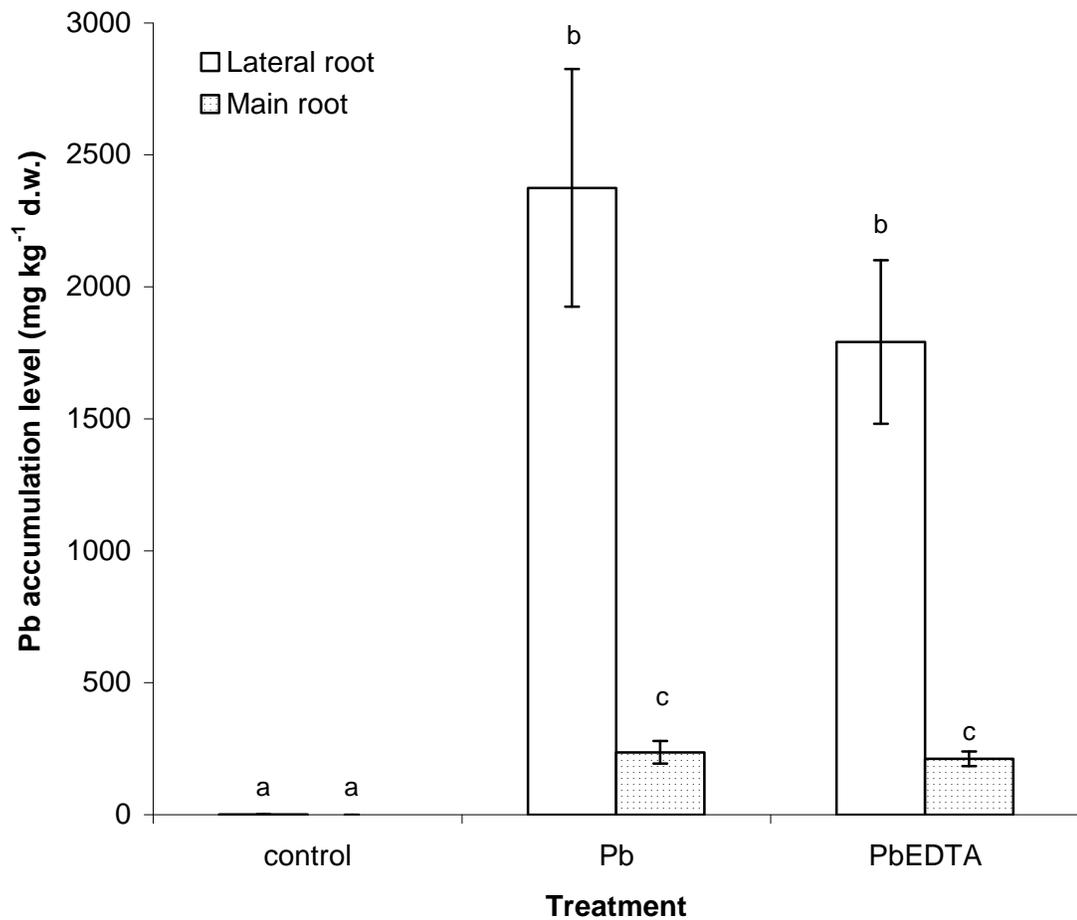


Figure 10B. Distribution and accumulation of Pb in 10-month-old *Symphytum officinale* roots after whole plants were treated for seven days in modified HC nutrient solution (pH 4.5) containing 0 (control), or 250 μ M Pb(NO₃)₂ with or without 125 μ M EDTA solution. Values are means \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.01$). HC = Huang and Cunningham.

In terms of the amount of Pb accumulated in the roots of plants exposed to unchelated Pb, high-polyphenol lateral roots accumulated ten times more Pb (on an average dry weight basis) than main root tissue ($P \leq 0.01$); lateral root tissue accumulated an average of 2375 mg kg^{-1} (d.w.) Pb, compared to an average of 236 mg kg^{-1} (d.w.) Pb in main root tissue (after desorption with EDTA). Although not significantly different ($P \geq 0.05$) this trend was slightly lower in chelated Pb treatments; Pb accumulation in the lateral root was only 10 times higher than the main root (on an average dry weight basis) (Figure 10B).

Lower temperatures are known to reduce heavy metal uptake (Baghour *et al.*, 2001). Since the temperature in the glasshouse used varies from 21°C to 35°C depending on the weather, this is a possible reason for the observed variation in Pb accumulation amongst the replicates (such as the decrease in Pb accumulation from replicate one to three). Despite these variations in Pb accumulation between replicates, a common trend in Pb distribution and accumulation levels was clearly identified.

4.3 Effect of lead treatments on polyphenol and tannin levels

In all treatments, lateral root polyphenol and tannin levels were at least three times higher (on an average dry weight basis) than the main root tissue.

The polyphenol (Figure 11A) and tannin (Figure 11B) level in the lateral root and main root tissue were not significantly affected by any of the three treatments (control and $250 \text{ M Pb(NO}_3)_2$ with or without 125 M EDTA). However relative to the control, lateral root polyphenol levels appeared to increase slightly with exposure to unchelated Pb (an average of 11% higher on a d.w. basis) and chelated Pb (an average of 16% higher on a d.w. basis). Similarly, lateral root tannin levels appeared to increase by 17% and 34% with unchelated Pb and chelated Pb treatments. The lower polyphenol containing main root tissues did not appear to elicit such a trend (Figure 11A). This study suggests that Pb exposure does not induce significant increases in polyphenol levels. It may be argued that Pb could have induced a large increase in polyphenol levels, but this was not observed because polyphenols are known to precipitate heavy metals (Evans, 1996, McDonald *et al.*, 1996). However if precipitation were to occur, one would expect that the polyphenol levels in Pb exposed plants to be less than that of controls because of the high Pb concentration used in this study. Since this was not the case, the suggestion that Pb exposure does not induce

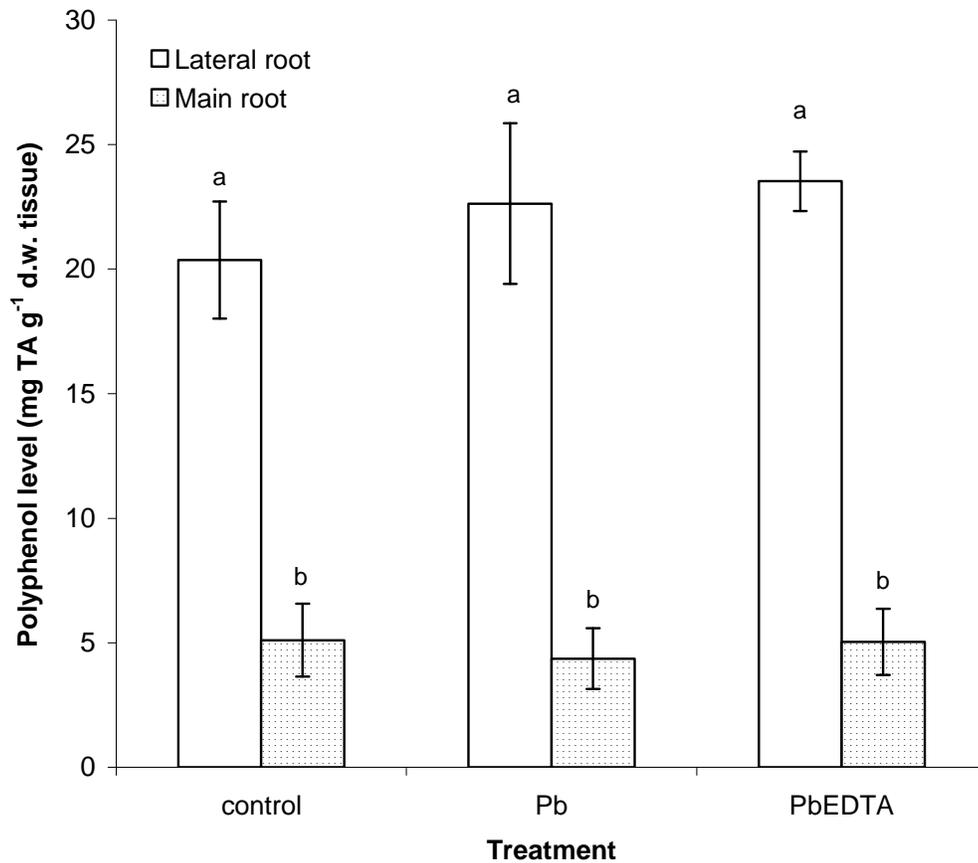


Figure 11A. Polyphenol levels extracted from 10-month-old *Symphytum officinale* roots after whole plants were treated for seven days in modified HC nutrient solution (pH 4.5) containing 0 (control), or 250 μ M Pb(NO₃) solution with or without 125 μ M EDTA solution. Values are means \pm S.E of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). HC = Huang and Cunningham.

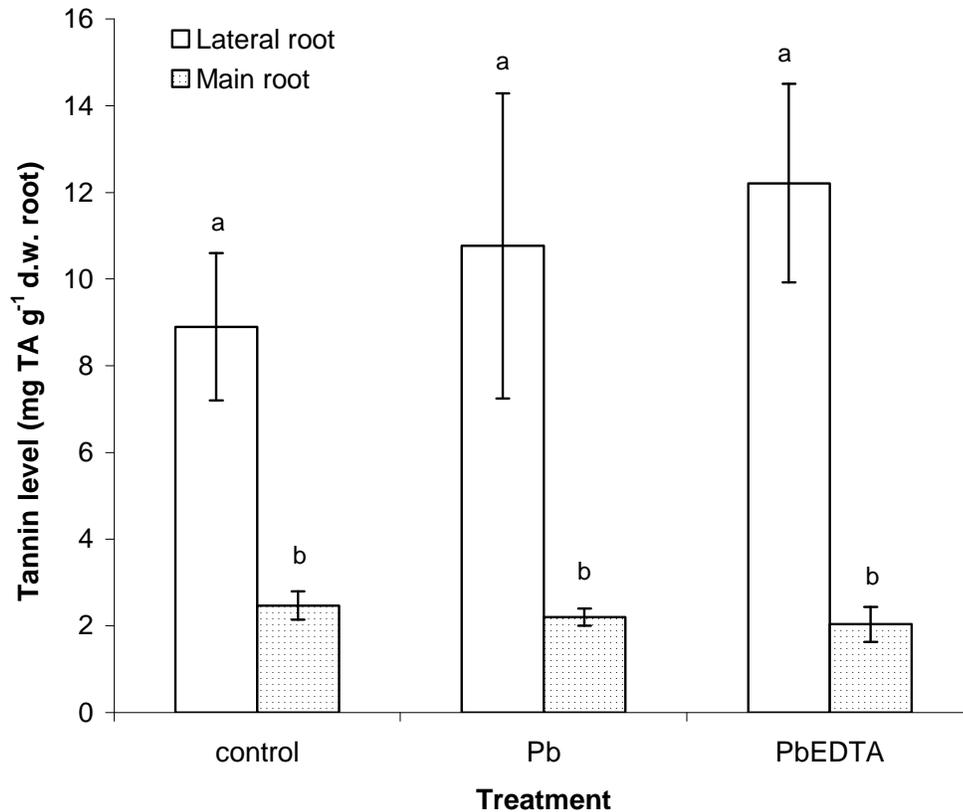


Figure 11B. Tannin levels extracted from 10-month-old *Symphytum officinale* roots after whole plants were treated for seven days in modified HC nutrient solution (pH 4.5) containing 0 (control), or 250 μ M $\text{Pb}(\text{NO}_3)_2$ solution with or without 125 μ M EDTA solution. Values are means \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). HC = Huang and Cunningham.

significant increases in polyphenol levels still holds well. Another reason is that after seven days of Pb treatment, polyphenol polymerisation may occur, causing a reduction in the extractability of polyphenols. The observation of stunted root growth and browning of roots after Pb treatment support this.

4.4 Transmission electron microscopy (TEM)

Sections for transmission electron microscopy were made from the entire lateral root cross-section or, in the case of the large main roots, towards the epidermis (Figure 12). All the TEM observations were performed on unstained root sections, as Pb particles were sufficiently electron dense to contrast with the tissue. Pb particles generally appeared as distinctive dark fine grains or as dark heavy deposits. In control plants, no Pb particles were seen (Plates 5A and B).

In the lateral root of plants exposed to unchelated Pb, Pb particles were easily found in sections. Most of the fine Pb grains were found in the middle lamella region (Plates 6, 7, 8), possibly due to chelation to the pectin carboxyl groups. Interestingly in contrast, all the dark heavy Pb deposits were found along (and some fusing to) the primary cell wall region/plasma membrane interface (Plates 7 and 8). Such locality is consistent with Pb deposits observed by TEM and confirmed by X-ray microanalysis in *Sesbania drumondii* root cells (Sahi *et al.*, 2002). Similar observations of Pb-laden dictyosomes vesicles fusing and discharging Pb into extracellular space have been reported (Malone *et al.*, 1974; Qureshi *et al.*, 1986). In main root tissues of unchelated Pb-treated plants, very little or no Pb could be detected in the primary cell wall region of the sections observed (Plate 9). However, a rare section did show the same location and level of Pb deposits as the lateral roots (Plate 10). Since the main root TEM sections were taken in from the epidermis region, it is likely that the heavy Pb deposits in this plate were from the root epidermis (where most polyphenols are found), whilst the observations with a lack of Pb were made from cells towards the inner root tissues (Figure 12). Overall, more Pb appeared to accumulate in each lateral root TEM section when compared to the main root tissue. This supports the Pb levels found in these tissues.

The apparent outward flow of Pb from the cytoplasm is supported by TEM micrographs as: (i) in unchelated Pb treatments, small vesicles containing heavy Pb deposits appeared to fuse or pass through the plasma membrane, into the primary cell

wall region of lateral roots (Plates 7 and 8) and (ii) this fusion appeared only in highly vacuolated cells with little cytoplasmic content remaining (Plate 10).

In chelated Pb treatments, Pb in both root types was more difficult to find (despite having similar Pb levels in roots as the unchelated Pb treatments). Malone *et al.*, (1974) also reported this observation. In their study with corn, no Pb precipitate was ever observed on the root's surface when EDTA was added to the medium, regardless of the amount of Pb in the solution. One possible reason is that in chelated form, less Pb particles coagulate into easily detectable heavy deposits.

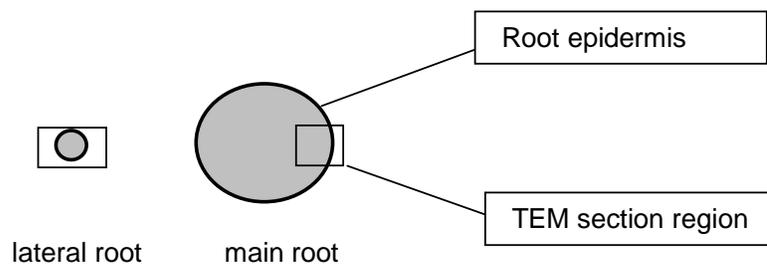


Figure 12. Region of TEM section in lateral and main root.

This more soluble Pb form would also be more effective for translocation to the shoots; Pb may have also moved deeper into the root tissue towards, or as supported by the Pb levels in shoots, into the xylem. Of the observations made, Pb appeared as fine particles in the middle lamella and cytoplasmic layer against the cell wall (Plate 11). Possible Pb particles were also observed in the vacuole (Plate 12) and in the cell wall (Plate 13).

What about tannin observations? Given their strong affinity to bind to proteins (Getachew and Becker 1998), tannins are mostly in the vacuole of cells and bound as esters in the primary cell wall/plasma membrane (see introduction section) in living plant tissues. As the cell ages (in this case due to Pb stress) and loses its cytoplasmic contents, tannins commonly become adsorbed onto the cell wall (Ximenes, 1998). Given their strong affinity for osmium, tannins usually appear as a continuous black layer along the cell wall or as large black shades in the vacuole in transmission electron micrographs (Parham and Kaustinen, 1976; Mueller and Beckman, 1976). In these results, heavy black staining was only observed in vesicles fusing to the primary cell wall/ plasma membrane interface. But as already mentioned earlier these were identified as Pb deposits. Definitive location of tannins, even in the controls, was not observed in

the root sections despite being clearly present in assays. This may be because (i) the concentration of the tannins were too low for microscopy detection without staining (lateral roots contained 0.8% d.w. tannins; main roots 0.4% d.w.) and/or (ii) the ethanol and acetone based dehydration step during TEM sample preparation may have reduced the tannin content of the roots prior to observation. However, we cannot exclude the possibility that the dark matter enclosed in vesicles is tannins or cytoplasmic Pb-tannin complexes fusing with cell wall. This confirmation would require transmission electron microscope with an EDX or X-ray microanalysis attachment and mass spectroscopy facilities, neither of which was available at the time of this experiment.

Although tannins were not clearly visible on the TEM micrographs, condensed tannins are known to occur in the primary cell wall and suberin layer (between the cell wall and plasma membrane) of plant cells (Fry 1984; Chalker-Scott and Krahmer, 1989). Thus, the location of heavy Pb deposits in this region could suggest that polyphenols (in addition to other compounds such as pectins) chelate Pb ions and is involved in accumulation and tolerance of Pb (chelation-based tolerance mechanism 1; see introduction).

Pb appears to be transported both apoplastically and symplastically in both forms of Pb treatment. The location of Pb in the cell wall, indicative of apoplastic Pb transport, is consistent with Pb exposure in *Allium cepa* L. (Wierzbicka, 1998) and *Chamecystis proliferus* (L.f.) (Jarvis and Leung, 2001). The occurrence of Pb in vesicles and vacuoles supports symplastic transport. Additionally, Pb in vacuoles and vesicles is consistent with isolation-based tolerance mechanism 1 (Clemens, 2001; Hall, 2002; Wierzbicka, 1995).

Overall these observations are meaningful as (i) TEM observations in other publications (see above) showed similar results and (ii) studies by Antosiewicz and Wierzbicka (1999) and Qureshi *et al.*, (1986) showed that Pb loss, and thus artifactual re-distribution of Pb during tissue preparation for TEM observations, was minimal during chemical (glutaldehyde and osmium tetroxide) or physical (freeze drying) sample fixation for TEM.

- Plate 5.** A. Transmission electron micrograph at 30,000 X magnification of an ultra-thin section of *Symphytum officinale* lateral root treated with 0 M $\text{Pb}(\text{NO}_3)_2$ (control) for seven days.

Key:

- 1 Plasmalemma
- 2 Middle lamella free of Pb particles
- 3 Intercellular space

- B. Transmission electron micrograph at 10,000 X magnification of an ultra-thin section of *Symphytum officinale* main root treated with 0 M $\text{Pb}(\text{NO}_3)_2$ (control) for seven days.

Key:

- 1 Plasmalemma
- 2 Middle lamella
- 3 Intercellular space

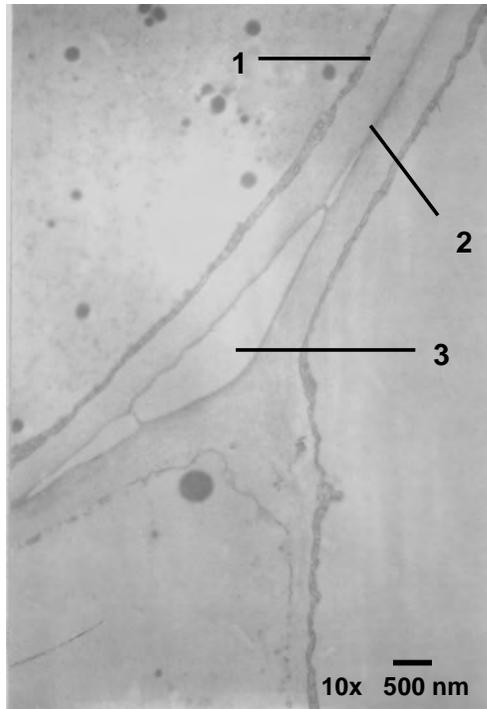
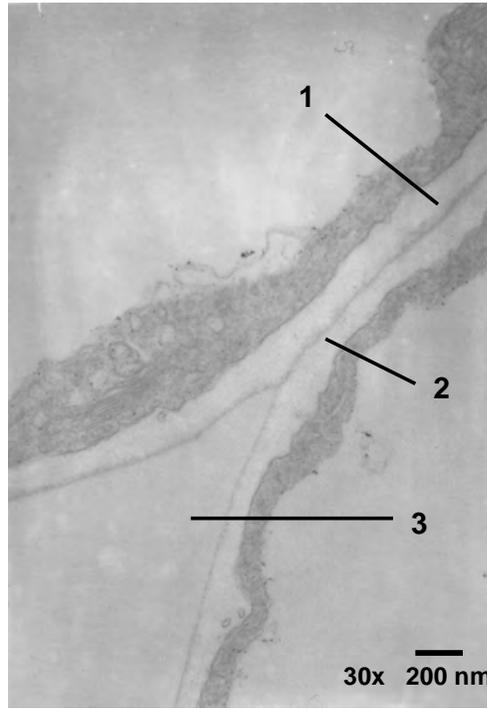


Plate 6. Transmission electron micrograph at 20,000 X magnification of an ultra-thin section of *Symphytum officinale* lateral root treated with 250 M $\text{Pb}(\text{NO}_3)_2$ for seven days.

Key:

- 1 Plasmalemma
- 2 Intercellular space
- 3 Pb particles concentrated along middle lamella

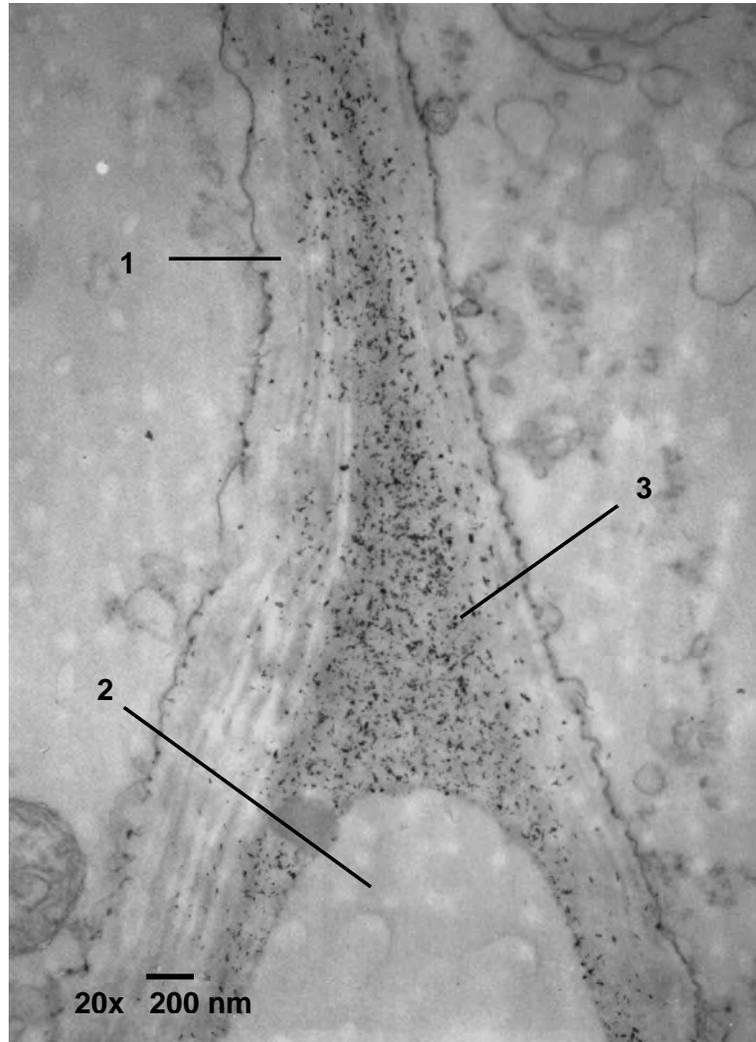


Plate 7. Transmission electron micrograph at 20,000 X magnification of an ultra-thin section of *Symphytum officinale* lateral root treated with 250 M $\text{Pb}(\text{NO}_3)_2$ for seven days.

Key:

- 1 Vesicle-like structures containing Pb deposits
- 2 Fine Pb particles concentrated along middle lamella
- 3 Vesicle-like structure in the cell wall region
- 4 Heavy Pb deposits along primary cell wall and plasmalemma
- 5 Lipid body
- 6 Mitochondrion

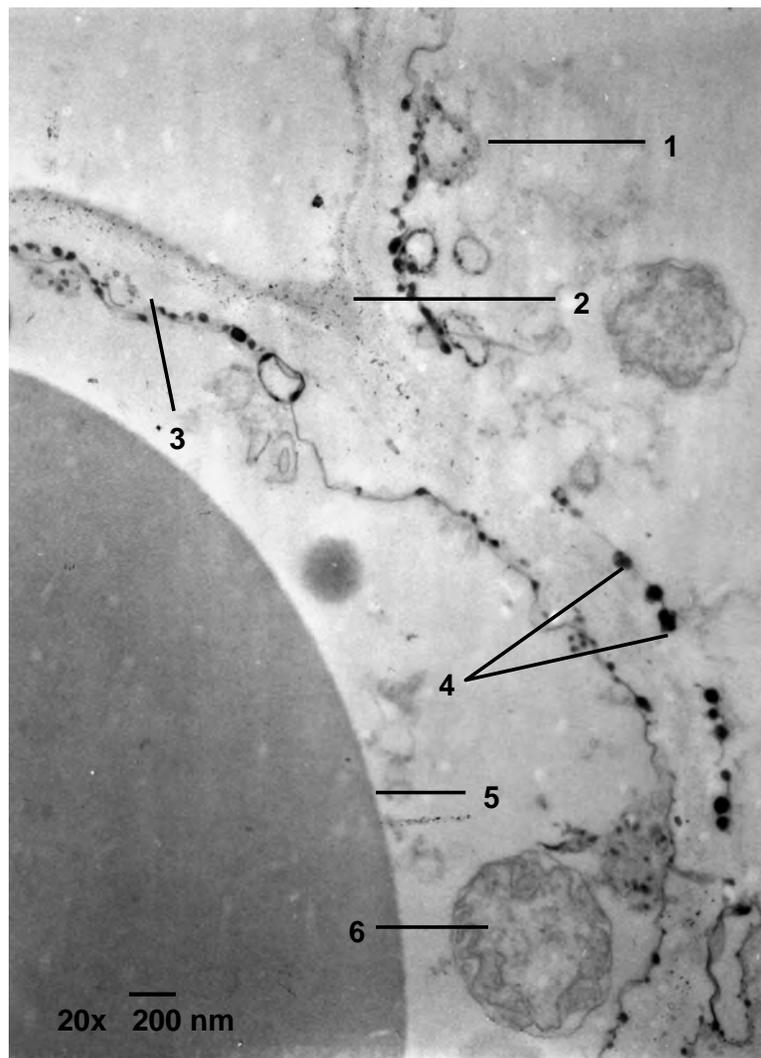


Plate 8. Transmission electron micrograph at 10,000 X magnification of an ultra-thin section of *Symphytum officinale* lateral root treated with 250 M $\text{Pb}(\text{NO}_3)_2$ for seven days.

Key:

- 1 Heavy Pb deposits along primary cell wall and plasmalemma
- 2 Fine Pb particles concentrated along middle lamella
- 3 Mitochondrion
- 4 Vesicle-like structures fused with plasmalemma

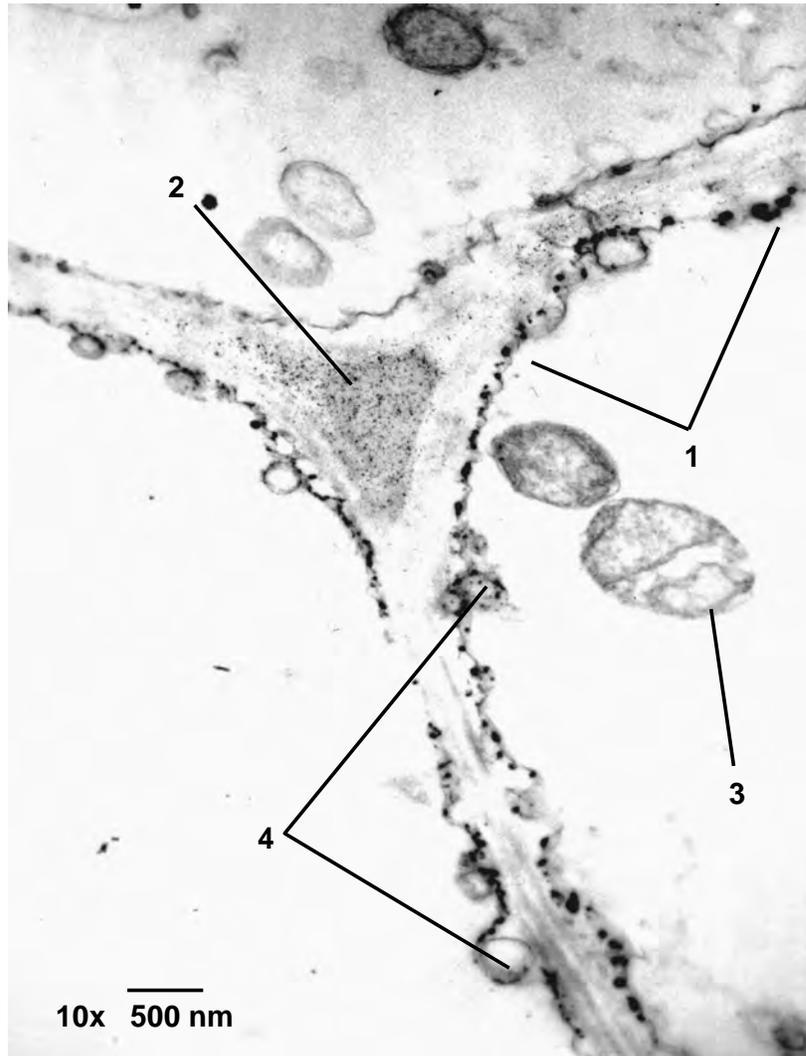


Plate 9.

Transmission electron micrograph at 6,000 X magnification of an ultra-thin section of *Symphytum officinale* main root treated with 250 M $\text{Pb}(\text{NO}_3)_2$ for seven days.

Key:

- 1 Starch grain
- 2 Lipid body
- 3 Pb particles along primary cell wall and plasmalemma
- 4 Middle lamella free of Pb particles

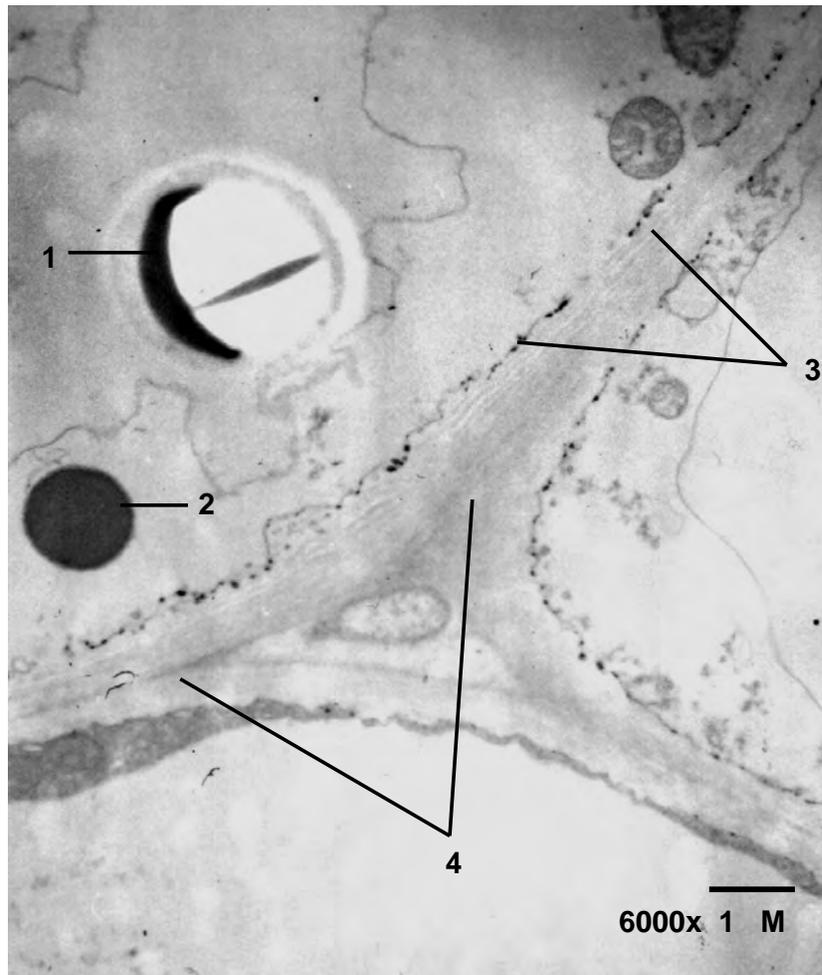


Plate 10. Transmission electron micrograph at 7,500 X magnification of an ultra-thin section of *Symphytum officinale* main root treated with 250 M $\text{Pb}(\text{NO}_3)_2$ for seven days.

Key:

- 1 Primary cell wall
- 2 Heavy Pb particles in middle lamella
- 3 Plasma membrane of cell with cytoplasmic contents
- 4 Plastid
- 5 Vacuole (larger circle) and vesicle (smaller circle)

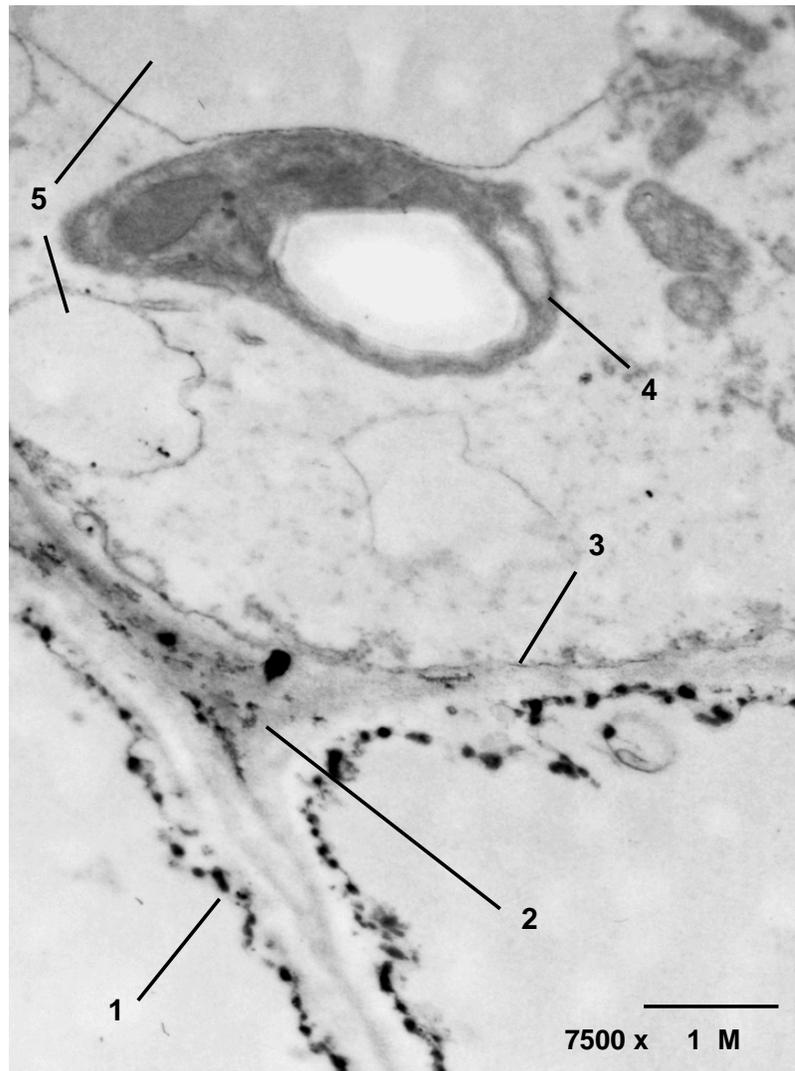


Plate 11. Transmission electron micrograph at 15,000 X magnification of an ultra-thin section of *Symphytum officinale* lateral root treated with 250 M $\text{Pb}(\text{NO}_3)_2$ plus 125 M EDTA for seven days.

Key:

- 1 Intercellular space
- 2 Vesicle-like structures in middle lamella
- 3 Pb particles deposited in middle lamella
- 4 Cytoplasmic material against cell wall; very fine Pb particles present

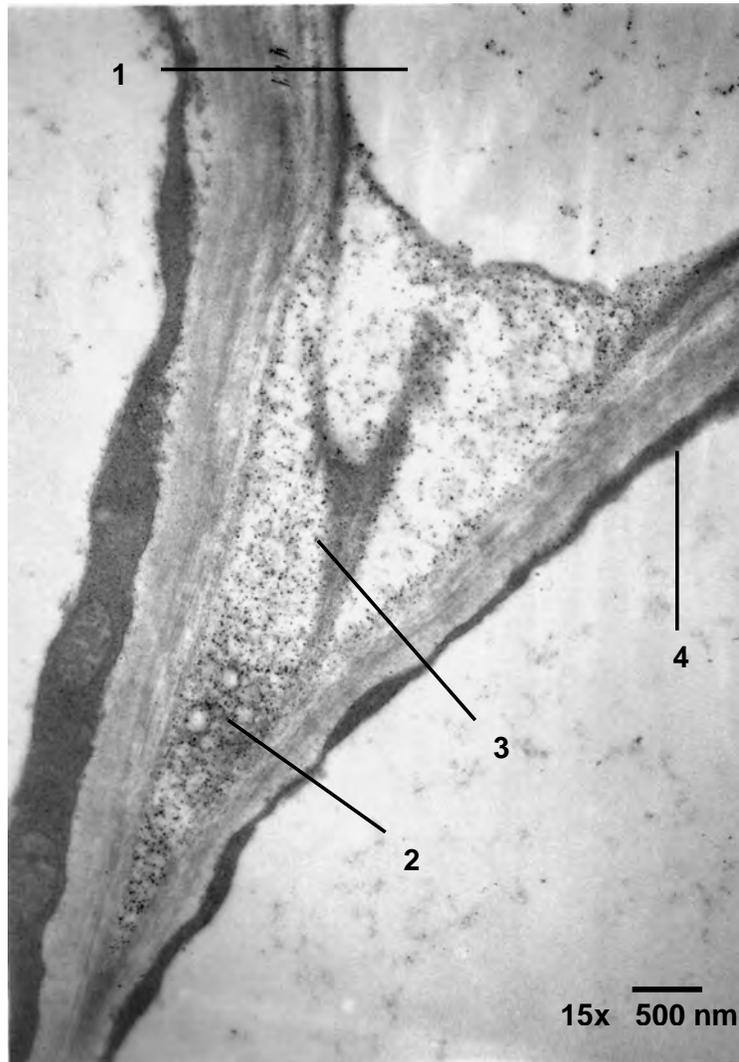


Plate 12.

Transmission electron micrograph at 7500 X magnification of an ultra-thin section of *Symphytum officinale* lateral root treated with 250 μ M Pb plus 125 μ M EDTA for seven days.

Key:

- 1 Heavy Pb particles in vacuole
- 2 Cytoplasmic contents
- 3 Cell wall

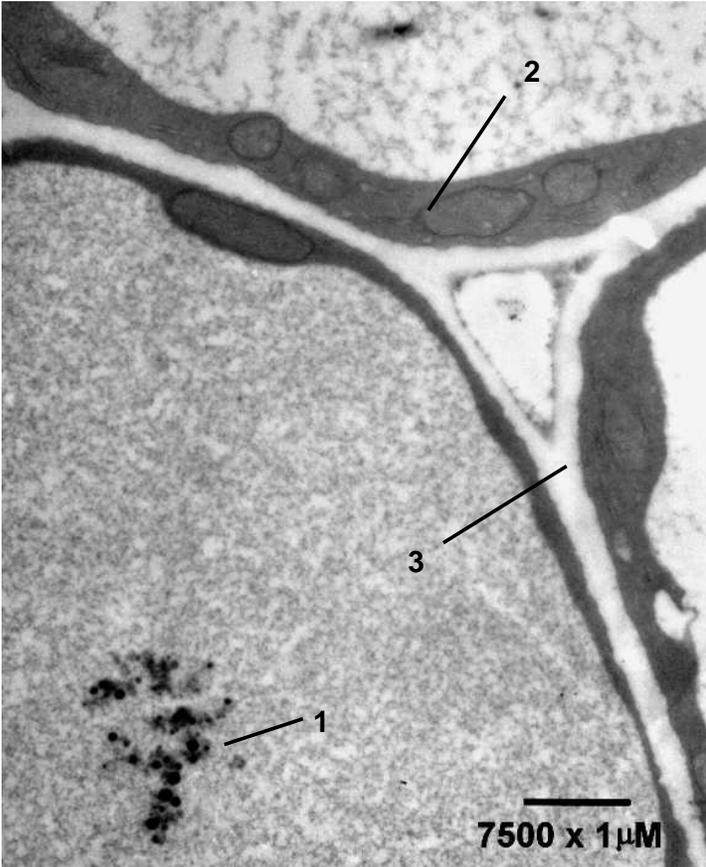
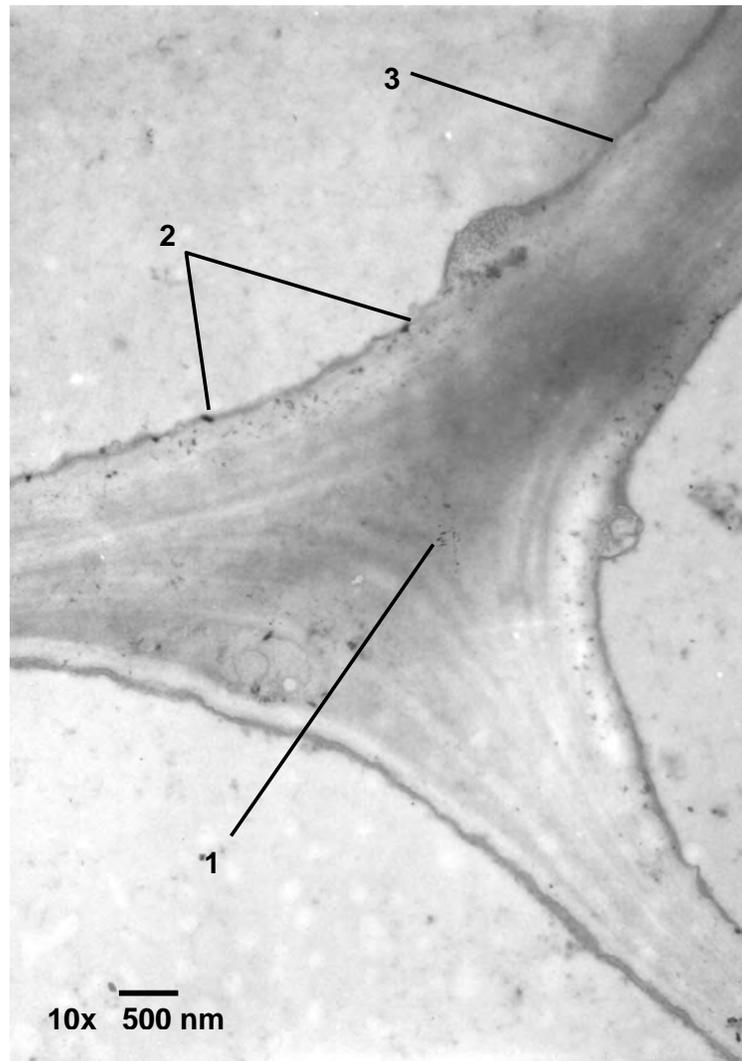


Plate 13. Transmission electron micrograph at 10,000 X magnification of an ultra-thin section of *Symphytum officinale* main root treated with 250 μ M Pb plus 125 μ M EDTA for seven days.

Key:

- 1 Plasmalemma
- 2 Pb particles
- 3 Middle lamella



5 CORRELATION BETWEEN TANNIN AND LEAD ACCUMULATION

Taking the Pb, polyphenol and TEM findings into consideration, it is tempting to conclude that there is an *in vivo* trend between pre-existing polyphenol and tannin levels with Pb accumulation; the root with the more polyphenols has more Pb-polyphenol chelating sites, and hence higher Pb levels. However, it is important to note that whilst this trend supports the idea that chelation of Pb to tannins may play an important role in Pb accumulation and tolerance, there is a possibility that other (perhaps more significant) factor(s) were responsible for the higher level of Pb found in the lateral roots. These include root volume in a hydroponic system, relative age of root tissue and other chelating compounds.

5.1 Other possible factors for lead accumulation trends

5.1.1 Root volume

At the whole plant level, the domination of lateral roots in the hydroponic system may lead to higher Pb accumulation in lateral roots than main roots. However if volume was a factor, one would have expected more Pb dilution and therefore lower Pb accumulation in the lateral root tissue (on a per gram dry weight basis). Dushenkov *et al.*, (1995) also showed that Pb accumulation in root tissue was not dependent on root mass: only the *rate* of removal was affected. From an opposing perspective, if the same volume of the roots was compared, the surface area of the lateral root is greater than the main root because of the smaller diameter of the roots (Figure 13). Since the surface area of the root (i.e. epidermal tissue) appears to be the prominent location for polyphenols, this would result in higher Pb levels in the lateral roots. Thus from this perspective, it is impossible to distinguish between the surface area and the polyphenol factor.

5.1.2 Root age

Young roots are often considered responsible for efficient nutrient uptake. They have root hairs and living epidermal cells that increase surface area and absorption of nutrients. Thus, this could be another reason why the younger lateral roots accumulated more Pb than the older main root (Maschner, 1995).

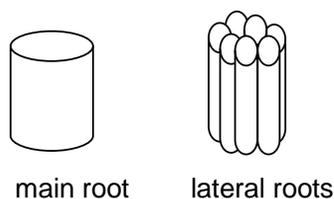


Figure 13. Surface area and volume of roots. When the same volume of root tissue is compared, the surface area of the lateral roots is greater than main roots.

6 IN VITRO LEAD CHELATION TO TANNINS

To add credence to the *in vivo* findings, *in vitro* evidence showing Pb chelation to isolated *S. officinale* tannin root extracts was required at two levels; lateral root tannins (higher-tannin) in comparison to main root tannins (lower-tannin) and increasing concentrations of tannins from just one root tissue.

It is important to stress that in this section of *in vitro* studies, it is not the source of tannins (lateral vs. main roots), but the relative levels of tannins in these root tissues and their ability to chelate Pb that are being compared – “do the roots with a higher level of tannins chelate more Pb?”

6.1 Lavid’s dialysis method (I)

6.1.1 Established protocol

In order to determine the metal chelation ability of complex polyphenols from *S. officinale* root extracts, our initial *in vitro* polyphenol chelation experiment was based on the Lavid *et al.*, (2001b) protocol (the only available plant-based protocol). Dialysis tubes, containing isolated high molecular weight polyphenol root extracts, were suspended in heavy metal solutions. In theory the higher the polyphenol concentration in the dialysis tube, the greater the amount of Pb trapped inside the tubes (due to polyphenol chelation). However, following this method for our experiment resulted in a contrary finding to our hydroponic studies (results section 4).

The polyphenol level in the lateral root (Figure 14) was 16% or 1.2 times higher (on an average fresh weight basis) than those in the main root extracts ($P \leq 0.05$) – much

smaller than compared with hydroponic experiments. This is possibly because tannins were extracted from fresh roots (following the Lavid dialysis method) and not freeze-dried roots. Despite the difference in extraction and tannin levels, the trend – higher polyphenol concentration in lateral roots – was the same. However unexpectedly, the dialysis tube with the least polyphenols (main root extracts) trapped four times more Pb ($P \leq 0.05$) than the tube with the most polyphenols (lateral root extracts) (Figure 15). An opposite trend was expected if high molecular weight polyphenols like tannins were involved in Pb accumulation. As discussed in results section 6.1.2, it is likely that interfering compounds, more of which were in the main root extracts, may be responsible for the observed trend.

6.1.2 Modified protocol

To make a better comparison between the Lavid dialysis method and the new *in vitro* chelation method designed below, the Lavid dialysis method was repeated so that the root tissue age (three months), method of extraction (using ball-mill ground freeze-dried tissues), and Pb concentration were approximately the same.

The polyphenol and tannin level in 3-month-old lateral roots were six times higher (on a dry weight basis) than in the main root tissue respectively ($P \leq 0.05$) (Figure 16). Dialysis tubes with main root polyphenol extracts accumulated on average 24% (or 1.2 times) more Pb than those from the lateral root, despite having six times less tannins than the lateral root (Figure 17). This was a smaller trend than the above experiment, possibly because there was less interference compound (fructan), as they were harder to extract from dry issue, or because of age, sample size, or natural variation between plants. Another possibility for this trend is that lateral roots contained more low-molecular weight polyphenols than main roots. Their accumulation and polymerisation outside the tube could have chelated to Pb ions, thus reducing the amount of Pb available for entry into the dialysis tube. This idea is supported by observations where, at the end of the experiment, the Pb solution surrounding dialysis tubes (containing lateral root polyphenols) were browner than those with main roots. However, it is likely to be only a small factor as most of the polyphenols (based on the intensity of the extracts) remained inside the tube (Plate 14). Despite this, the results were consistent with the first Lavid dialysis experiment (results section 6.1.1) in which the tannin level did not correlate to the Pb accumulation level.

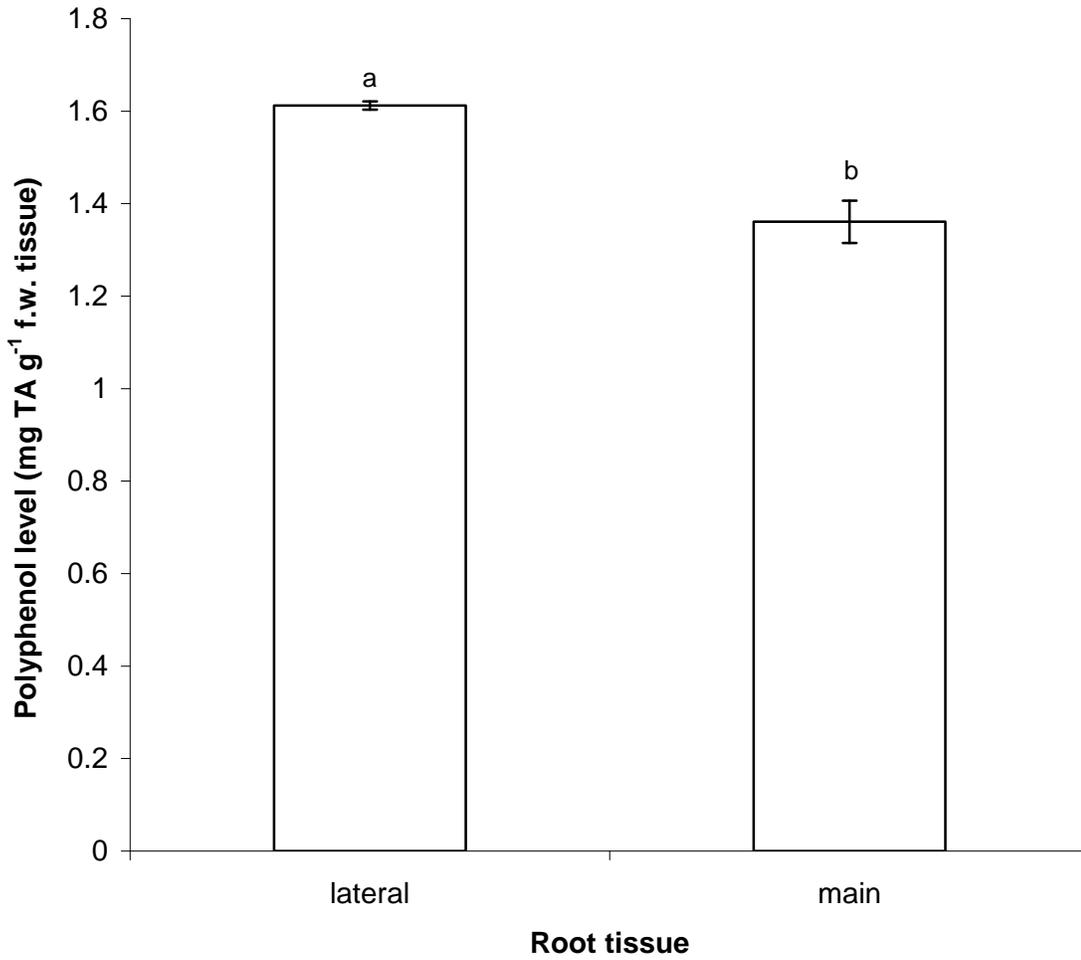


Figure 14. *In vitro* Pb-polyphenol chelation (Lavid's dialysis method): Polyphenol levels extracted from 10-month-old *Symphytum officinale* root tissues. Values are means \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). TA = tannic acid.

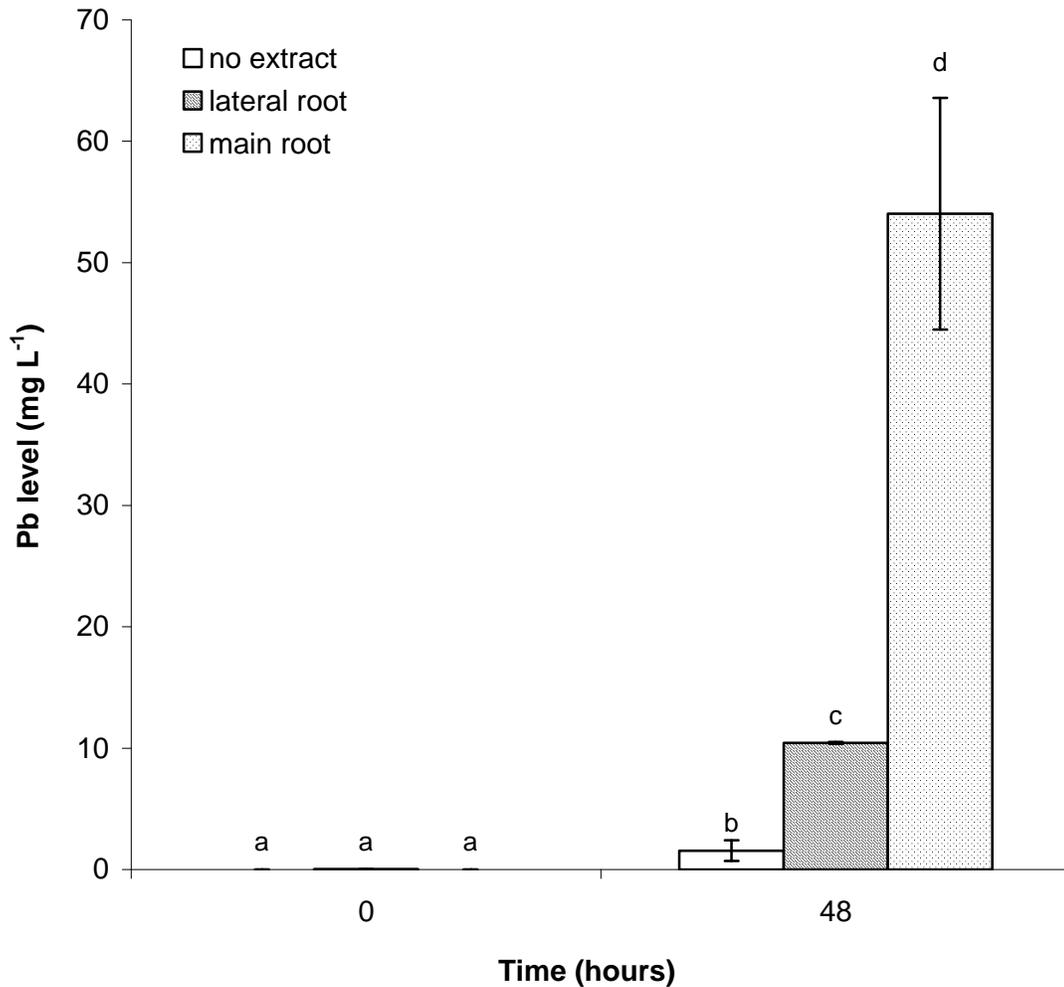


Figure 15. *In vitro* Pb-polyphenol chelation (Lavid's dialysis method): Level of Pb trapped in dialysis tubes (containing *Symphytum officinale* polyphenols) after 48 hours in 250 M Pb(NO₃)₂ salt solution at pH 4.5. Values are means ± S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$).

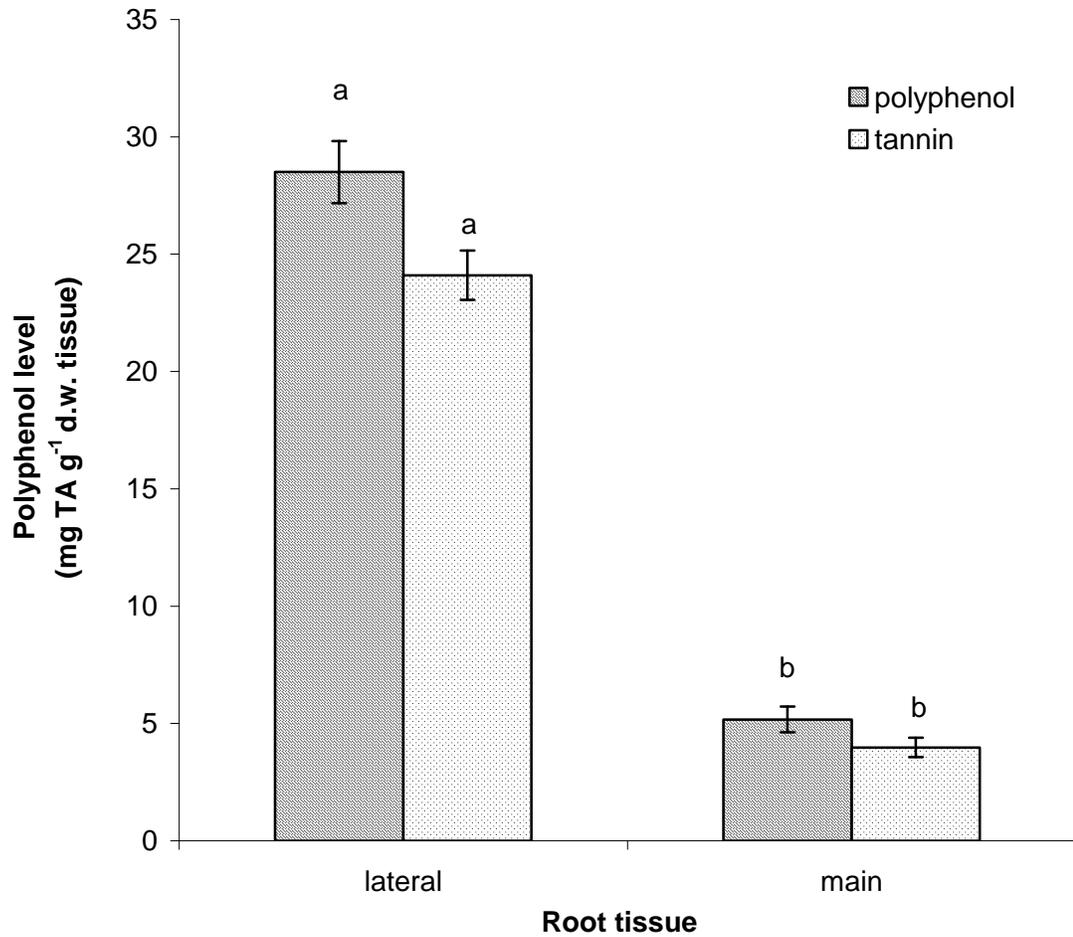


Figure 16. *In vitro* Pb-polyphenol chelation (modified Lavid's dialysis protocol): Polyphenol levels extracted from 3-4 month-old *Symphytum officinale* root tissues. Values are means \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). TA = tannic acid.

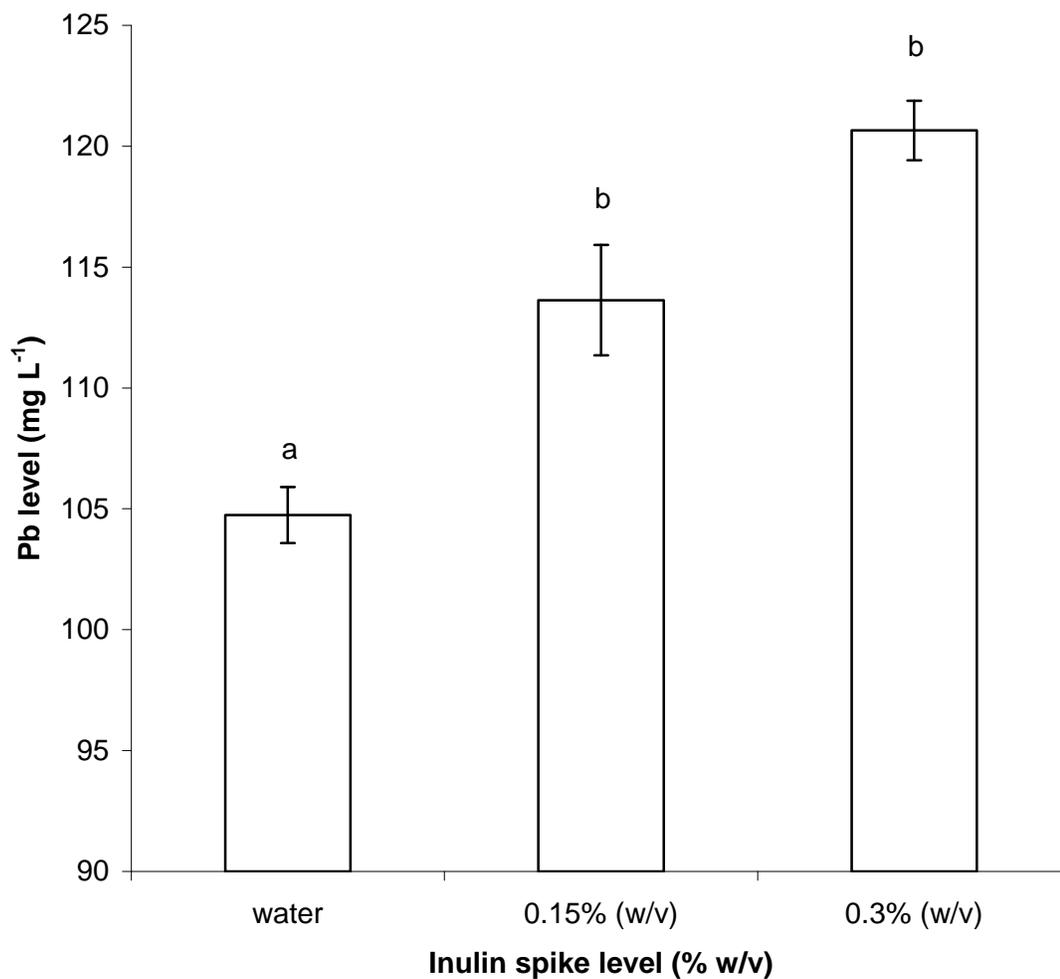


Figure 17. *In vitro* Pb-polyphenol chelation (modified Lavid's dialysis protocol): Level of Pb trapped in dialysis tubes (containing *Symphytum officinale* polyphenols) after 48 hours in 250 mg L⁻¹ Pb(NO₃)₂ salt solution at pH 4.5. Values are means ± S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$).

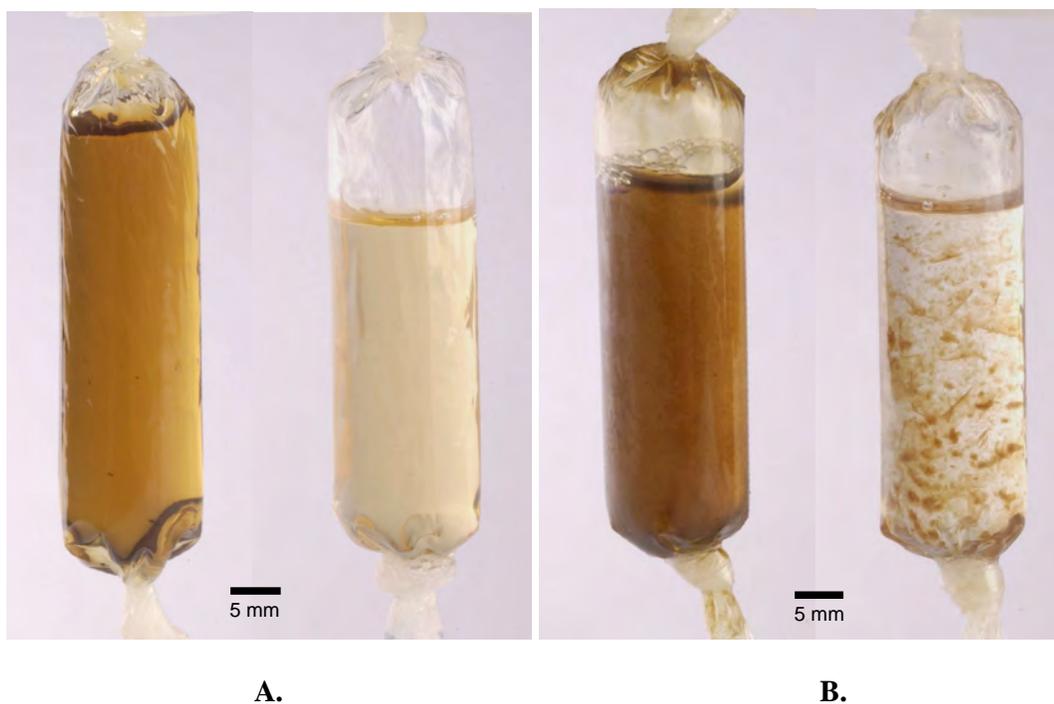


Plate 14. *In vitro* Pb-polyphenol chelation (modified Lavid's dialysis protocol).

Dialysis tubes containing high molecular weight polyphenols extracted from 3-4 month-old *Symphytum officinale* lateral root (left, darker brown) and main root (right, lighter brown) after 48 hours in:

A. Control (no Pb in external solution)

B. Pb treatment ($250 \text{ mg L}^{-1} \text{ Pb}(\text{NO}_3)_2$ salt solution), pH 4.5

6.1.3 Fructan interference (I)

The Lavid dialysis method above may be subject to interferences from other high molecular weight Pb chelating compound(s) in the root (especially main root) polyphenol extracts; the most obvious being fructans.

Fructans are fructose-based polysaccharides (Koster, 1996; Ritsema and Smeekeens, 2003) with numerous potential Pb-chelating hydroxyl groups. Quantitatively fructans are typically between 10 to < 29% d.w. (AbouMandour *et al.*, 1987), which is up to five times higher than tannin levels (4-6% d.w) in *S. officinale* roots. However, their location *in planta* suggests that their role in Pb accumulation and tolerance may be smaller compared to polyphenols. Unlike polyphenols that are in the cell wall and vacuole of most cells, fructans are reserve polysaccharides, which are found in vacuoles of only photosynthetic and storage cells (Cairns, 2003). During the polyphenol extraction process fructans may be released from vacuoles of these specific cells, allowing fructans to be more readily available for Pb chelation *in vitro* than *in vivo*. Thus, the content of the dialysis tubes could reflect the total concentration of high molecular weight Pb-chelating compounds extracted and trapped in the dialysis tube (e.g. fructans and complex polyphenols), rather than a particular type of chelating compound (i.e. complex polyphenols) and their true level of availability for Pb chelation *in planta*.

In terms of the two root tissues, it is suspected that main root extracts may have a higher concentration of these interfering fructans (but lower tannin level) than lateral root extracts. If so, this would explain why a greater level of Pb chelation was observed in the lower-tannin main root polyphenol extracts. Observations supporting the presence of fructans, especially in main root tissues, were:

- Main root tissues were much thicker in appearance than lateral roots, indicating their role as polysaccharide storage roots.
- A gelatinous layer (possibly indicative of polysaccharides) was only observed along the dialysis membrane containing the main root extracts.
- Pb chelation within the dialysis tube was noticeably different: Pb chelation to lateral root polyphenols appeared to be colloidal, whereas Pb chelation to main root polyphenols appeared as sheets (Plate 14).
- Using the HBH glucose assay and KI/I₂ spot plate test (data not shown), both main and lateral root polyphenol extracts gave a negative test for starch and

other glucose-based polysaccharides. Starch was not detected because these compounds were removed by centrifugation.

6.1.4 Fructan interference (II): Direct experimental evidence

To add credence to the possibility of fructan interference above (6.1.2), dialysis bags containing purified chicory inulin (Sigma) was tested. Inulin is a fructan made from a linear chain of fructose units bound to a glucose starter molecule (Ritsema and Smeekens, 2003). It was used as a model fructan in this experiment as it was the most common and only purified fructan available from our suppliers. Inulin levels were set at 0.15% and 0.30% (w/v) to represent the level of fructans expected if 50-100% of the known *S. officinale* root fructan content was released into the polyphenol extract. At this inulin level, the Pb level trapped in the dialysis bag increased by an average of 8-15% ($P \leq 0.05$) compared to the control (water). There was no statistical difference between the two levels of inulin (Figure 18). The elevated levels of Pb trapped in the dialysis tube containing inulin, indicates that fructans is an interfering factor in the Lavid dialysis method.

However, if the Pb accumulation levels (6.1.1) were corrected against this level of interference, the higher tannin lateral roots would still have less Pb trapped in the tube than the lower tannin main roots (in this case by 8-16%). This is still not consistent with the hydroponic trends. What if higher concentrations of inulin were used in the dialysis tube? It is possible that (similar to section 6.4.1. with tannins) a higher threshold concentration of inulin is required before significantly more Pb can be trapped in the dialysis tube. However, if this type of inulin were the main fructan in *S. officinale*, such concentrations would exceed the known amount of fructan in *S. officinale* (as tested in this study). Thus higher concentrations of inulin are unlikely to produce a meaningful result. It seems more likely that other unidentified compounds or different fructans (in size and structure) in *S. officinale* may be involved. Inulin from chicory is the simplest linear fructan, made of 35 units of fructose units linked in a β (1-2) manner. Fructans, with a chain length of a few hundred fructose units long linked in β (1-2) and/or β (2-6) manner also exist (Ritsema and Smeekens, 2003; Koster, 1996). For example, inulin from globe artichoke is made of 200 units of fructose (Velisek and Cejpek, 2005). The types of fructans in *S. officinale* is unknown, but it is likely that these larger and structurally different fructans are present in *S. officinale* roots and may better reflect the

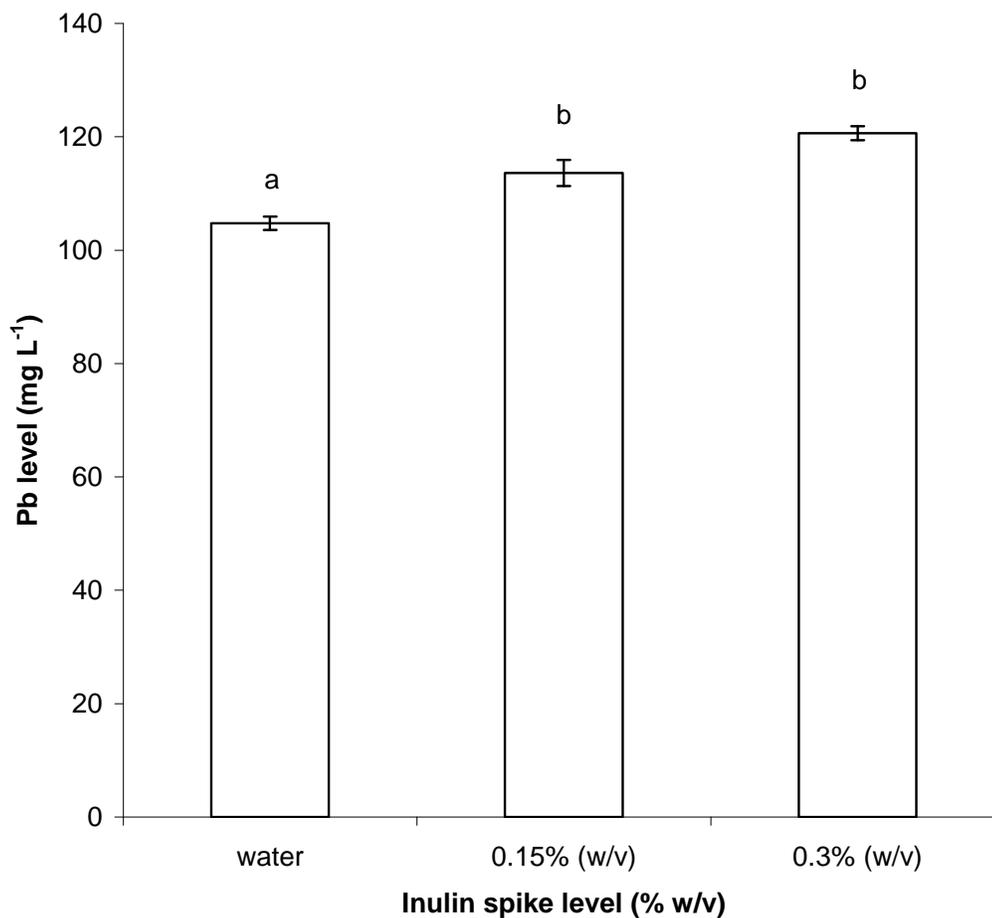


Figure 18. *In vitro* Pb-inulin chelation (Lavid's dialysis protocol): Level of Pb trapped in dialysis tubes (containing purified chicory inulin) after 48 hours in 250 mg L⁻¹ Pb(NO₃)₂ salt solution at pH 4.5. Values are means ± S.E. of three replicates. Values not sharing the same letter are significantly different (P ≤ 0.05).

degree of interference observed in the Lavid dialysis results above. The lack of viscosity observed in the 0.15%-0.30% (w/v) chicory inulin solution when compared to the highly viscous main root polyphenol extracts supports this possibility. A smaller difference may be also due to the mg tannin:mg Pb ratio, as explained in 6.2.2 below.

6.2 New approach: Lead chelation to immobilised tannins

6.2.1 Approach to experimental design

To minimise or avoid the possible interferences, particularly from the main root, alternative approaches were considered. These included removing polysaccharides after extraction and isolating tannins from the polyphenol extracts. Firstly, enzymatic treatment was considered as this method has been used to remove polysaccharide contaminants from DNA extracts (Barnwell *et al.*, 1998). However, enzymatic treatment of polyphenol extracts to degrade co-extracted polysaccharides was not deemed possible because the methanol and polyphenol content of the extracts would inhibit the polysaccharide-degrading enzymes. Secondly, investing in the cost of endo- and exo-inulinase enzymes to degrade polysaccharides could not be justified (over 150 Euros), especially if it was the incorrect type of fructans or was not the sole interference compound. The use of purified *S. officinale* tannin extracts from each of the roots in the Lavid dialysis method may be plausible if the purification process was not a time-consuming process.

The best approach was to try to isolate tannins from the polyphenol extracts and the interfering factor. This idea came from a similar problem encountered with DNA extraction from starchy plant tissue (Wulff *et al.*, 2002). In order to isolate DNA, they used a combination of ethanol and salt solutions to keep starch in the supernatant whilst precipitating DNA. In this experiment, possible fructan interferences were kept in solution by keeping the polyphenol extracts above 4°C, whilst the tannins were isolated and separated. Insoluble PVPP is a synthetic polyproline polymer that binds to tannins via numerous hydrogen bonds, between the carbonyl groups on PVPP (C=O) and polyphenolic hydrogen of the hydroxyl (-OH) functional groups. It is routinely used to bind tannins in the Folin-Ciocalteu assay for tannins and during enzyme extractions to reduce enzyme inhibition by polyphenols. Tannins bound to PVPP, called PVPP-immobilised tannins, would allow separation from interfering compounds. The only

question remaining was if the immobilised tannin still had enough OH functional groups to bind Pb after being bound to PVPP; the preliminary investigation using 50 mg L⁻¹ Pb(NO₃)₂ showed that up to 86% of the available Pb in the solution chelated to the PVPP-immobilised tannins from the root extracts. Clearly, this Pb chelation ability was sufficient for further studies.

6.2.2 *In vitro* lead chelation by lateral and main root tannins

Figure 19 shows the extractable polyphenol and tannin level in 3-month-old *S. officinale* roots; on a dry weight basis, lateral root tissue contained 8 times more polyphenols and 13 times more tannins than main root tissue. Based on the tannin assay results and the volume of extract used per tube, the amount of immobilised tannin in each tube (expressed as mg tannic acid-TA) was calculated to be on average 2.618 mg TA and 0.1907 mg TA for the lateral and main root polyphenol extracts respectively.

In contrast to the Lavid dialysis method, the trend presented here correlated well with the hydroponic experiments; the higher tannin lateral roots removed more Pb than the lower-tannin main roots. At 50, 250 and 500 mg L⁻¹ Pb(NO₃)₂ concentrations, an average of 1.1, 3.0 and 3.5 times more Pb was *removed* from the upper layer Pb solution respectively by chelation to lateral root immobilised tannins (Figure 20). The level of Pb *gained* by chelation to the PVPP-immobilised tannin pellet (lower layer) was also higher in the lateral root than the main root samples (Table 4). However, the lower layer data were more variable (i.e. did not pass F (max) test) because two different matrices were involved; one solid, one liquid. Hence only the upper layer was assessed in subsequent experiments.

In terms of percentages, the highest percentage of Pb chelated occurred at the 50 mg L⁻¹ Pb(NO₃)₂ concentration. At this concentration 86% (on average) of the total Pb (element) chelated to PVPP-immobilised lateral root tannins.

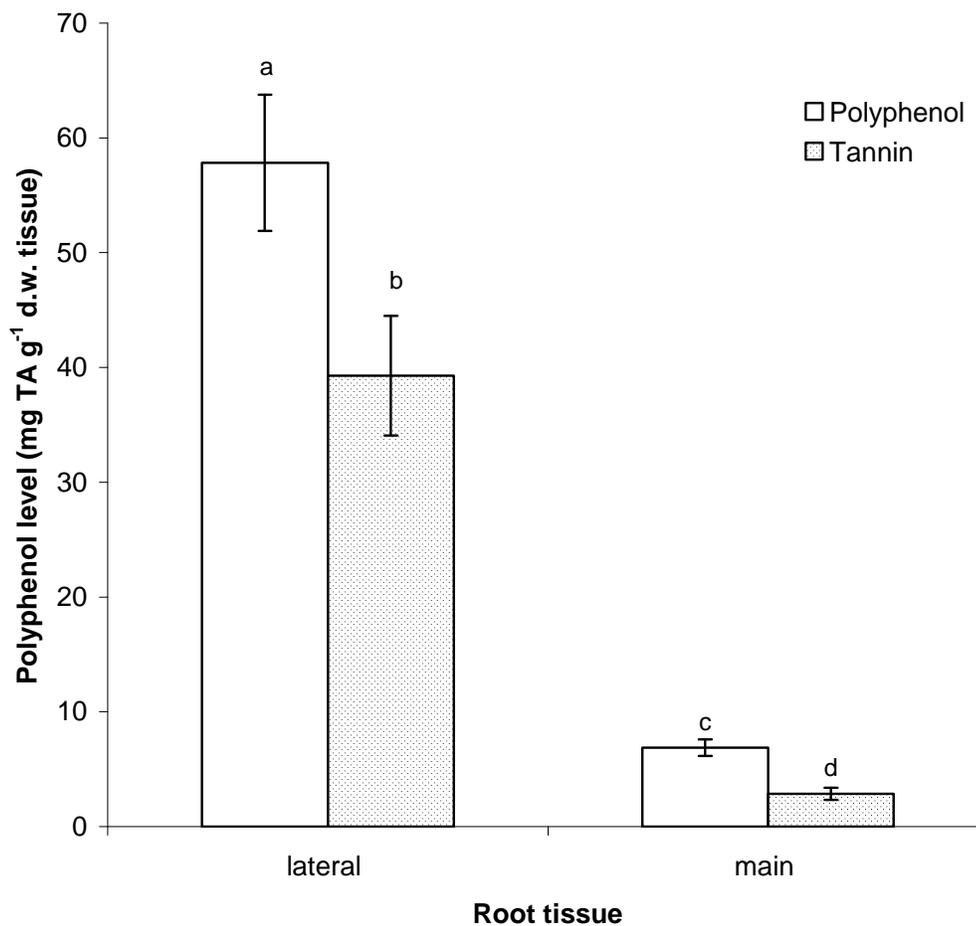


Figure 19. *In vitro* Pb-tannin chelation (immobilised tannin method): Polyphenol and tannin levels extracted from 3-month-old *Symphytum officinale* root tissue. Values are means \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). TA = tannic acid.

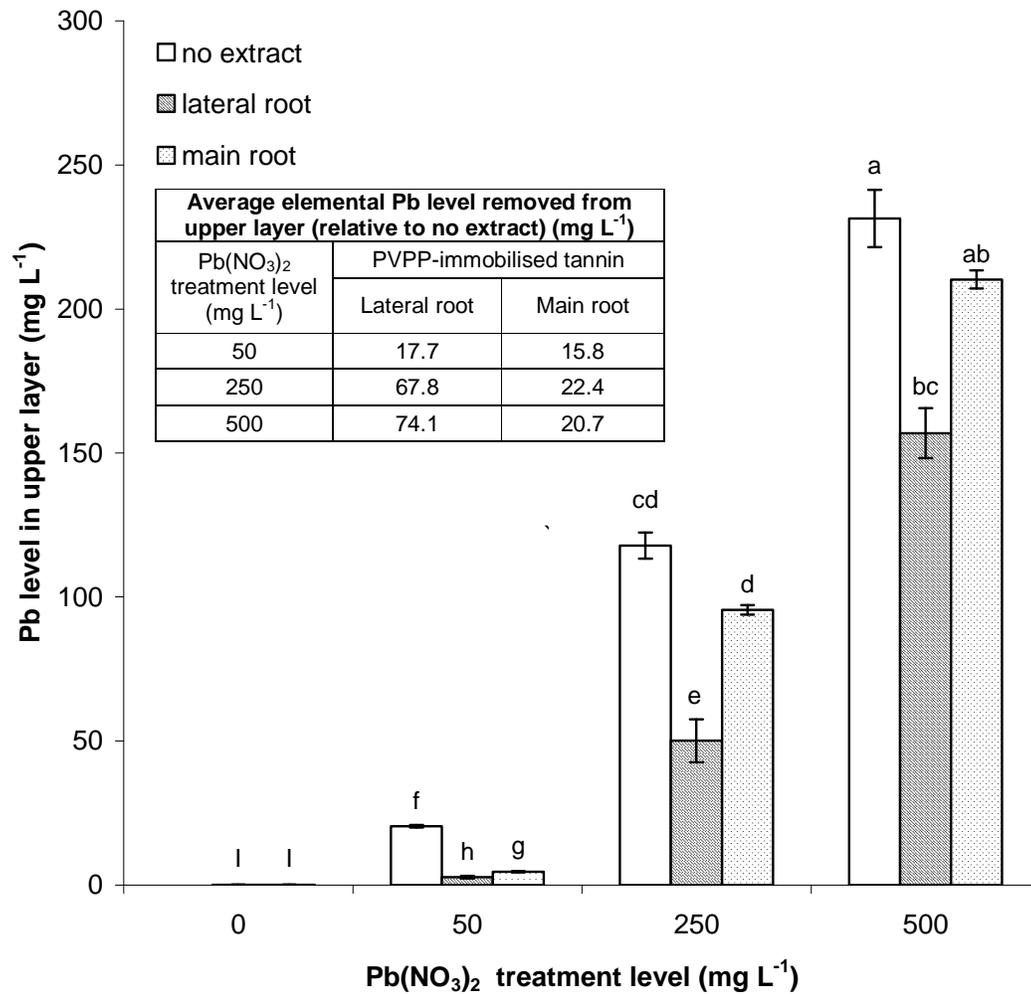


Figure 20. *In vitro* Pb-tannin chelation (immobilised tannin method): Level of Pb remaining in the upper layer after treatment with PVPP, with or without immobilised tannins from 3-month-old *Symphytum officinale* lateral root or main root tissues, after four hours. Solutions were all at pH 4.5. Values are means \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$).

NB: Pb treatment level (x-axis) refers to mg L⁻¹ of Pb(NO₃)₂. The Pb level in the upper layer refers to mean level of Pb element (mg L⁻¹) measured by AAS.

Table 4. *In vitro* Pb-tannin chelation (immobilised tannin method): Level of Pb in the lower layer after treatment with PVPP, with or without immobilised tannins from 3-month-old *Symphytum officinale* lateral root or main root tissues, after four hours. Values are means \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). Treatments with no letters are data that did not pass F (max) test and could not be compared to other treatments.

Pb(NO₃)₂ treatment level (mg L⁻¹)	PVPP-immobilised tannin	Pb level in lower layer (mg L⁻¹)	Level of significance
0	No extract	Not tested	Not tested
	Lateral root	0.087 \pm 0.072	A
	Main root	0.090 \pm 0.090	A
50	No extract	24.263 \pm 1.171	-
	Lateral root	31.103 \pm 11.970	-
	Main root	28.750 \pm 9.550	-
250	No extract	135.710 \pm 0.794	C
	Lateral root	195.333 \pm 7.401	A
	Main root	157.333 \pm 0.0761	B
500	No extract	275.200 \pm 4.203	B
	Lateral root	335.120 \pm 13.096	A
	Main root	283.947 \pm 18.651	AB

NB: Pb treatment level refers to mg L⁻¹ of Pb(NO₃)₂. The Pb level in the lower layer refers to mean level of Pb element (mg L⁻¹) measured by AAS.

In theory, the **mg Pb:mg TA ratio** of the lateral and main roots samples (from Figure 20) should be equal if tannins are involved in Pb accumulation and tolerance. However the mg Pb:mg tannin ratio calculated was far from equal; the lower tannin main root chelated 12.6, 13.6 and 4.9 times more Pb (at the respective concentrations) than the higher tannin lateral root samples (Table 5). This suggests that tannin levels are not responsible for the *in vivo* hydroponic results. But, as explained below and with the aid of Figure 21A and B and Table 6 there are two factors which make the Pb:tannin ratio outcome invalid.

Firstly, similar to enzyme activity calculations, Pb-tannin chelation should be linear for ratio-based calculations. However, the Pb-tannin chelation was not linear in both roots; when calculated under such circumstances, the Pb:tannin ratio is relatively higher in the main root than the lateral roots (Table 5).

Secondly, the Pb:tannin ratio calculates the amount of Pb bound to *each* tannin. For this calculation to be valid, tannins must exist individually, unbound to other compounds such as sugars or other polyphenols. Consequently, all the Pb-binding groups on tannins would be available for direct Pb chelation, making the Pb:tannin ratio the same in the two root tissues (Figure 21A and Table 6). However, unless tannins are at low concentrations, most tannins in reality are unlikely to all exist individually. Tannins are likely to be bound to other tannins (defined by the degree of polymerisation) or other compounds in the crude extracts. When these Pb-chelating groups are unavailable for direct Pb chelation, the amount of Pb bound to *each* tannin (efficiency) decreases. With increasing tannin levels this effect is multiplied as more polymerisation occurs. As a result, a decreasing trend in the Pb:tannin ratio is observed with increasing tannin levels (Table 5).

Thus comparing the Pb:tannin ratio of the lateral and main root data from (Table 5) is misleading as it does not accurately reflect the actual amount of *individual* tannins that bind Pb. Rather it reflects a *group* of tannins that can bind Pb.

Instead, under such circumstances a general correlation – i.e. “do the tissues with higher tannin levels bind more Pb?” – should be used to interpret the results, as it is *this factor* that governs the overall concentration of Pb bound by tannins, not the ratio. Main root tannins may bind more Pb *per* tannin because the lower tannin level in the extract results in less polymerisation and more efficient Pb-binding tannins. However, it does not have enough of these to override the high level of less efficient Pb-binding tannins (due to polymerisation at high tannin concentrations) from the lateral root.

Table 5. Incorrect usage of Pb:tannin ratio of data from *in vitro* Pb-tannin chelation experiment using tannins from 3-month-old *Symphytum officinale* lateral root or main root tissues immobilised to PVPP. Values are means \pm S.E. of three replicates.

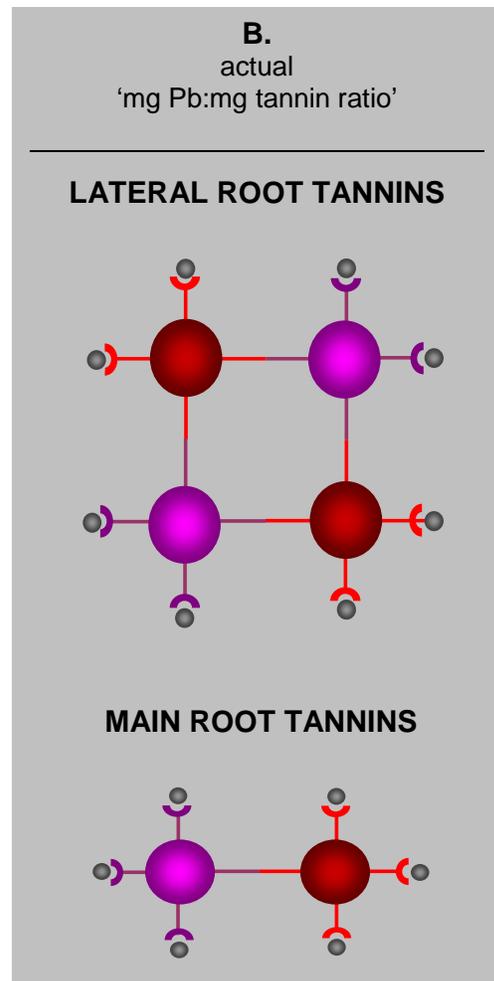
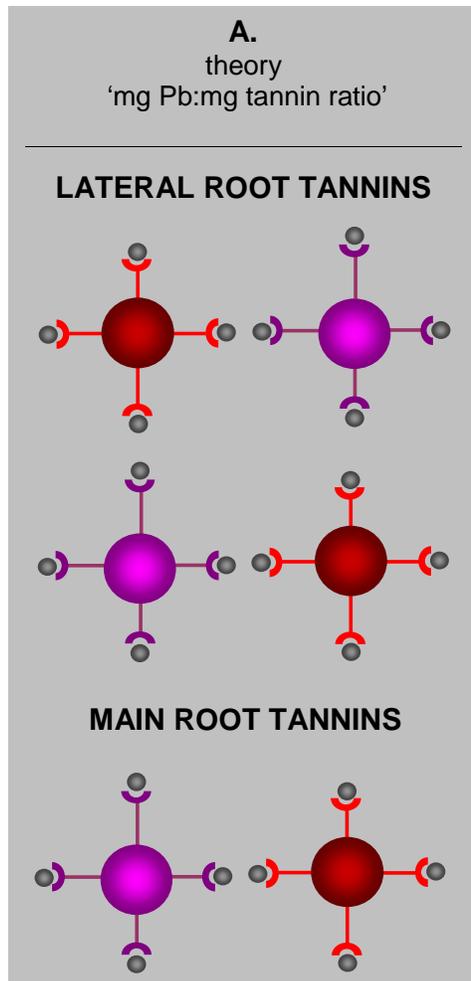
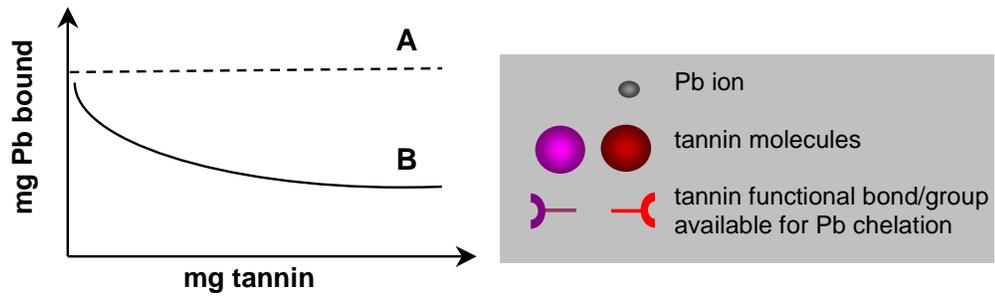
Pb(NO ₃) ₂ level (mg L ⁻¹)	Pb:tannin ratio (mg Pb:mg tannin)		MR ratio > LR ratio?
	lateral root tannins	main root tannins	Y= yes, N= no
50	0.033 \pm 0.004	0.422 \pm 0.032	Y, P<0.05
250	0.033 \pm 0.004	0.451 \pm 0.004	Y, P<0.05
500	0.129 \pm 0.031	0.626 \pm 0.122	Y, P<0.05

Figure 21 (opposite page). *In vitro* Pb-tannin chelation: Ratio of Pb to tannin.

- A. Theory involves individual, unpolymerised tannin molecules (equal Pb:tannin ratio).
- B. Actual experimental data takes account of tannin polymerisation. Generally, lateral and main tannins have the same degree of polymerisation. The root with more tannins has a lower Pb:tannin ratio but still has a higher overall Pb chelation level.

Table 6. Schematic of data and ratios from Figure 21A and B.

Schematic	Number of Pb ions bound to tannins (Pb:tannin ratio)	Number of tannins	Pb:tannin ratio
A. lateral root tannins	16	4	4:1
A. main root tannins	8	2	4:1
B. lateral root tannins	8	4	2:1
B. main root tannins	6	2	3:1



Using this general correlation, the immobilised lateral root tannins (with higher-tannin levels) did bind more Pb than the lower tannin main roots, suggesting that tannins are involved in the chelation-based tolerance mechanism, Pb accumulation and the trends observed in the *in vivo* hydroponic experiment.

6.2.3 Linear *in vitro* chelation

Using a micro-scale version of the experiment above (6.2.2), increasing concentrations of immobilised tannins were prepared from only the lateral root polyphenol extracts. This was to provide evidence of tannin involvement in the observed Pb chelation (i.e. removal from solution) trends, rather than factors related to the differences in root tissue e.g. interference compound in main roots compared to lateral roots. Additionally, the Pb:tannin ratio can be applied here because the Pb-chelation trends were linear.

The same trend as 6.2.2 was observed, except linearity in Pb chelation was observed; the higher the tannin concentration, the greater the level of Pb removal from solution ($P \leq 0.05$). In this case, 0, 6, 10, 24 and 60% of the initial Pb in 50 mg L^{-1} $\text{Pb}(\text{NO}_3)_2$ (23 mg L^{-1} Pb on average recovered by AAS) was removed by increasing concentrations of the lateral root immobilised tannins (0, 12.5, 25, 50, and 100% (v/v) of the initial extract containing an average of 0, 0.028, 0.058, 0.113 and 0.225 mg TA respectively) (Figure 22).

Whilst not significantly different ($P \geq 0.05$), a slight increase in the Pb:tannin ratio, indicative of a small amount of interference from the crude tannin extracts, was also apparent (Figure 23). However, the degree of error due to the low tannin concentrations used (to achieve linearity in Pb chelation) or small amount of fructan interference (see below) may have also contributed to this apparent trend. Overall under these linear Pb-chelation trends, this ratio-based analysis further supports the idea that tannins are involved in the chelation-based tolerance mechanism and Pb accumulation in *S. officinale*.

6.3 Lead chelation to immobilised tannins: general points

In this chelation method, there are two important points relating to the functional groups involved in Pb-tannin chelation. Firstly, whilst the immobilisation of tannins to PVPP would remove some chelating functional groups, clearly enough remained to allow a

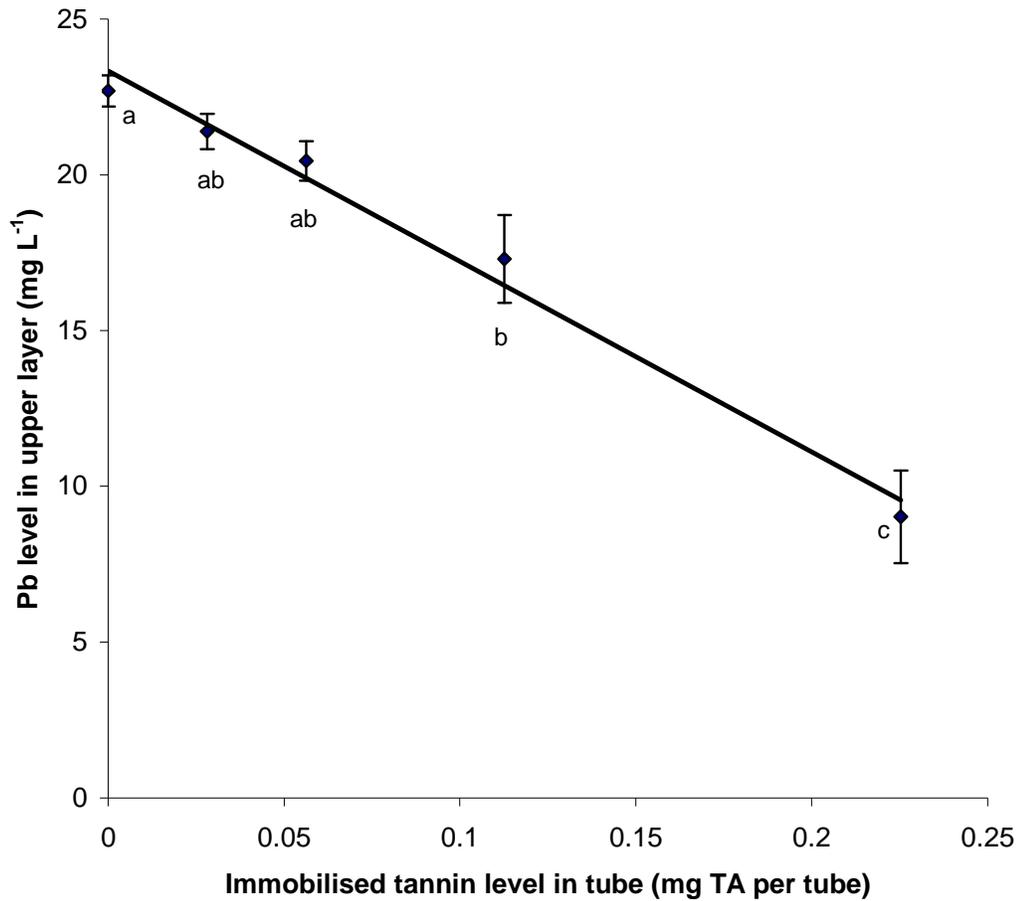


Figure 22. *In vitro* Pb-tannin chelation (immobilised tannin method): Level of Pb removed from the upper layer of 50 mg L⁻¹ Pb(NO₃)₂ salt solution (pH 4.5) after treatment with increasing concentrations of 3-month-old *Symphytum officinale* lateral root tannins immobilised to PVPP for two hours. Values are means ± S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$).

NB: Note that mg L⁻¹ Pb refers to elemental Pb level (mg L⁻¹) in upper layer measured by AAS level.

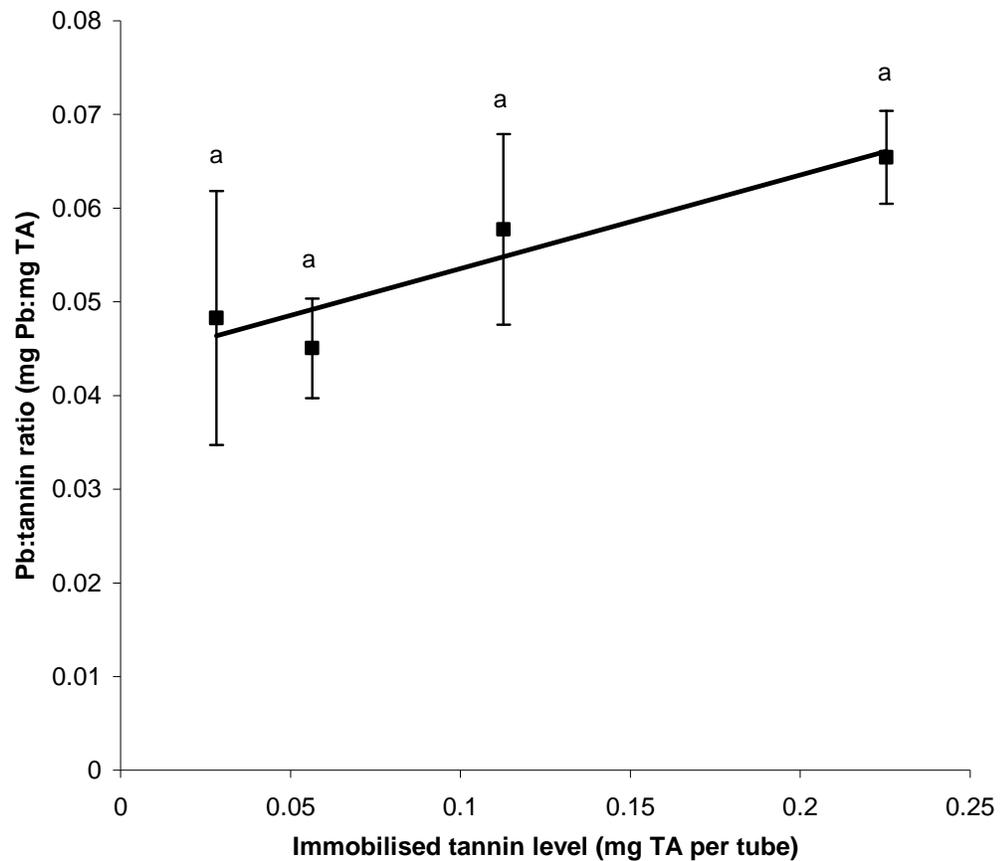


Figure 23. Pb:tannin ratio trend from *in vitro* Pb chelation experiment using tannins from 3-month-old *Symphytum officinale* lateral root immobilised to PVPP (Figure 22). Values are means \pm S.E. of three replicates. Values sharing the same letter are not significantly different ($P \geq 0.05$). TA = tannic acid.

significant difference in Pb chelation between high tannin-lateral and low tannin-main roots to be observed. Secondly, some Pb could chelate to tannins *indirectly*. Since a crude extract was used, it is possible that Pb chelation to tannins occurred via a bridge with other compounds such as iron oxides (indirect Pb-tannin chelation). As lateral roots had higher tannin levels than main roots, the amount of bridging in the lateral root immobilised tannins would be greater than the main root. However, despite this additional variable, the over-riding point is that for Pb to be removed from the solution (via direct or indirect chelation), immobilised tannins must be involved in the first instance.

6.4 Validating immobilised tannin method: purified tannins

6.4.1 *In vitro* lead chelation: purified tannic acid

Using purified tannic acid (tannins) from Sigma and the micro-scale *in vitro* chelation method, this section demonstrates that PVPP-immobilised tannins have the ability to *directly* chelate and remove Pb from solution.

A range finding experiment was conducted to determine: (i) the Pb-binding capacity of purified tannin immobilised to PVPP, and (ii) how these levels reflect the trends observed with the *S. officinale* Pb-tannin chelation studies.

Initially, a purified tannin concentration between 0-0.4 mg mL⁻¹ was immobilised to PVPP to reflect the level of *S. officinale* lateral root tannins immobilised to PVPP (i.e. between about 0.2 to 0.4 mg tannic acid). Since immobilised *S. officinale* lateral root tannins (results section 6.2) were already capable of removing up to 86% the Pb in a solution of 50 mg L⁻¹ Pb(NO₃)₂, the Pb concentration used here needed to be higher than this to ensure enough Pb remained in the solution for any additional Pb chelation by fructan spikes (see 6.4.2 below). Hence, the immobilised purified tannins were initially exposed to 100 mg L⁻¹ Pb(NO₃)₂. The maximum immobilised tannin concentration in this range, 0.4 mg mL⁻¹, removed only 7% of the Pb (element) found in 100 mg L⁻¹ solution of Pb(NO₃)₂. When the immobilised tannin concentration range was multiplied 10 times to 0-4 mg mL⁻¹, only 34% of the Pb (element) found in 100 mg L⁻¹ Pb(NO₃)₂ solution was removed. When the tannin concentration range was further increased (up to 20 mg mL⁻¹), the highest level of Pb removed from the 100 mg L⁻¹

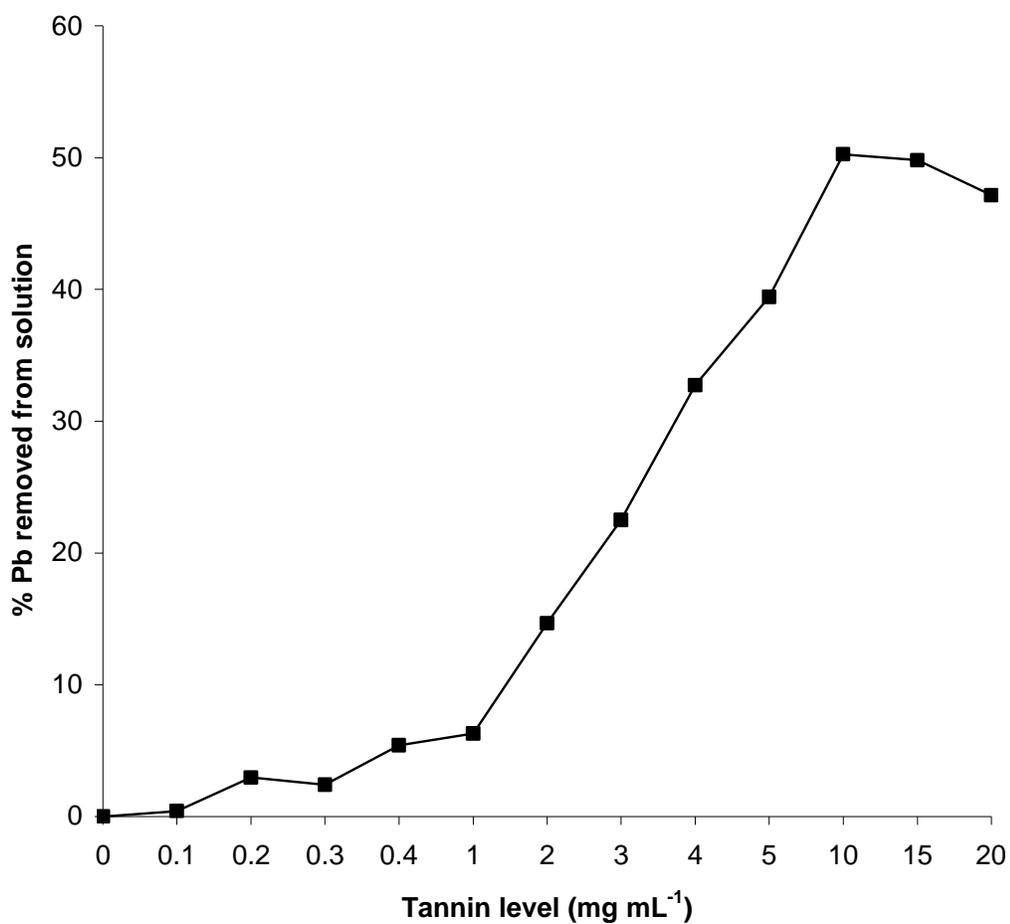


Figure 24. *In vitro* Pb-tannin chelation (immobilised tannin method): Percentage of Pb (element) in the upper layer of 100 mg L⁻¹ Pb(NO₃)₂ salt solution (pH 4.5) removed by chelation to increasing concentrations of purified tannic acid (tannin) immobilised to PVPP. Values are from one replicate.

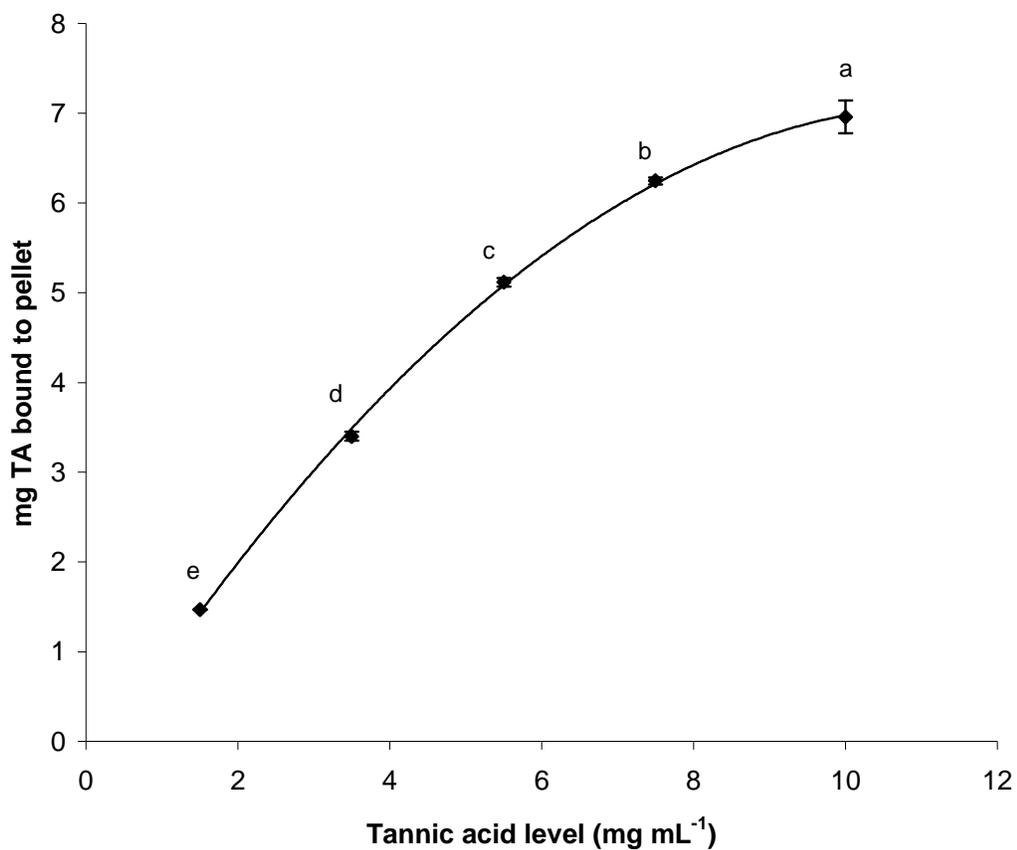


Figure 25. *In vitro* Pb-tannin chelation (immobilised tannin method): Level of purified tannic acid (tannin) bound to 0.02g of PVPP pellet. Values are means \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). TA = tannic acid.

Pb(NO₃)₂ solution was achieved (Figure 24). At 10 mg mL⁻¹ tannic acid, where an average of 7 mg tannic acid was immobilised to the PVPP pellet, 50% of the Pb (element) in the solution was removed (Figure 25).

There are three key points from these findings. Firstly, the percentage of Pb removed was only five times higher, despite the ten times higher tannin concentration in the pellet. This is evidence that the mg Pb:mg tannin ratio is inaccurate. Secondly, the percentage Pb removed from solution leveled off at 10-15 mg mL⁻¹ tannic acid. Clearly saturation and chelation kinetics are involved in the capacity of PVPP-immobilised tannins to bind Pb. Finally, the level of Pb removed from solution was much less than expected, when compared to the level of Pb lost with the *S. officinale* tannins bound to the PVPP pellet.

To match the Pb-binding capacity of PVPP-immobilised *S. officinale* root tannins, the Pb(NO₃)₂ concentration had to be lowered and the concentration range of purified tannin set to between 0-10 mg mL⁻¹ tannic acid. At 10 mg mL⁻¹ tannic acid (where 7 mg tannic acid was immobilised to the PVPP), 70% of Pb (element) in the 50 mg L⁻¹ Pb(NO₃)₂ (P≤0.05) was removed (Figure 26). *S. officinale* root tannins immobilised to PVPP removed a similar amount of Pb but using only between 0.2-0.4 mg mL⁻¹ tannic acid. The steep increase in the level of purified tannic acid (about 25 times) needed to remove the equivalent amount of Pb from solution may be in part due to: (i) the different plant origins of the tannin involved (*S. officinale* vs. purified Sigma tannin), (ii) use of purified and crude extracts, and (iii) oxidised tannins extracted from the *S. officinale* root surface; this would mean that 0.4 mg mL⁻¹ of crude *S. officinale* tannin is actually an under-estimation of the actual amount of tannin in the pellet. It is also worth noting that the Pb:tannin ratio also exponentially decreased (Figure 27), supporting section 6.2.2 explanations for not endorsing the use of this ratio when Pb chelation is not-linear.

6.4.2 *In vitro* lead chelation: fructan-spiked purified tannic acid

To determine if the fructans interfere (i.e. enhance Pb removal from solution) with this new method, purified tannic acid was spiked with purified chicory inulin. The level of tannins and fructan found in *S. officinale* roots is 4-6% (d.w.) and 10 to < 29% (d.w.) respectively: this is an approximate ratio of 1:5. Thus, the fructan spike levels were set at two, three and six times the level of tannic acid bound to PVPP to reflect the range of

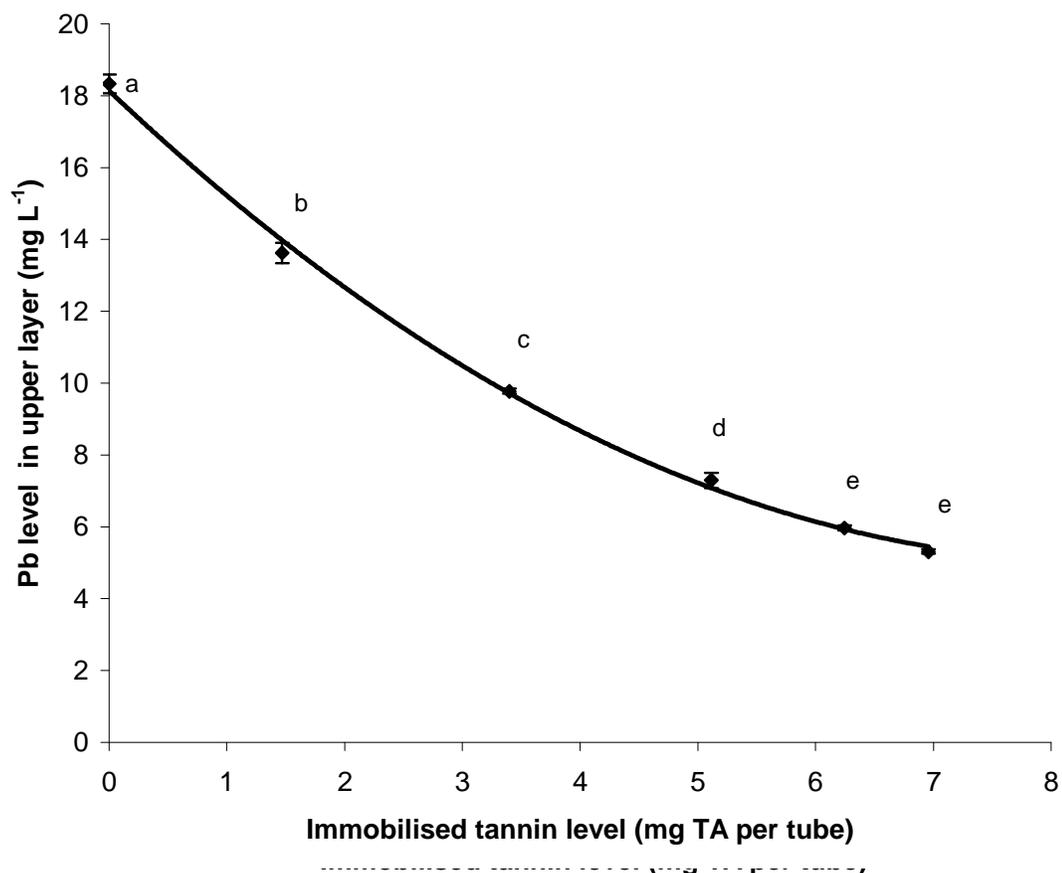


Figure 26. *In vitro* Pb-tannin chelation (immobilised tannin method): Level of Pb remaining in the upper layer of 50 mg L⁻¹ Pb(NO₃)₂ salt solution (pH 4.5) after two hours of treatment with increasing concentrations of purified tannins immobilised to PVPP. Values are means ± S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). TA = tannic acid.

NB: The Pb level in the upper layer refers to mean level of Pb element (mg L⁻¹) measured by AAS.

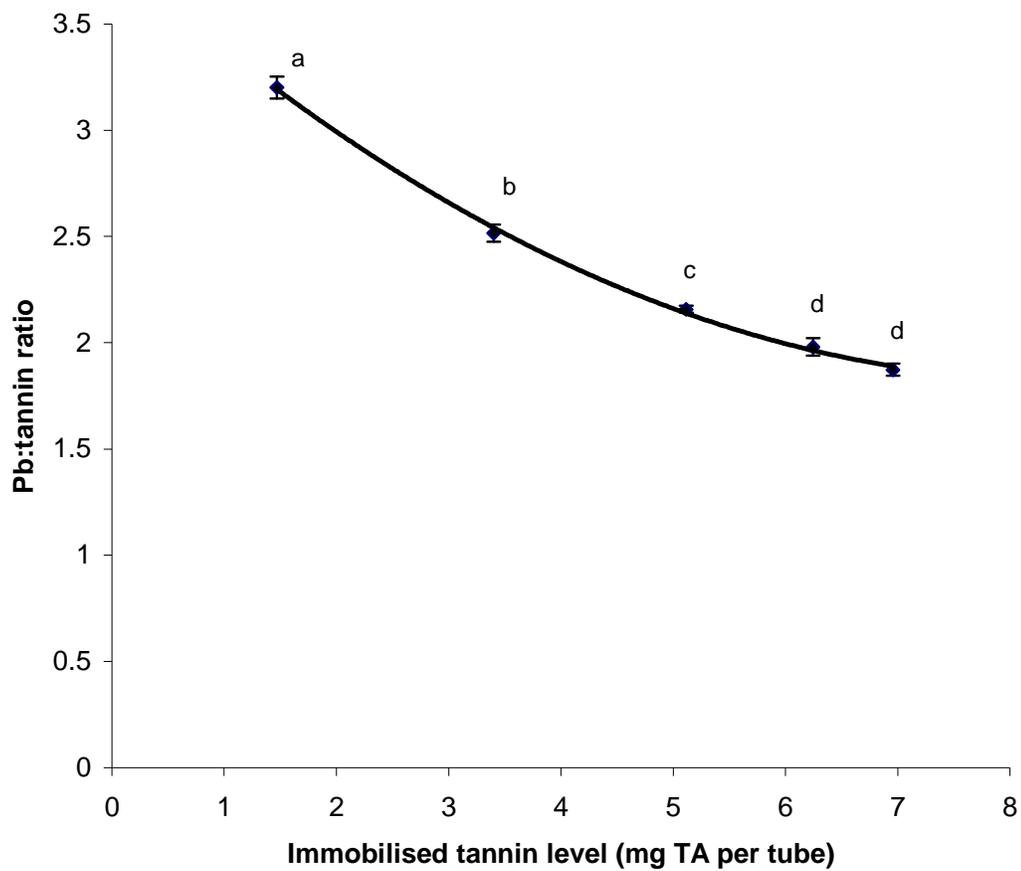


Figure 27. *In vitro* Pb-tannin chelation (immobilised tannin method): Exponential decrease in Pb:tannin ratio (mg Pb:mg tannic acid) of values from Figure 26. Values are means \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). TA= tannic acid.

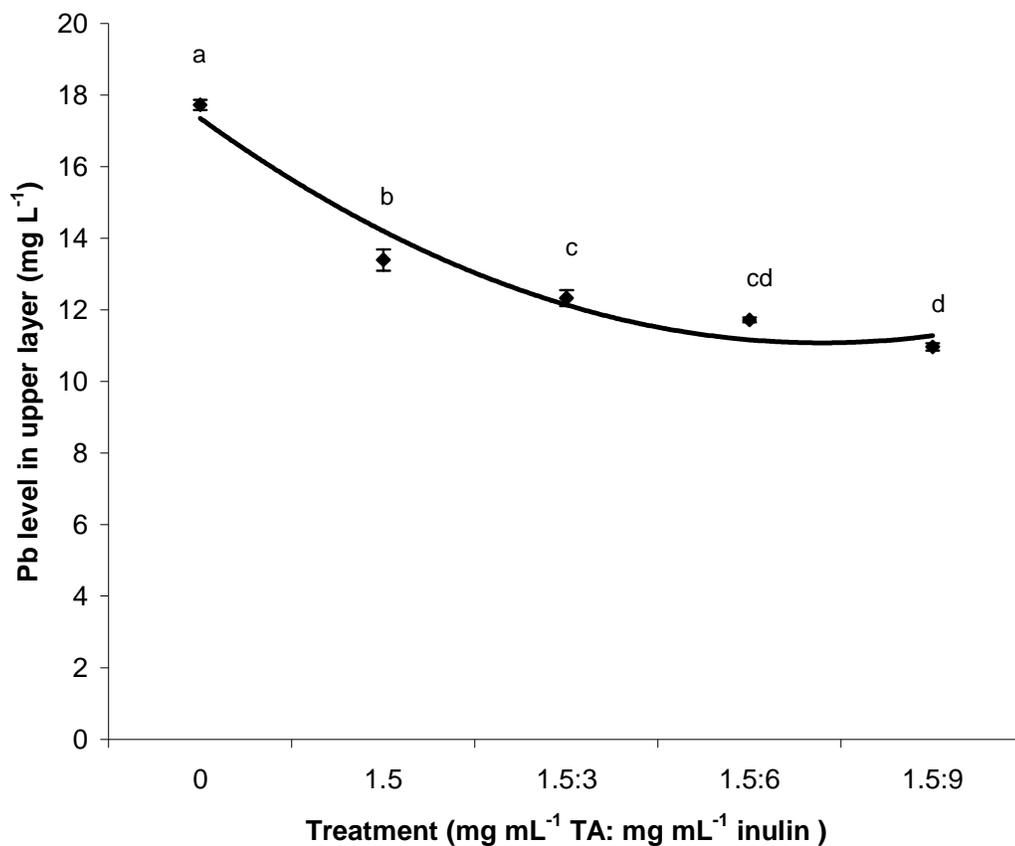


Figure 28. *In vitro* Pb-tannin chelation (immobilised tannin method): Level of Pb remaining in the upper layer of 50 mg L⁻¹ Pb(NO₃)₂ salt solution (pH 4.5) after two hours of treatment with increasing concentrations of purified tannins immobilised to PVPP and spiked with purified chicory inulin. Values are means ± S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$).

NB: The Pb level in the upper layer refers to mean level of Pb element (mg L⁻¹) measured by AAS.

fructans that could have been co-extracted. The level of Pb removed from solution increased by average of 13%, demonstrating that this new method is also subject to fructan interferences (Figure 28). This may account for the apparent increase in the Pb:tannin ratio observed with lateral root immobilised tannins 6.2.3. However unlike the dialysis method, this degree of interference did not interfere with the immobilised tannin method because the trends were consistent with the correlation between Pb accumulation and tannin levels from the hydroponic experiment (4.2).

6.4.3 *In vitro* chelation observations

Insoluble-PVPP granules were white coloured. However, once immobilised with *S. officinale* root tannins they became yellow/brown, with PVPP-immobilised lateral root tannins being visibly darker than those from the main root. It is not a 'masking effect' from the colour of the surrounding lateral root polyphenol supernatant (which was significantly darker than those from the main root) because the yellow/brown PVPP-immobilised tannin colour remained fixed to the PVPP after rinsing the PVPP pellet. The colour of increasing lateral root tannin levels immobilised to PVPP in 50 mg L⁻¹ Pb(NO₃)₂ (pH 4.5) solution is shown in Plate 15.

The yellow intensity of the PVPP pellets was a good indicator of the amount of tannins immobilised to PVPP and the level of Pb removed from solution; the yellower pellets removed more Pb from solution. This was the best factor to help estimate the level of immobilised purified tannic acid required for Pb removal. Only when the purified tannin level was increased to 10-20 mg mL⁻¹, was the pellet colour sufficiently yellow to remove a significant and comparable level (to the 0-0.2 mg mL⁻¹ of crude *S. officinale* lateral root tannins) of Pb from solution.

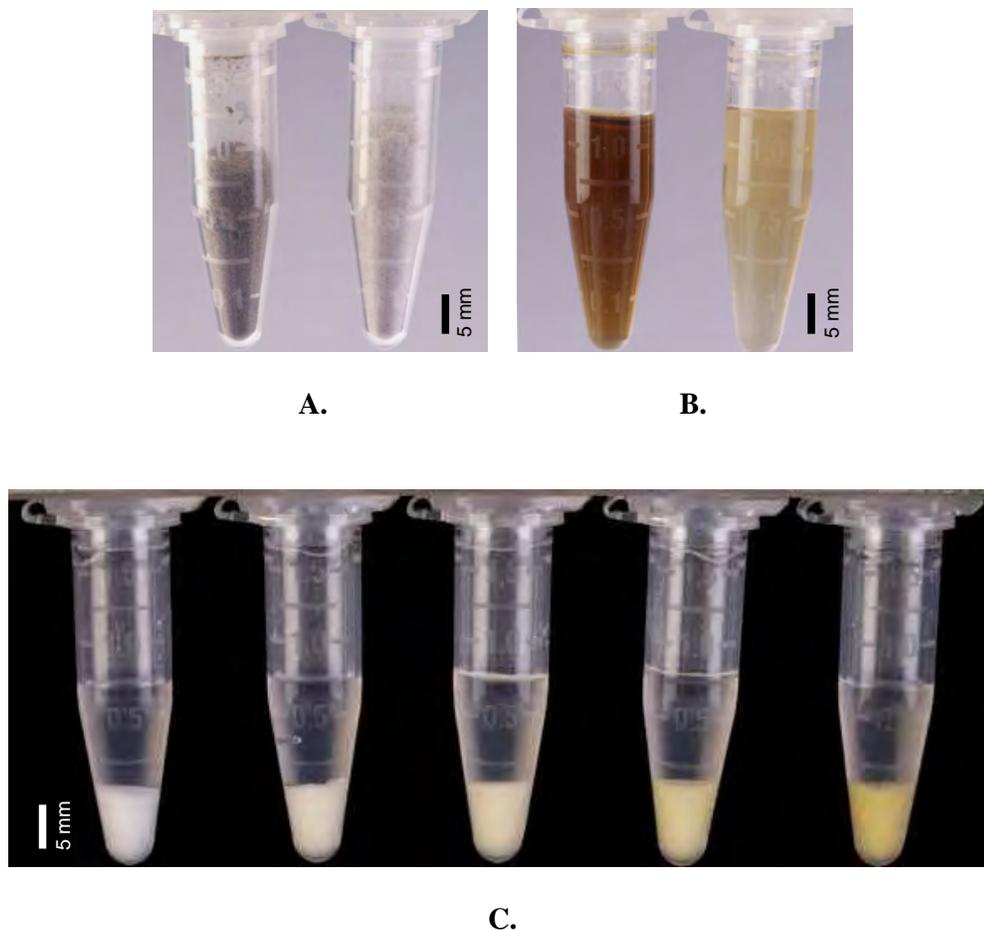


Plate 15. The colour of 3-month-old *Symphytum officinale* root powder, polyphenol extracts and immobilised root tannins.

- A. Colour of lateral and main root powder prior to polyphenol extraction (left to right).
- B. Colour of lateral and main root polyphenol extract. The same colour trend was observed immediately after addition of 50% (v/v) methanol (left to right).
- C. *In vitro* Pb-tannin chelation using micro-scale method: Increasing levels of lateral root tannins (left to right: 0, 0.028, 0.058, 0.113, 0.225 mg tannic acid per tube) immobilised to PVPP in 50 mg L⁻¹ Pb(NO₃)₂ solution. Increasing yellow colour was indicative of immobilised tannin level.

Objective 2

*Phytoremediation potential of Symphytum officinale:
Hydroponic studies with sand-grown Symphytum officinale*

7 LEAD ACCUMULATION STUDIES (II)

Many chemical-based parameters affect the level of Pb accumulation in plants. These include Pb concentration, and the type and concentration of chelating agent application. Thus to determine the phytoremediation potential of *S. officinale*, Pb and EDTA concentrations were increased as there may be a threshold level of these chemicals that is required to induce Pb transport and accumulation in the shoots. Additionally, the effect of another chelating agent was evaluated.

7.1 Effect of increasing EDTA concentration on lead accumulation

In chelate-assisted phytoextraction of Pb, chelating agents have two functions: (i) to increase the bioavailability of Pb for increased root uptake and accumulation, and (ii) to increase the translocation of Pb from roots to the shoots.

Three concentrations of EDTA were tested in this study: 250, 500, and 750 μM . These were equal to, or at two or three times the molar concentration of 250 μM $\text{Pb}(\text{NO}_3)_2$. The control treatment did not contain $\text{Pb}(\text{NO}_3)_2$ or EDTA. Compared to the $\text{Pb}(\text{NO}_3)_2$ solution with 250 μM EDTA, the 500 μM and 750 μM EDTA concentrations did not increase Pb levels in the shoot. At root level, these two EDTA concentrations decreased the Pb accumulation level (Figure 29).

A decrease in Pb accumulation in roots is normally expected, given the translocation function of EDTA. Interestingly however, the amount of Pb loss from the roots was *not* all accounted for by translocation to the shoots. This was unexpected. With increasing EDTA concentrations, similar levels of Pb were translocated to the shoots ($P \geq 0.05$), yet at root level Pb concentrations decreased ($P \leq 0.05$). This is unlikely to be due to differences in root and shoot biomass because as shown later, the root and shoot biomass are approximately the same (Appendix G). Moreover, this effect also occurred in detached roots (8.1).

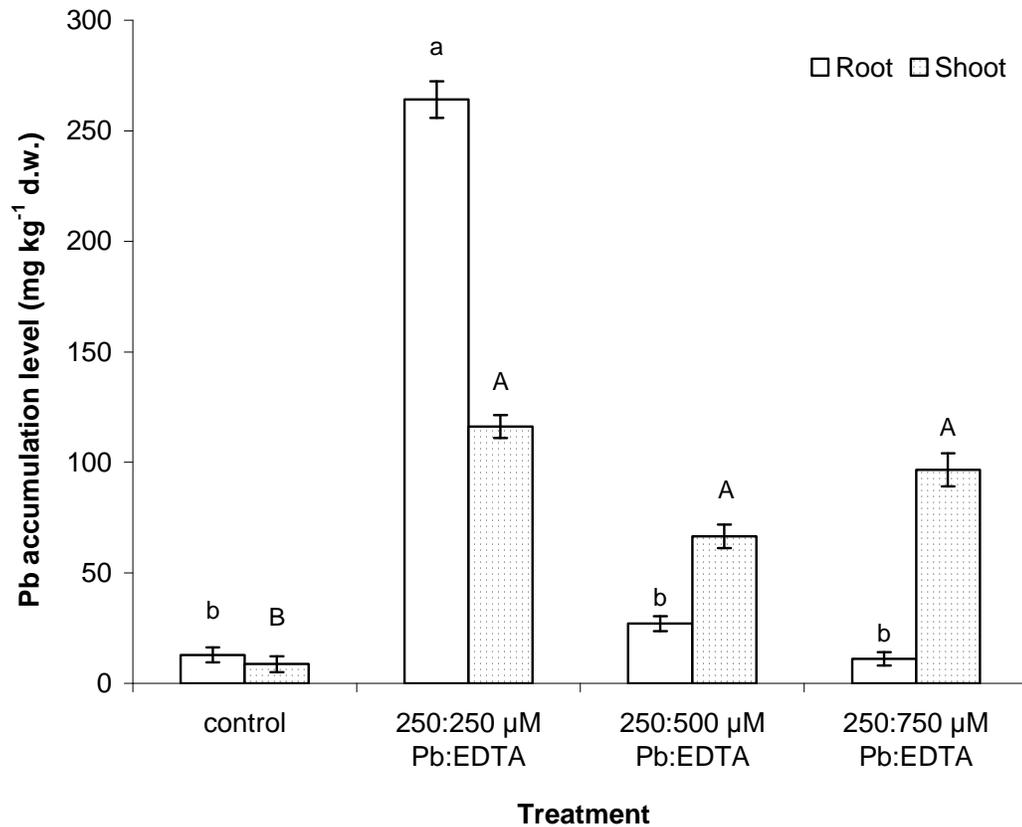


Figure 29. Effect of increasing EDTA concentrations on Pb accumulation level in 3-month-old *Symphytum officinale* roots and shoots of whole plants treated for seven days in modified HC nutrient solution (pH 4.5) containing 0 (control), or 250 μM $\text{Pb}(\text{NO}_3)_2$ with 250, 500 or 750 μM EDTA. Values are mean \pm S.E. of three replicates. Statistical analysis were performed within tissue type only, hence the different case of letters between tissues. Values not sharing the same letter are significantly different ($P \leq 0.05$). HC = Huang and Cunningham.

7.2 Effect of 500 M Pb(NO₃)₂ exposure level

In *unchelated* Pb treatments (500 M Pb(NO₃)₂), most of the Pb accumulated in the root tissue; a mean of 21,319 mg kg⁻¹ (d.w.) of Pb accumulated in the root tissue (after root desorption with 1 mM EDTA), compared to just a mean of 71 mg kg⁻¹ (d.w.) in the shoot tissue (Figure 30A and 30B). Of all the unchelated Pb treatments performed thus far, these were the highest shoot and root Pb accumulation levels recorded after seven days of treatment.

In *chelated* Pb treatments containing 500 M Pb(NO₃)₂ and 500 M EDTA (1:1 PbEDTA), shoots accumulated over seven times the amount of Pb compared to the unchelated Pb treatment (on an average dry weight basis, P≤0.05). In the chelator treatment, shoots contained twice the concentration of Pb than roots (Figure 30A and 30B). This confirms our previous 250 M Pb(NO₃)₂ uptake studies with EDTA, where EDTA increases translocation of Pb to shoots.

However similar to section 7.1, Pb accumulation levels in roots with PbEDTA were drastically reduced to only 18% of the unchelated Pb treatments. The majority of the reduction of Pb level in the roots was also not accounted for by translocation to shoots (Figure 30B).

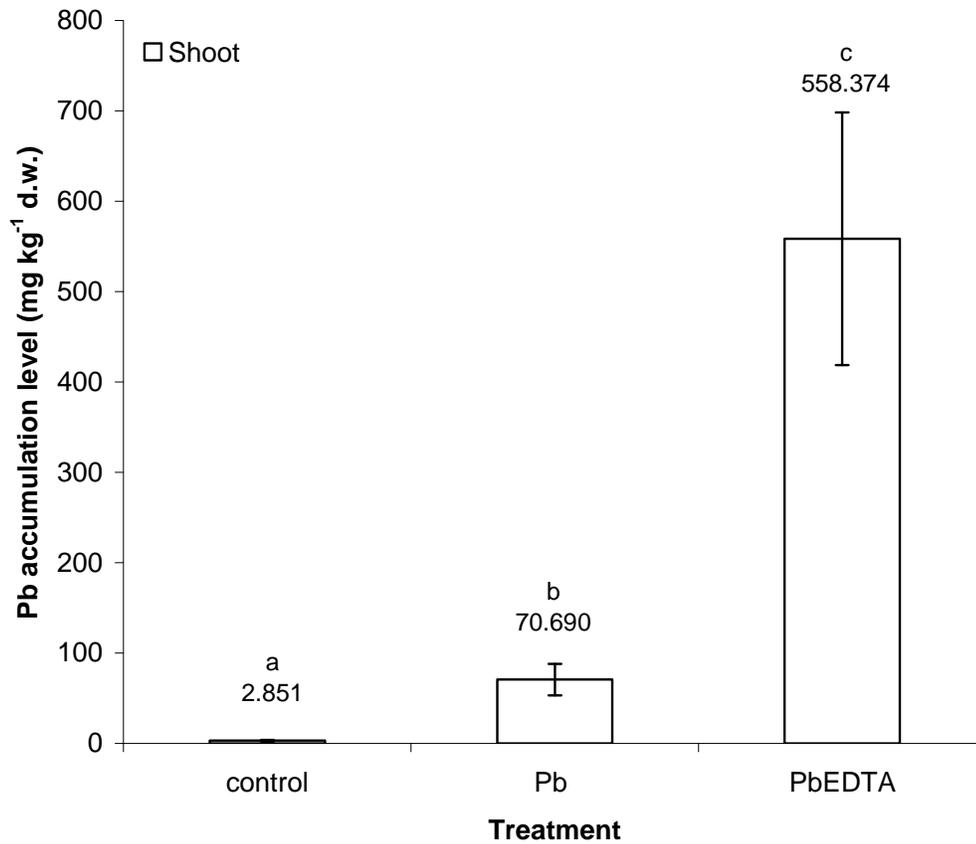


Figure 30A. Pb accumulation level in 3-month-old *Symphytum officinale* shoots of whole plants treated for seven days in modified HC nutrient solution (pH 4.5) containing 0 (control), or 500 μ M Pb(NO₃)₂ with or without 500 μ M EDTA solution. Values are mean \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). HC = Huang and Cunningham.

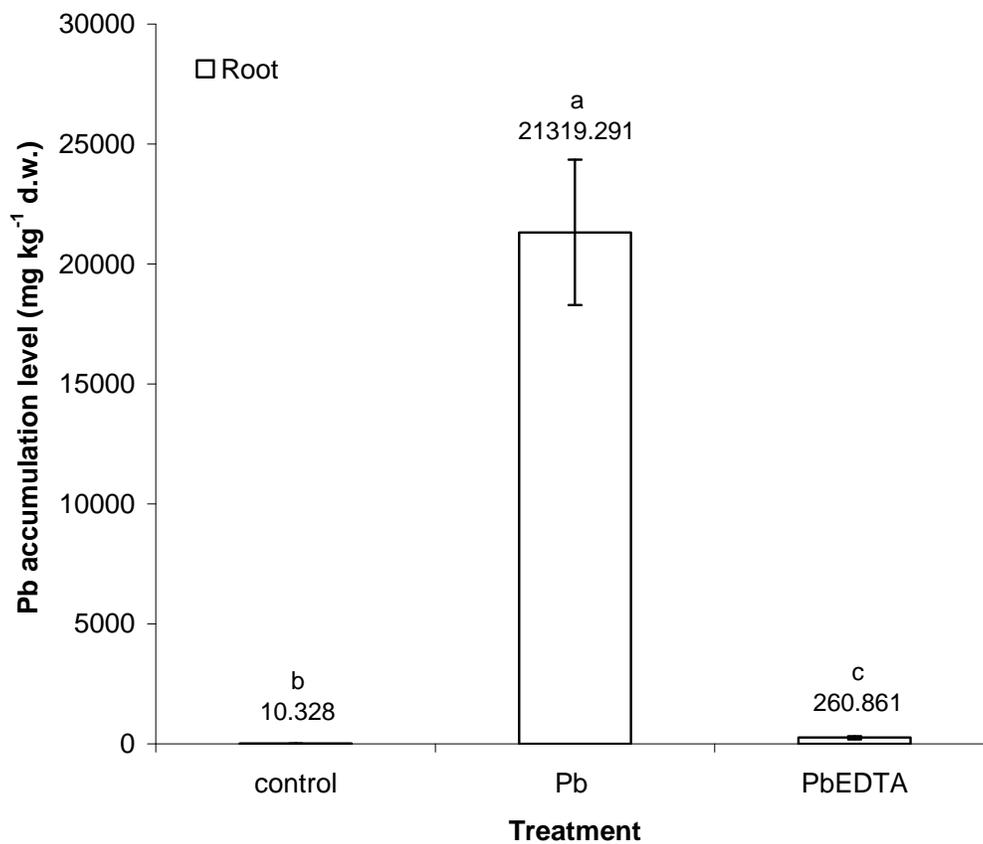


Figure 30B. Pb accumulation level in 3-month-old *Symphytum officinale* roots of whole plants treated for seven days in modified HC nutrient solution (pH 4.5) containing 0 (control), or 500 M $\text{Pb}(\text{NO}_3)_2$ with or without 500 M EDTA solution. Values are mean \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). HC = Huang and Cunningham.

8 WHY DOES EDTA REDUCE LEAD ACCUMULATION IN ROOTS?

The majority of phytoextraction studies published to date (e.g. Huang *et al.*, 1997b; Vassil *et al.*, 1998) reported that EDTA increased or had no effect on heavy metal accumulation in roots and shoots. In our studies, EDTA similarly increased Pb levels in shoots. However, contrary to Vassil *et al.*, (1998), increasing EDTA concentrations had no additional positive effect on Pb shoot levels. Additionally, compared to unchelated Pb treatments, all Pb levels in the roots of PbEDTA treatments decreased. Movement of Pb to the shoots (translocation) *did not* account for most of the Pb lost from the roots*.

This section attempted to address why EDTA has this effect on Pb accumulation (in the PbEDTA form) in *S. officinale*. This is important for phytoextraction because, perceivably, if the concentration of Pb in the roots of chelated Pb-treated plants (at a 1:1 molar concentration) were higher, more Pb would accumulate in the shoots.

From the above Pb studies (results section 7.1 and 7.2) addition of EDTA to Pb treatments resulted in a distinct loss in root colour, from dark to light brown. Did this EDTA-induced colour loss contribute to a reduction in Pb accumulation at root level, and consequently limit Pb accumulation in shoots? To address this question, pre-treatment of root segments and whole plants with EDTA prior to Pb treatments (results section 8.2 and 8.3) was planned. An alternative chelator (ADA) (results section 8.4) was also planned.

Two possible explanations arose from these experiments:

- (i) Replulsion of PbEDTA complex (by root polyphenols)
- (ii) EDTA is not a lead specific chelating agent

Only explanation (ii) appeared to directly involve the role of colour loss in reduction of Pb accumulation. Since a combination of experiments are involved in these explanations, experimental results are presented first followed by a discussion of how these experiments relate to each of the two explanations above.

* Note that root and shoot values are comparable as separate tissues as the biomass of these tissues are approximately the same (Appendix G).

8.1 EDTA pre-treated root segments

Two tests were performed on excised lateral roots (root segments) from 3-4 month-old *S. officinale* plants: (i) a three day test: 18 hours EDTA pre-treatment followed by 24 hours Pb (with or without EDTA) treatment, and (ii) a seven day test: 72 hours EDTA pre-treatment followed by 96 hours Pb treatment (with or without EDTA). After three days the root segments appeared alive, but by seven days they were visibly dead (i.e. browned throughout root section).

8.1.1 Effect of EDTA pre-treatment on lead accumulation level

Lead accumulation in the root segments treated with 500 μ M Pb(NO₃)₂ (unchelated Pb) appeared to increase over time (from the three day to seven day test), reaching over 80,000 mg kg⁻¹ (8% d.w.) (Figure 31). Pre-treatment with 500 μ M EDTA had no effect on Pb accumulation (Figure 31). However, a completely opposite trend was observed for 500 μ M PbEDTA treatments (Figure 32). With time Pb accumulation decreased further. The EDTA pre-treatment of roots exacerbated this effect. All Pb levels were corrected against controls. It was also interesting to note that regardless of EDTA pre-treatment, the level of Pb accumulated in PbEDTA root tissue (Figure 32) was at least six times lower at day three and 37 times lower at day seven than unchelated Pb treatments (Figure 31). Although only root segments were used, this trend was also the same as experiments on whole plants (section 7.1 and 7.2 above). This is contrary to Crist *et al.*, (2004), who observed a reduction in Pb accumulation in excised root segments but not intact whole seedlings of *Brassica juncea* with PbEDTA treatments.

8.1.2 Effect of EDTA pre-treatment on root appearance

Pre-treatment of root segments with 500 μ M EDTA resulted in a loss of brown colour from the roots (Figure 33A), leaving light brown roots that darkened slightly with time (24 to 72 hours). This effect of EDTA was not as apparent after Pb treatments (photo not shown).

The initial lightening of root colour may represent the loss of polyphenols due to EDTA. However, quantitative analysis of polyphenol levels after the three day test (Figure 33B) showed no significant loss in polyphenol levels to support this observation

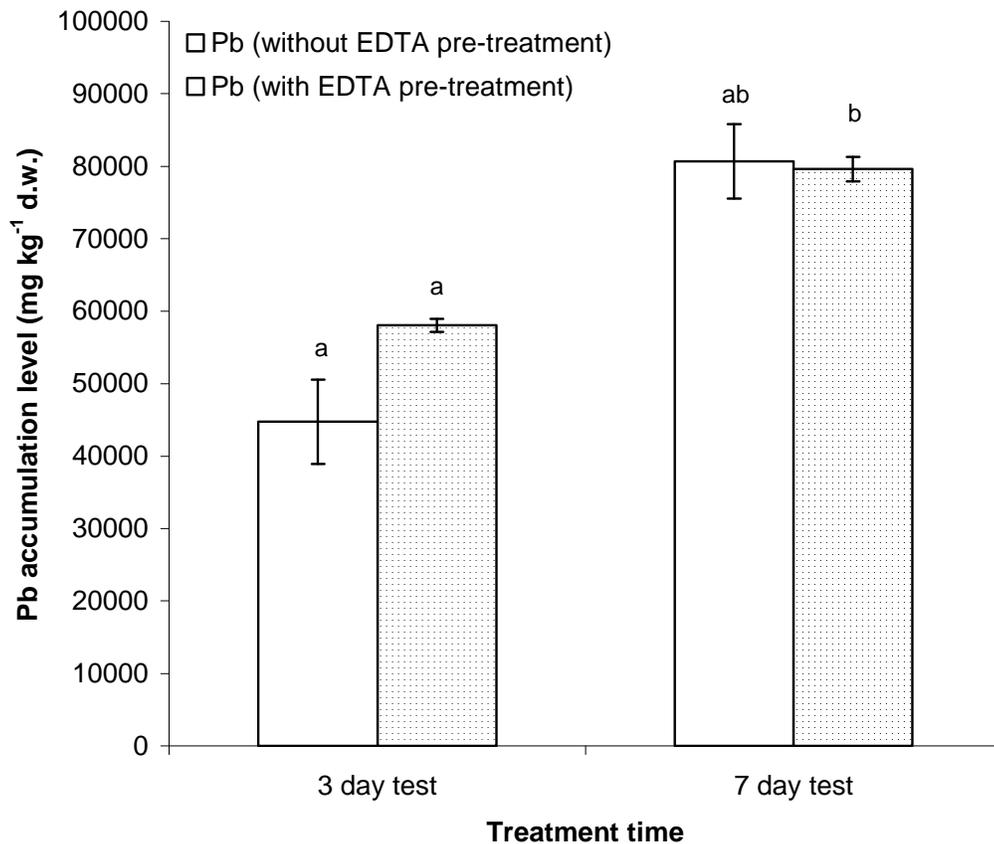


Figure 31. Pb accumulation level in *Symphytum officinale* lateral root segments after the three and seven day test with $\text{Pb}(\text{NO}_3)_2$. In the three day test, lateral root segments were pre-treated for 18 hours in modified HC nutrient solution (pH 4.5), with or without 500 μM EDTA, followed by 24 hours in modified HC nutrient solution (pH 4.5) containing 0 (control) or 500 μM $\text{Pb}(\text{NO}_3)_2$. The seven day test was the same, except the hours were extended to 72 hours for the EDTA pre-treatment and 96 hours for the $\text{Pb}(\text{NO}_3)_2$ treatment. Values are mean \pm S.E. of three replicates and corrected with controls. Values sharing the same letter are not significantly different ($P \leq 0.05$). HC = Huang and Cunningham.

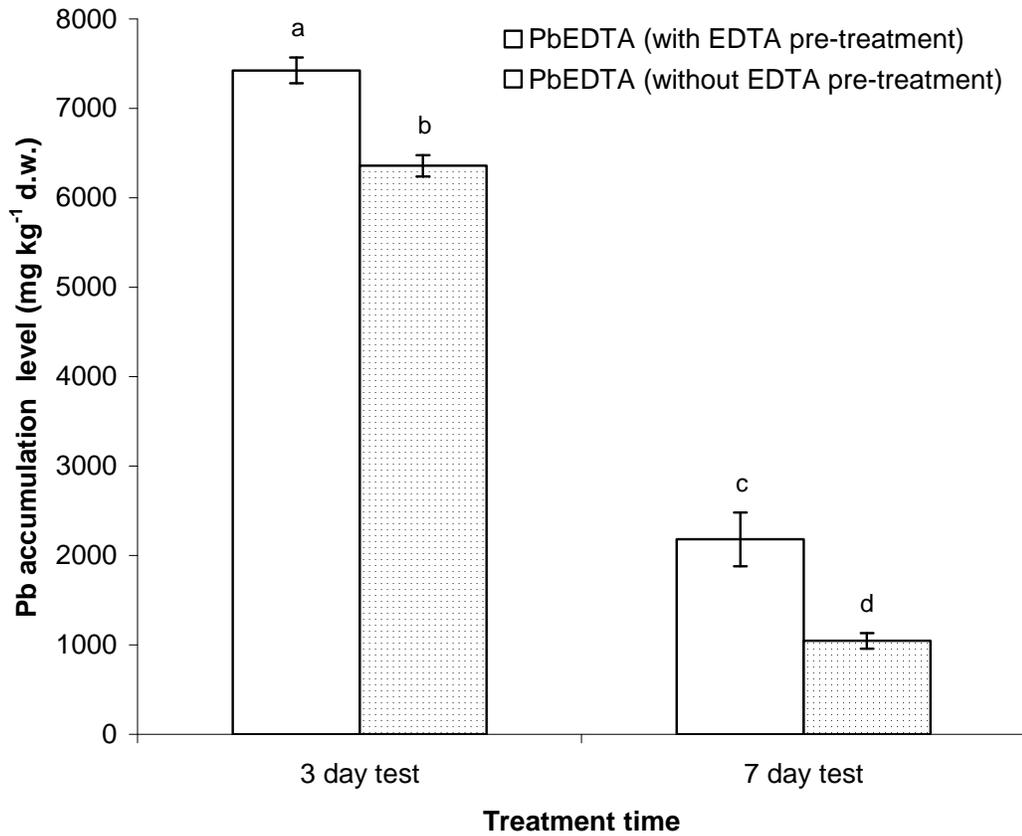


Figure 32. Pb accumulation level in *Symphytum officinale* lateral root segments after the three and seven day test with PbEDTA. In the three day test, lateral root segments were pre-treated for 18 hours in modified HC nutrient solution (pH 4.5), with or without 500 μ M EDTA, followed by 24 hours in modified HC nutrient solution (pH 4.5) containing 0 (control), or 500 μ M Pb(NO₃)₂ and 500 μ M EDTA. The seven day test was the same, except the hours were extended to 72 hours for the EDTA pre-treatment and 96 hours for the PbEDTA treatment. Values are mean \pm S.E. of three replicates and corrected with controls. Values sharing the same letter are not significantly different ($P \leq 0.05$). HC = Huang and Cunningham.

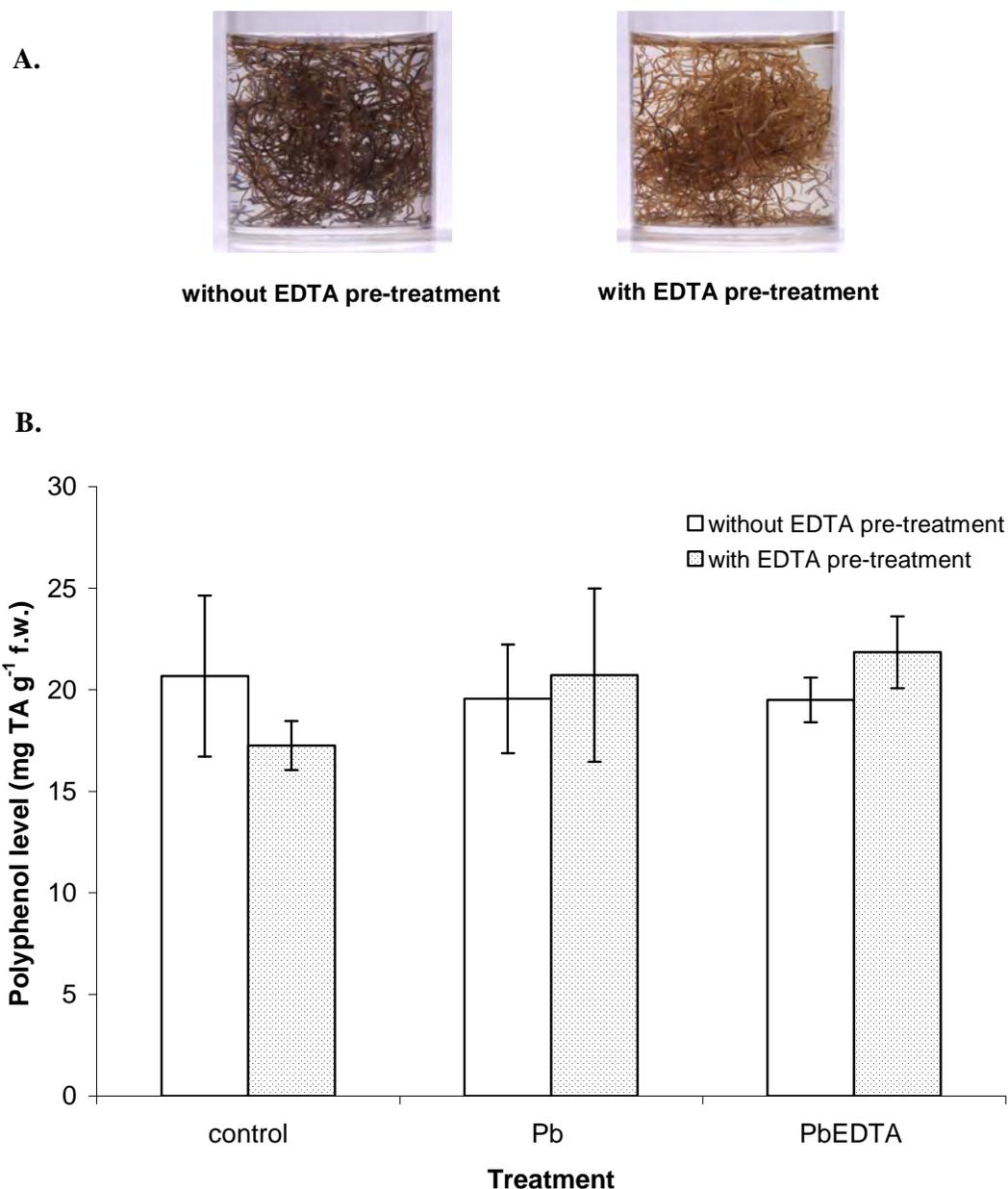


Figure 33.

- A. Colour of *Symphytum officinale* lateral root segments roots after 72 hours pre-treatment in modified HC nutrient solution (pH 4.5) with or without 500 μ M EDTA (part of seven day test).
- B. Polyphenol levels extracted from *Symphytum officinale* lateral root segments after the three day test. Lateral root segments were pre-treated for 18 hours in modified HC nutrient solution (pH 4.5), with or without 500 μ M EDTA, followed by 24 hours in modified HC nutrient solution (pH 4.5) containing 0 (control), or 500 μ M Pb(NO₃)₂ with or without 500 μ M EDTA. Values are means \pm S.E. of three replicates. All means were not significantly different ($P \geq 0.05$).

HC = Huang and Cunningham. TA= tannic acid.

($P \geq 0.05$). Root wounding may have masked any effect of EDTA. No polyphenol levels were measured after 96 hours because the roots were visibly dead.

8.2 EDTA pre-treated whole plants

The root segment test provided valuable information on the effect of EDTA pre-treatment and time on Pb accumulation. However with respect to polyphenol levels, trends could not be observed because root wounding may have masked EDTA treatment effects. Thus the goal of this experiment was to run a similar test but using roots (lateral and main roots) from *non-wounded living* plants. *S. officinale* plants were pre-treated with 500 μ M EDTA for three days before treatment with 500 μ M $\text{Pb}(\text{NO}_3)_2$ (unchelated Pb) or PbEDTA (chelated Pb), for four days.

8.2.1 Effect of EDTA pre-treatment on lead accumulation level

Despite an apparent decreasing trend, EDTA pre-treatment did not statistically ($P \geq 0.05$) affect root accumulation of Pb in either Pb treatments (500 μ M $\text{Pb}(\text{NO}_3)_2$ and PbEDTA) (Figure 34A).

Interestingly, Pb reduction in shoots of EDTA pre-treated plants treated with 500 μ M PbEDTA was significant ($P \leq 0.05$) and was only observed in this experiment. In this treatment, the level of Pb in the shoot decreased by 35% on an average dry weight basis ($P \leq 0.05$) (Figure 34B). It is important to note that the comparison of the relative Pb concentration in roots and shoots separately was possible as root and shoot biomass were not significantly ($P \geq 0.05$) different (Appendix G).

8.2.2 Effect of EDTA pre-treatment on root appearance

The EDTA pre-treatment appeared to increase the number of lateral roots in plants treated with 500 μ M $\text{Pb}(\text{NO}_3)_2$. However, this EDTA pre-treatment effect was not apparent in 500 μ M PbEDTA treatments. Even without EDTA pre-treatment, PbEDTA caused more lateral roots to form compared to plants treated with 500 μ M $\text{Pb}(\text{NO}_3)_2$. All Pb treatments appeared to reduce root growth when compared to the control (Plate 16A). These observations resulting from EDTA exposure, in pre-treatment or part of Pb treatment, seem to reflect the effect of EDTA on Pb levels in the root tissues. The EDTA pre-treated and PbEDTA-treated roots appeared to accumulate less Pb,

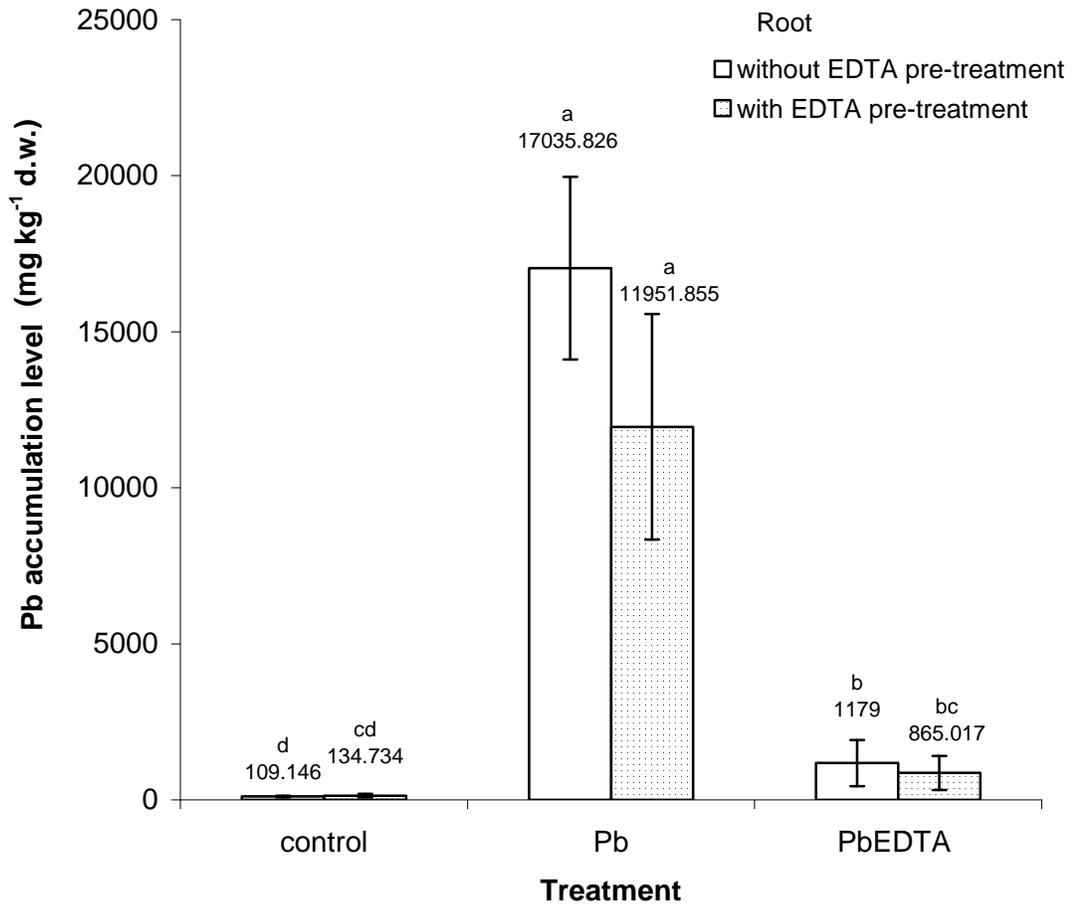


Figure 34A. Pb accumulation levels in 3-month-old *Symphytum officinale* roots of whole plants pre-treated for three days in modified HC nutrient solution (pH 4.5), with or without 500 μ M EDTA, followed by four days in modified HC nutrient solution (pH 4.5) containing 0 (control), or 500 μ M Pb(NO₃)₂ with or without 500 μ M EDTA. Values are mean \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). HC = Huang and Cunningham.

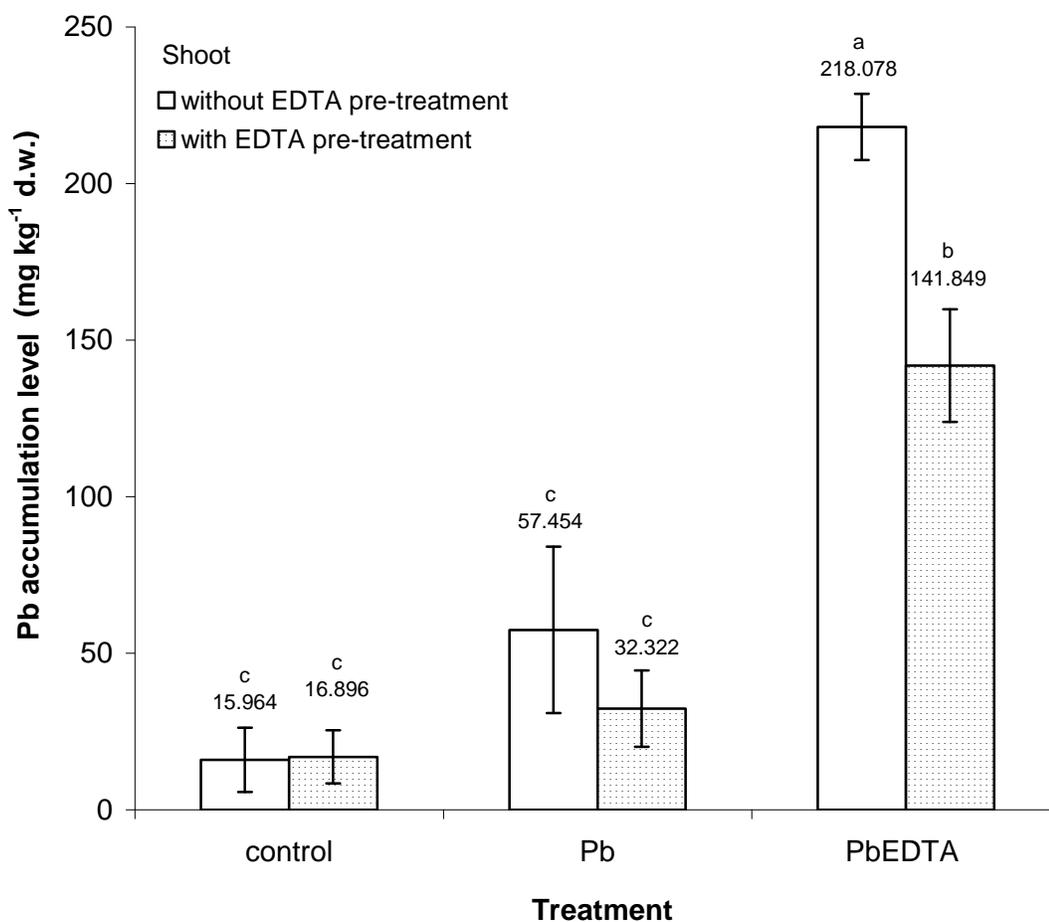


Figure 34B. Pb accumulation levels in 3-month-old *Symphytum officinale* shoots of whole plants pre-treated for three days in modified HC nutrient solution (pH 4.5), with or without 500 μ M EDTA, followed by four days in modified HC nutrient solution (pH 4.5) containing 0 (control), or 500 μ M Pb(NO₃)₂ with or without 500 μ M EDTA. Values are mean \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). HC = Huang and Cunningham.

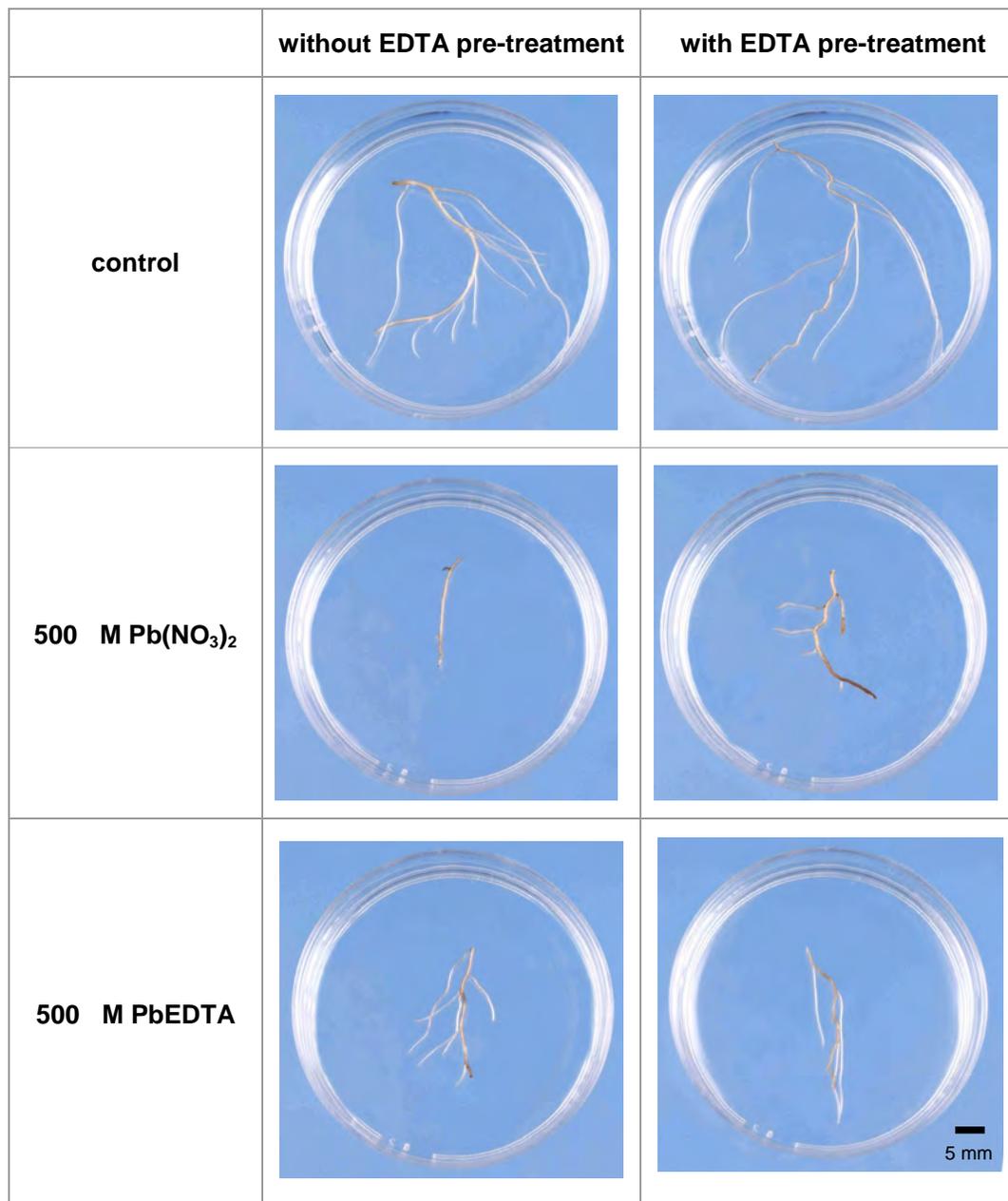


Plate 16A. Structure of 3-month-old *Symphytum officinale* roots after whole plants were pre-treated for three days in modified HC nutrient solution (pH 4.5), with or without 500 M EDTA, followed by four days in modified HC nutrient solution (pH 4.5) containing 0 (control), or 500 M Pb(NO₃)₂ with or without 500 M EDTA. HC = Huang and Cunningham.

thus reducing root growth inhibition.

In terms of root colour, EDTA pre-treatment clearly resulted in a loss of brown colour from the roots, with the most pronounced effect in PbEDTA treatments (Plate 16B). However despite the clear loss in root colour, the apparent reducing trend in polyphenol levels was not significant (Figure 35A). There were no trends in tannin levels (Figure 35B). The other and more significant reason for the loss in root colour was Fe. When analysed, a decrease in Fe levels was also apparent, but again was not significant at a statistical level (Figure 36).

8.3 Alternative chelating agents

8.3.1 Why choose ADA?

In the present work, EDTA pre-treatment appeared to reduce Pb, Fe and polyphenol levels at root level. However despite the apparent trends, the results were not significant ($P \geq 0.05$). Thus a strong link between the role of polyphenols and Fe reduction with how EDTA may have reduced Pb accumulation in roots could not be drawn.

Polyphenol levels were not measured in this experiment as the changes between the chelated Pb and control plants from the previous experiments were not large enough to warrant further investigation. Instead the possible role of polyphenols in charge repulsion of the PbEDTA complex was investigated by changing to a chelating agent with a neutrally charged chelated Pb complex. Since there was quite a strong decrease in Fe levels, the Fe levels in this section were re-tested. Co-incidentally, **ADA** (N-[2 acetamido] iminodiacetic acid) was able to test both of these factors. At equimolar Pb:ADA concentrations, PbADA is neutrally charged (Chen and Hong, 1995). The same authors also note that ADA is a better Pb chelating agent than EDTA because it favours chelation with Pb ions over other competing cations, such as Fe and Ca in the soil. Additionally, ADA also had a comparable Pb soil extraction ability. It ranked a close second to EDTA in its ability to extract Pb from soils; 84% of Pb in soil is solubilised vs. 86% for EDTA (Steele and Pitchel, 1998). Moreover, ADA is not an entirely different chelator to EDTA. Both are analogs of iminodiacetic acid (IDA) (Xie and Marshall, 2001).

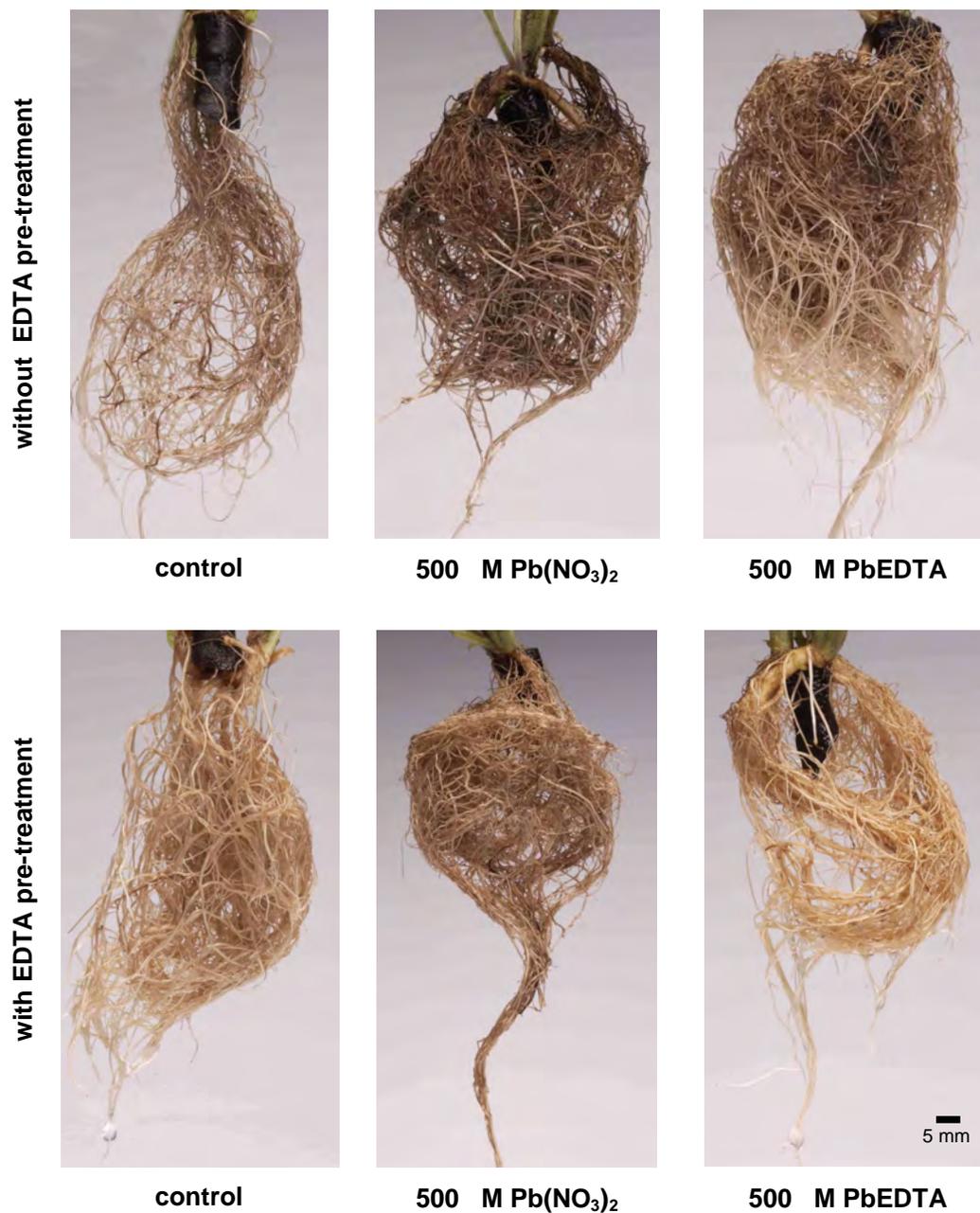


Plate 16B. Colour of 3-month-old *Symphytum officinale* roots after whole plants were pre-treated for three days in modified HC nutrient solution (pH 4.5), with or without 500 M EDTA, followed by four days in modified HC nutrient solution (pH 4.5) containing 0 (control), or 500 M Pb(NO₃)₂ with or without 500 M EDTA. HC = Huang and Cunningham.

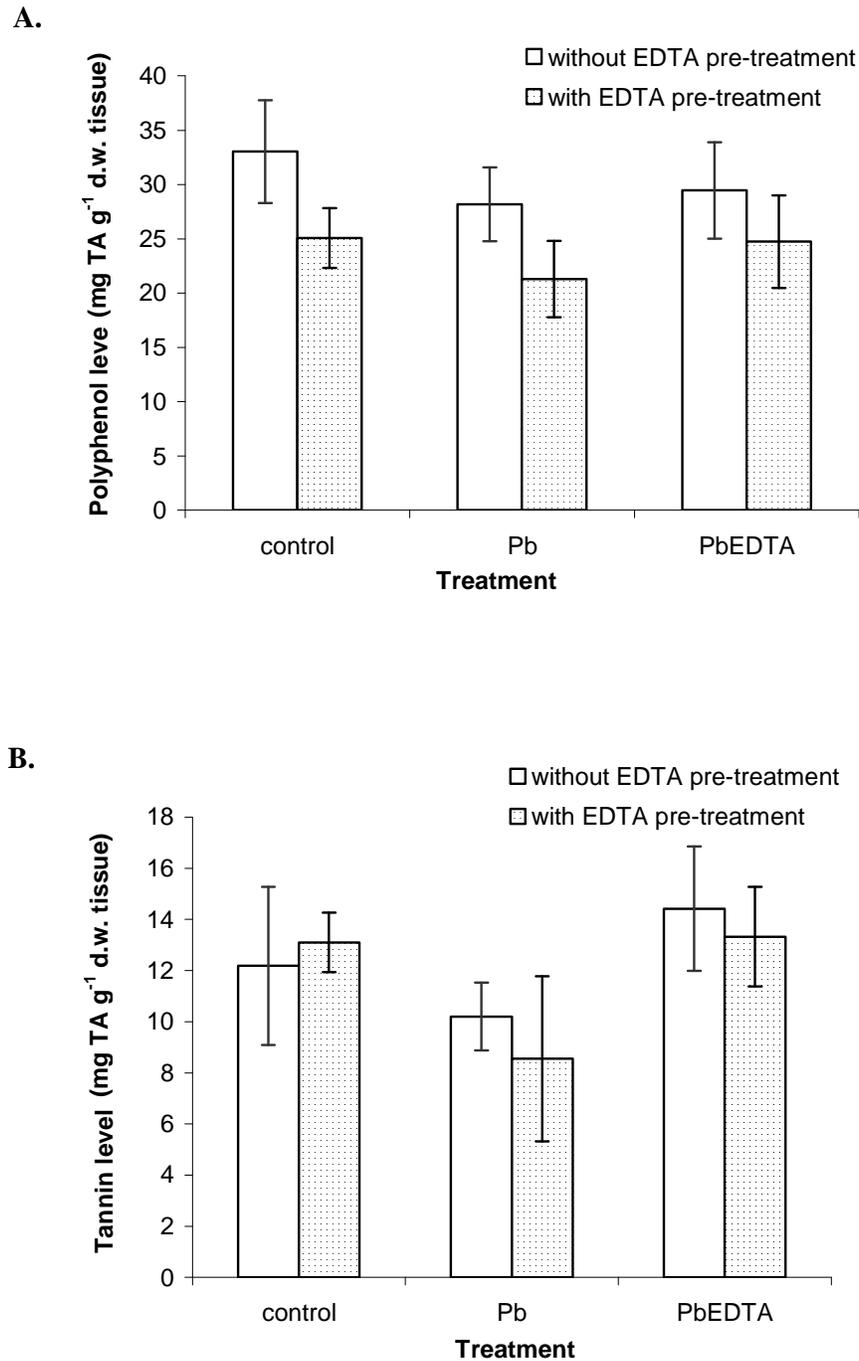


Figure 35. Polyphenol levels (A) and tannin levels (B) extracted from 3-month-old *Symphytum officinale* roots of whole plants pre-treated for three days in modified HC nutrient solution (pH 4.5), with or without 500 μ M EDTA, followed by four days in modified HC nutrient solution (pH 4.5) containing 0 (control), or 500 μ M Pb(NO₃)₂ with or without 500 μ M EDTA. Values are mean \pm S.E. of three replicates. All values are not significantly different ($P \geq 0.05$). HC = Huang and Cunningham.

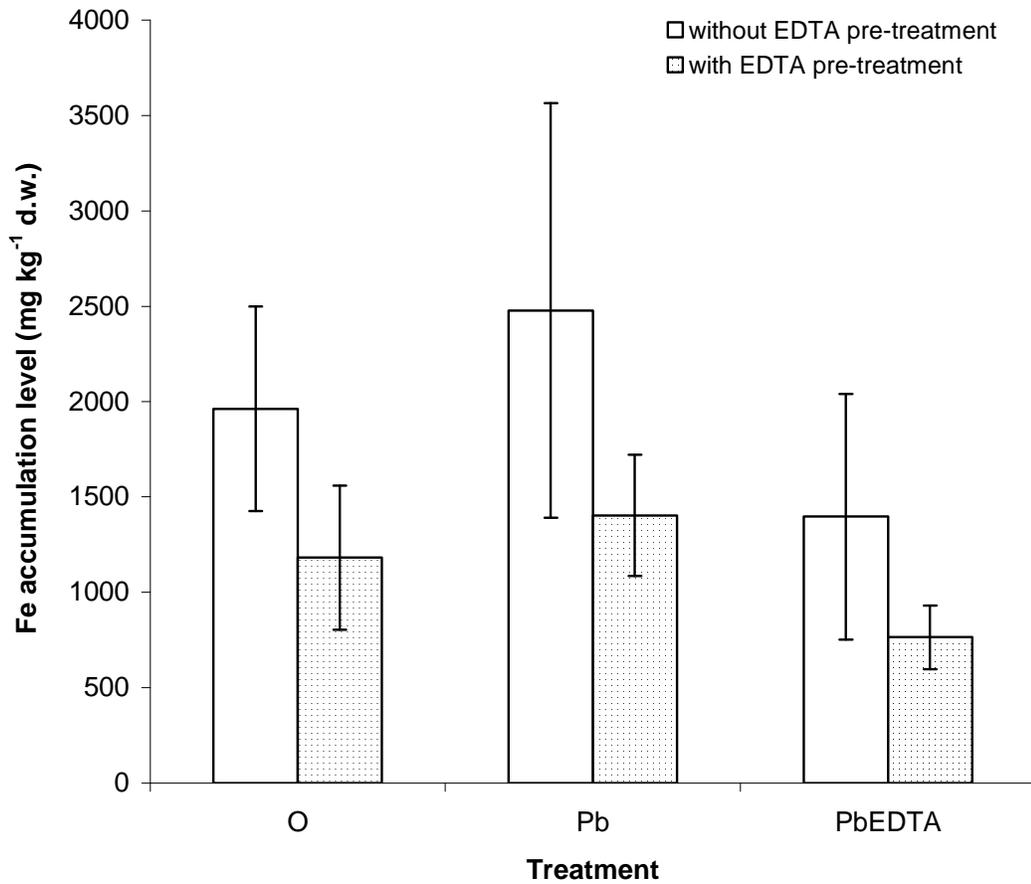


Figure 36. Fe levels in 3-month-old *Symphytum officinale* roots of whole plants pre-treated for three days in modified HC nutrient solution (pH 4.5), with or without 500 μ M EDTA, followed by four days in modified HC nutrient solution (pH 4.5) containing 0 (control), or 500 μ M Pb(NO₃)₂ with or without 500 μ M EDTA. Values are mean \pm S.E. of three replicates. All values are not significantly different ($P \geq 0.05$). HC = Huang and Cunningham.

ADA was recommended as a potentially better chelating agent to test in phytoextraction (Lasat, 2002), but as far as the author is aware demonstrations of its use in phytoextraction have not been reported. Thus, simultaneously this experiment was performed to determine if the phytoextraction ability of *S. officinale* could be improved with ADA.

8.3.2 EDTA vs. ADA: comparison of chelating agents

These experiments were performed at pH 7.0 because this is the optimal working pH for ADA and is the recommended pH (i.e. above 6.0) for EDTA to complex Pb in preference to Fe ions (Kim and Ong, 2000; Kim *et al.*, 2003).

After seven days exposure to modified HC nutrient solution (pH 7.0) containing 500 μ M Pb(NO₃)₂ and 500 μ M ADA, Pb root levels was 2.4 times higher on a dry weight basis than the EDTA treatments ($P \leq 0.05$) (Figure 37A). This also corresponded to a higher degree of root growth inhibition for PbADA compared to PbEDTA treated *S. officinale*. It is also important to mention that the root Pb levels are much higher than previous studies, such as those in results section 7.2; this is because no EDTA desorption was performed to avoid cross contamination of different chelating agents. Although not significant, shoot levels also appeared to be higher by 1.5 times (Figure 37B).

Although pH levels were set above 6.0, to avoid EDTA chelation to Fe ions, the PbEDTA treatment still significantly reduced Fe levels. The level of Fe in PbEDTA treated roots was 53% less than control plants ($P \leq 0.05$). In contrast, ADA did not remove significant levels of Fe relative to the roots of the control ($P \geq 0.05$) (Figure 38).

8.4 Explanations for reduction of lead in roots treated with PbEDTA

8.4.1 Explanation 1: Replulsion of PbEDTA complex (by root polyphenols)

In this research when Pb ions are complexed with EDTA, the same anionic polyphenols that attract Pb²⁺ ions may *repel* the negatively charged [PbEDTA]²⁻ complex (Figure 39A). This may limit accumulation of Pb (in the PbEDTA form) in the root. The idea of repulsion of EDTA-chelated metals and the use of the more neutral complexes initially came from Dekock and Mitchell (1957). Experiments with mustard and

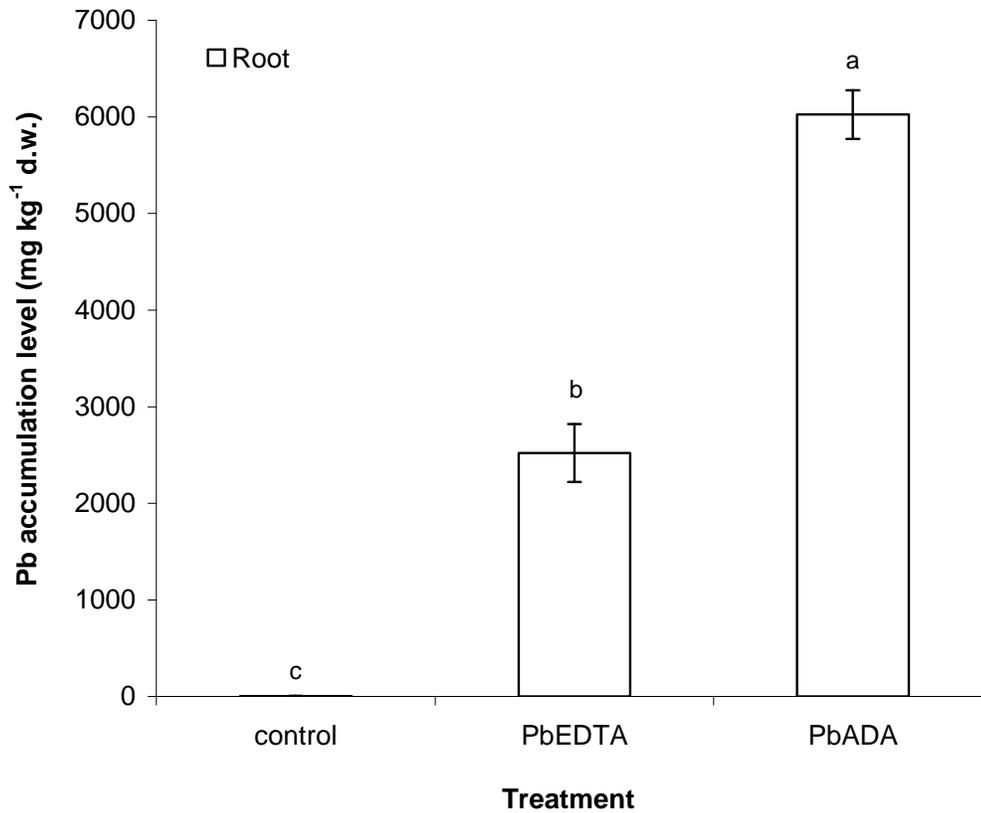


Figure 37A. Pb accumulation levels in 3-month-old *Symphytum officinale* roots of whole plants treated for seven days in modified HC nutrient solution (pH 7.0) containing 0 (control), 500 μ M Pb(NO₃)₂ with or without 500 μ M EDTA or ADA. Values are mean \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). HC = Huang and Cunningham.

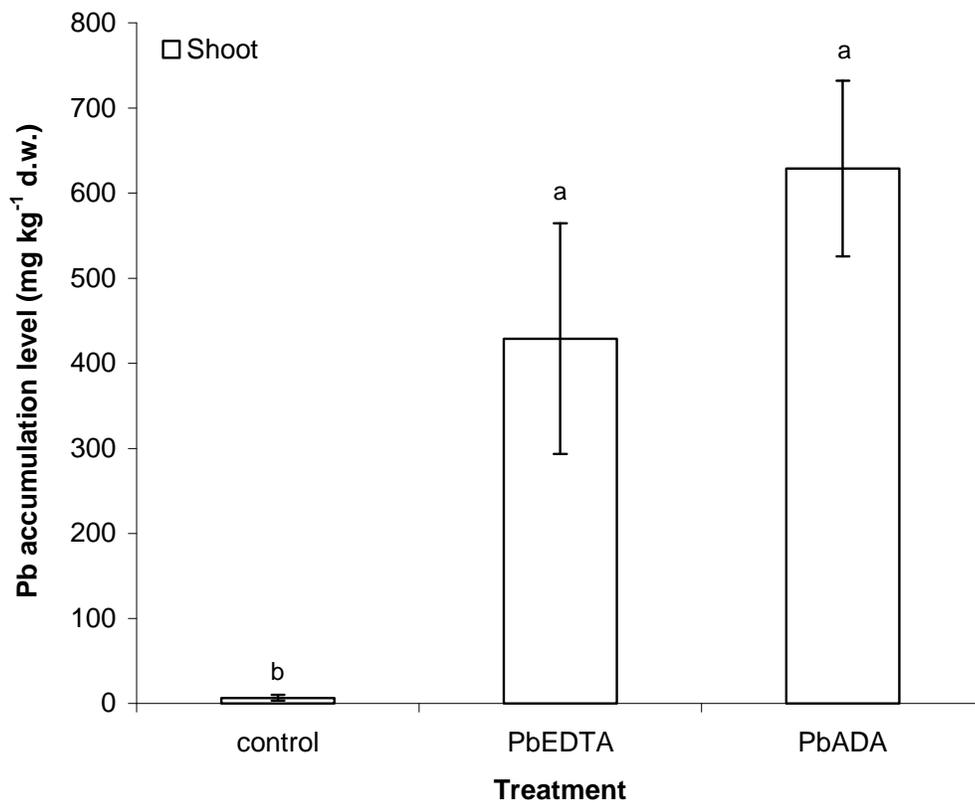


Figure 37B. Pb accumulation levels in 3-month-old *Symphytum officinale* shoots of whole plants treated for seven days in modified HC nutrient solution (pH 7.0) containing 0 (control), 500 μ M Pb(NO₃)₂ with or without 500 μ M EDTA or ADA. Values are mean \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). HC = Huang and Cunningham.

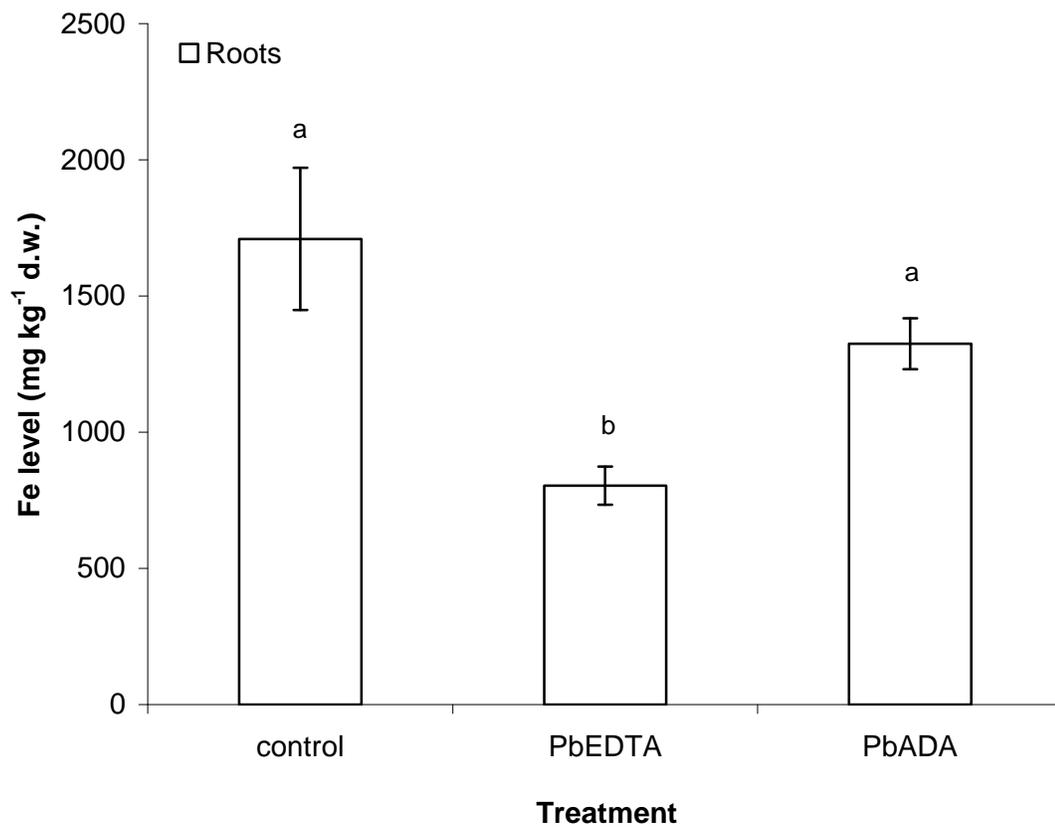


Figure 38. Effect of different chelating agents on Fe levels in 3-month-old *Symphytum officinale* roots of whole plants pre-treated for seven days in modified HC nutrient solution (pH 7.0) containing 0 (control), or 500 M $\text{Pb}(\text{NO}_3)_2$ with or without 500 M EDTA or 500 M ADA. Values are mean \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). HC = Huang and Cunningham.

tomatoes suggested that EDTA complexes of trivalent ions (e.g. Fe^{3+}) facilitate uptake of the cations, whereas EDTA complexes with divalent cations (e.g. Cu^{2+}) has the opposite effect. This is because of the difference in overall charge of the complexes. Trivalent ions form complexes with no charge or a single negative charge and can be taken up, whilst divalent cations form EDTA complexes with two negative charges and are repelled. From a medical standpoint Shulman and Dwyer (1964) stated that EDTA and some of its analogs form hydrophilic anions, and as a result penetrate cells poorly. Sattelmacher (2001) also stated that negative charges of the root cell wall favoured the accumulation of cations and repelled anions.

EDTA pre-treatment may exacerbate this charge-based repulsion by increasing **polyphenol polymerisation**, a reaction that creates a more concentrated region/barrier of anionic charge. Polymerisation may also reduce the number of ionic binding sites and the rate of Pb accumulation (Figure 40).

The mechanism for such polymerisation activation is unknown, but it is possible that EDTA induced a loss in polyphenols and/or removed cations (e.g. Ca^{2+}) stabilising the cell wall. Although at much higher concentrations (0.1M), EDTA has been documented to cause separation of root cell tips by removing cell wall/plasma membrane stabilising ions (e.g. Mg^{2+} and Ca^{2+}) during the development of TEM sample preparation methods (Klein and Ginzburg; 1959). Vassil *et al.*, (1998) also suggested that EDTA could remove these plasma membrane stabilising ions. But in this phytoextraction study it was suggested that this may induce $[\text{PbEDTA}]^{2-}$ access to roots. Additionally, PbEDTA may also induce oxidative stress (Geebelen *et al.*, 2002), a process that can also lead to polymerisation.

In the results above, this like-charge repulsion due to increased polyphenol levels or polymerisation was possibly supported by:

- (i) Whole plant test: The polyphenol trends from this section were slightly clearer. EDTA pre-treatment appeared to reduce the polyphenol levels possibly reflecting polyphenol polymerisation. Polyphenol polymerisation reduces polyphenol extraction and levels (Strycharz and Shetty 2002a and b), a part of the lignification process that is involved in root growth inhibition (Hose *et al.*, 2001, see Chapter 4 for more discussion). This could explain the reduction in Pb accumulation levels in EDTA pre-treated plants.

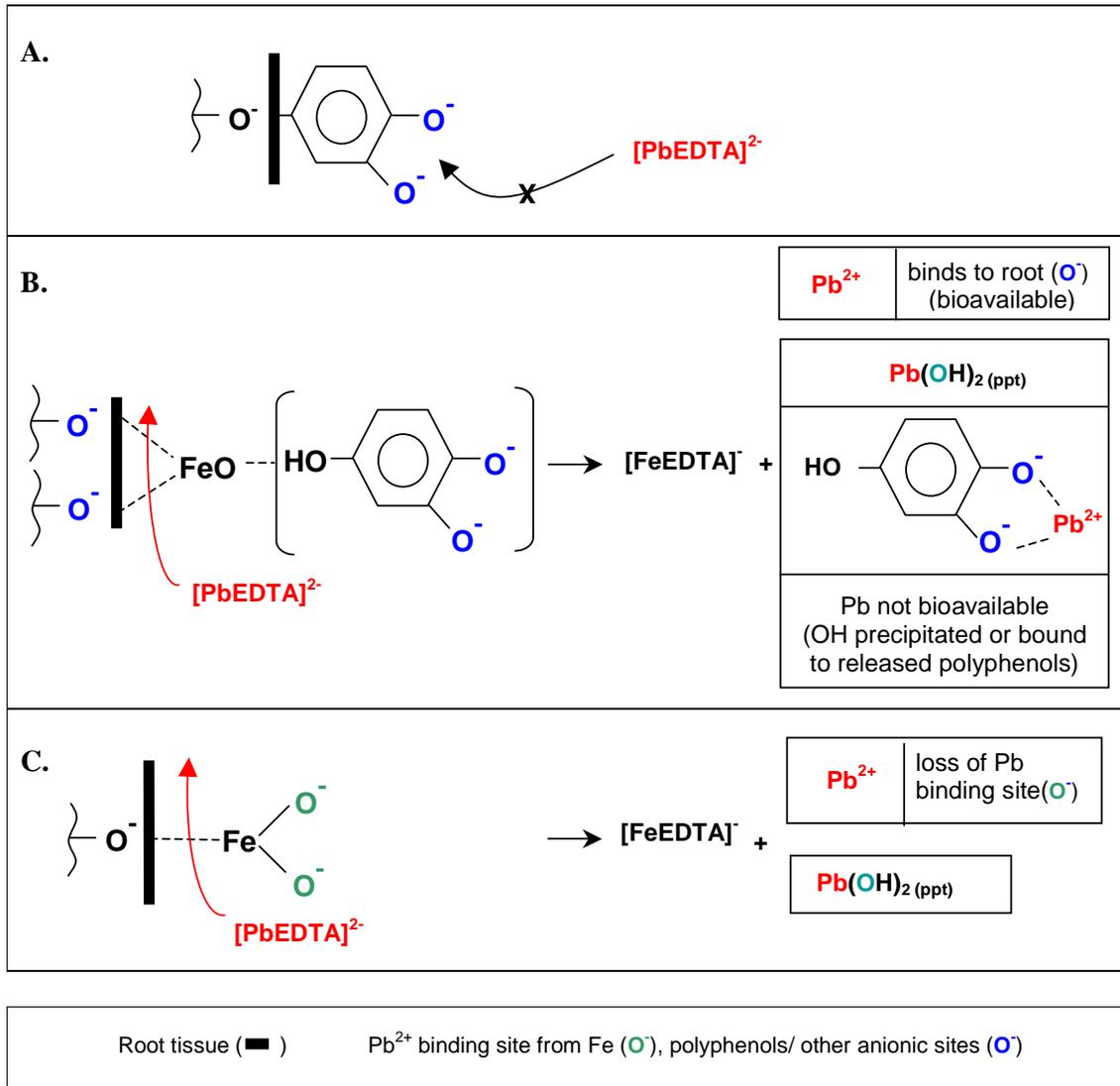


Figure 39. A. $[PbEDTA]^{2-}$ ion repulsion by polyphenols.

B and C. $[PbEDTA]^{2-}$ interaction with iron oxides. Removal of iron (hydr)oxides from root. This may also indirectly remove polyphenols bound to iron, or via other ions on the root, especially at the surface.

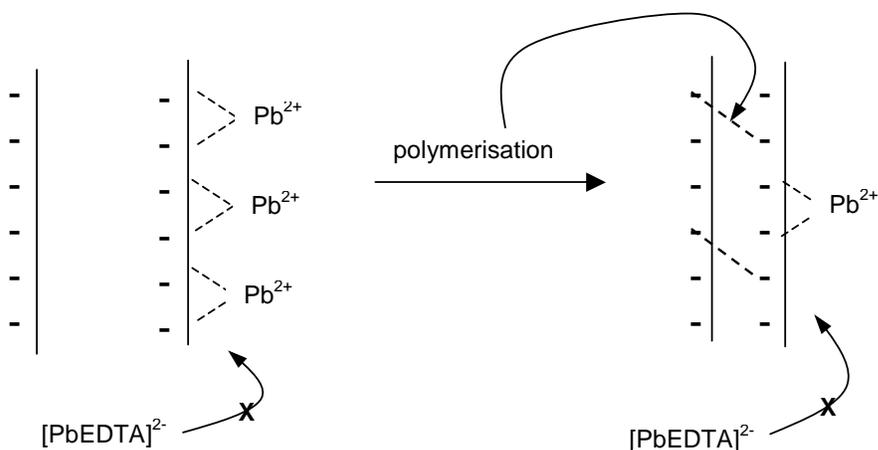


Figure 40. How increased polyphenol polymerisation in the root may reduce both Pb^{2+} and $[\text{PbEDTA}]^{2-}$ accumulation: decreased binding sites for Pb^{2+} and more repulsion at the root surface $[\text{PbEDTA}]^{2-}$.

- (ii) Alternative chelating agent test: The neutrally charged PbADA complex (at a 1:1 ratio, pH 7.0) accumulated over two times more Pb than the charged PbEDTA complex. This could have been even higher (by about 30%) as the ratio of Pb to ADA was not exactly 1:1. Only 70% of the calculated Pb was available in the medium. This would lead to a higher portion of ADA to Pb, and according to Chen and Hong (1995), negatively charged Pb:ADA complexes (i.e. at 1:2 molar ratios of PbADA , complexes are $[\text{PbADA}]^{2-}$). Caution also should accompany this interpretation. Although the pH between the two chelating agents was the same, the pH was higher relative to unchelated Pb treatments to meet optimal working pH of ADA. If unchelated Pb treatments are also considered, a pH effect also cannot be excluded. Hence there is a limitation in interpreting the effect of changing the chelating agent on repulsion.

8.4.2 Explanation 2: EDTA is not a lead specific chelating agent

Iron oxides and polyphenols may play a role in EDTA's non-specificity for Pb. Both are potential Pb binding sites that can be removed by EDTA. Brown iron oxides typically form and bind to Pb on the soil (Kim and Song, 2000; Liu and Huang 2003; Nowack and Sigg, 1996), or root surfaces (Hansel *et al.*, 2001; Lavid *et al.*, 2001a) of aerated environments via oxidation of Fe^{2+} to Fe^{3+} (Figure 41A). Simple polyphenols also bind to iron or other divalent metals (Blum, 1997; Dalton *et al.*, 1987; Kaminsky and Muller,

1977) (Figure 41B). As objective 1 demonstrated, polyphenols could also be potential Pb binding sites.

In the presence of iron (Fe) or iron hydr(oxides) and especially at pH less than 6.0, EDTA binds to Fe in preference to Pb (Kim and Ong, 2003; Kim *et al.*, 2000). High concentrations of EDTA (0.1M) have been used to remove simple polyphenols like ferulic acid bound to soils via metal ions (Blum, 1997; Dalton *et al.*, 1987) within hours. If either of these potential Pb binding sites were lost from the root, this would decrease the amount of Pb binding sites on the roots. The bioavailability of Pb could be also decreased if Pb ions, dissociated from EDTA in preference to iron, binds and precipitates with hydroxides (from iron (hydr)oxides) or simple polyphenols released from the root surface (Figure 39B and C).

This would explain the reduction in the amount of Pb in the root and the threshold concentration that may be required to drive Pb into the shoots. This was potentially demonstrated by the following experiments:

- (i) Root segment and whole plant test: A loss in brown root color (indicative of Fe) was very fast (within 24 hours) and noticeable with EDTA pre-treatment.
- (ii) Whole plant test: A noticeable (but not statistically significant) reducing trend in Fe level was present in all plants (including control) pre-treated with EDTA. An apparent decrease polyphenol levels but not tannins was observed. The latter is consistent with the notion that only simple polyphenols might be lost.
- (iii) Alternative chelating test: PbEDTA was found to significantly reduce Fe levels. No loss in Fe levels with the more Pb-specific ADA chelating agent was observed.

It is also worth noting a few points about Fe: (i) Fe was an indicator of specificity of the chelating agent, (ii) the positive charge on any Fe ion is not a binding site for $[\text{PbEDTA}]^{2-}$ because Fe was loss upon PbEDTA treatment, and (iii) most of the root color loss from the root is probably Fe, not simple polyphenols because a gain in brown root colour after unchelated Pb treatments correlated to an increase in Fe levels relative to the control (unlike polyphenol trends).

8.4.3 Other explanations

Another explanation for the loss of Pb in PbEDTA treatments is the EDTA desorption step. In conducting the alternative chelating agent test, it was noticed that replacing the EDTA desorption step (to remove apoplastic Pb of roots) with just a dH₂O rinse step lead to a 20-fold increase in the amount of Pb accumulated in the root tissue. An average of 5451 mg kg⁻¹ Pb (d.w.) (without EDTA desorption) compared to 261 mg kg⁻¹ Pb d.w. (with desorption) was observed (Table 7). However, these levels were still 70% lower than in the unchelated Pb treatment. Shoot levels did not account for this loss as only an average of 558 mg kg⁻¹ (d.w.) Pb (with desorption) and 283 mg kg⁻¹ (d.w.) Pb (without desorption) was recorded for shoots. The difference in desorption did not affect root Pb levels in unchelated Pb treatments. This indicates that a significant amount of PbEDTA is apoplastically bound (exactly how is unknown) and less strongly than unchelated Pb ions. Moreover, it indicates that ADA-chelated Pb ions are more strongly bound to the roots. Perhaps this is another example of repulsion of EDTA complexes.

Whilst the results do support each of the explanations presented, their roles appeared to be small as Pb accumulation in roots of chelated (EDTA or ADA) Pb treatments were still markedly reduced compared to unchelated Pb levels. Despite this, the explanations presented here do support the idea that if Pb levels in the roots are reduced (particularly below a possible threshold concentration in the root), then levels reaching the shoots are likely to be limited. The major reason(s) for the loss of Pb accumulation in roots, upon addition of chelating agents, remains elusive. The other possible reasons are discussed in Chapter 4.

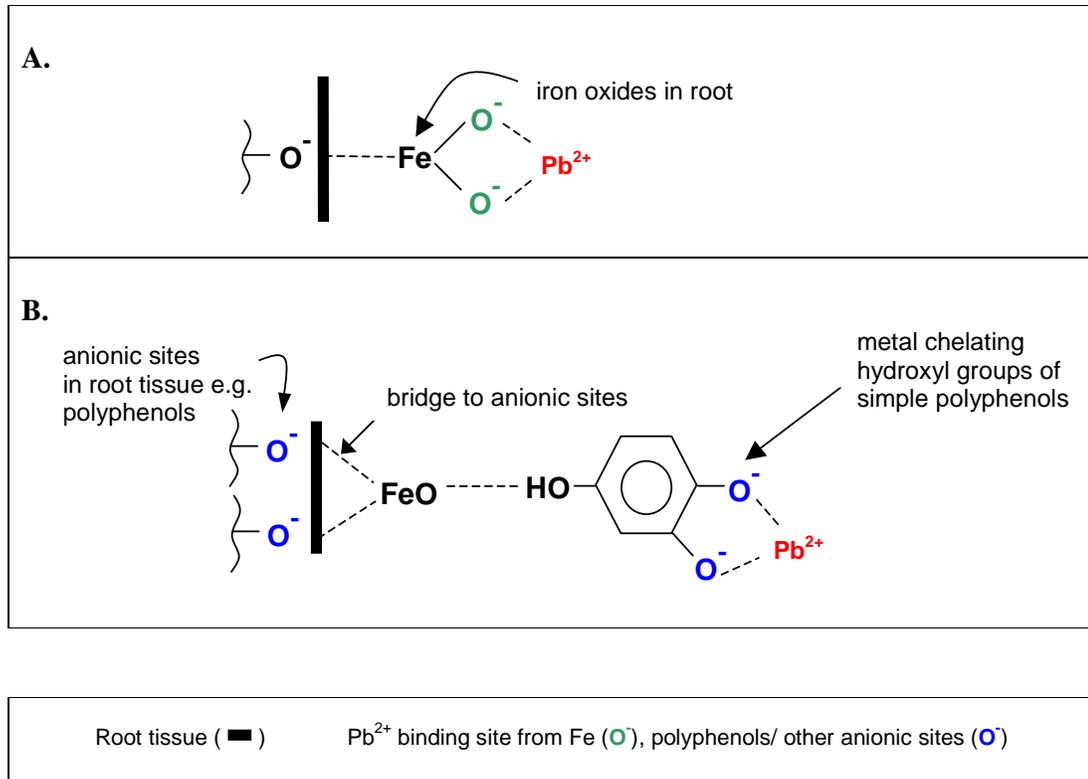


Figure 41. Chelation of free Pb ions to iron oxides (A) and polyphenols (B).

Table 7. Effect of EDTA desorption* on Pb accumulation levels in 3-4 month-old *Symphytum officinale* roots of whole plants treated with modified HC nutrient solution (pH 4.5) containing 0 (control), or 500 M Pb(NO₃)₂ with or without 500 M EDTA for seven days**. Values are mean ± S.E. of three replicates. Values not sharing the same letter are significantly different (P≤0.05). HC = Huang and Cunningham.

	Pb accumulation level (mg kg ⁻¹ d.w.)	
Treatment	with EDTA desorption*	without EDTA desorption
control	10.328 ± 4.851 ^c	4.958 ± 3.627 ^c
PbEDTA	260.861 ± 68.650 ^b	5451.228 ± 470.719 ^a

	Pb accumulation level (mg kg ⁻¹ d.w.)	
Treatment	with EDTA desorption*	without EDTA desorption**
control	10.328 ± 4.851 ^b	4.958 ± 3.627 ^b
Pb(NO ₃) ₂	21,319.291 ± 3026.682 ^a	18,676.504 ± 1673.164 ^a

*EDTA desorption involves immersing roots (attached to the whole plant) in modified HC nutrient solution (pH 4.5) supplemented with 1 mM EDTA for 30 minutes.

** Plants that were not desorbed with EDTA were treated with Pb treatments under different growth conditions. Glasshouse conditions were used (like objective 1) for desorption experiment because whiteflies contaminated the growth room facility. However, these are valid trends as the unchelated Pb treatments the same under different conditions.

Objective 3

*Pb mutant plant studies:
Symphytum officinale tissue culture*

9 DEVELOPING IN VITRO SYMPHYTUM OFFICINALE CULTURES

9.1 Somaclonal variation approach

In order to develop somaclonal variants, the traditional callus approach was initially followed. This involved:

- (i) developing a sterile line of undifferentiated callus tissue
- (ii) exposing the callus tissue to Pb
- (iii) inducing shoot growth from the Pb-exposed callus (to develop whole plants)

However as discussed below, the callus approach was later replaced with inducing shoot growth directly from Pb-exposed petioles. Sections below discuss the development of *in vitro S. officinale* tissues and whole plants.

9.2 Explant and sterilisation

9.2.1 Explant choice

Petioles of *S. officinale* plants were used for sterilisation tests due to:

- (i) abundance
- (ii) uniformity of tissue
- (iii) callus induction speed (within two weeks of culture)
- (iv) ease of tissue handling and robust nature after sterilisation

Sterile cultures from root explants were unsuccessful. The contamination was likely to stem from internal mycorrhiza fungi, which was observed within the stained roots (not shown).

9.2.2 Sterilisation and placement on media

Two sets of petiole sterilisation solutions were tested:

Set I: 10, 20, and 100% (v/v) commercial bleach for 10 and 20 minutes

Set II: 5, 10, and 20% (v/v) commercial bleach for 10 and 20 minutes

At all concentrations of bleach, callus and shoots developed within four weeks on MS (Murashige and Skoog, 1962) basal media. Shoots developed the best with the 10% (v/v) bleach sterilisation regime. Callus and shoots developed mostly on the petiole explants with polarity; callus developed on the petiole closest to the root, whilst shoots formed on the opposite end. Roots developed from the callus end after three weeks. Even after surface sterilisation with commercial bleach at 100% (v/v) (equivalent to 5% (w/v) sodium hypochlorite as used by Huizing *et al.*, 1983), surprisingly shoot and callus were still formed, but was the slowest. Overall 10% (v/v) bleach for 10 minutes was the chosen sterilisation regime for subsequent experiments as it achieved 95-100% sterile explants with the ability to produce *de novo* tissue.

Over the course of the sterilisation tests, two additional observations were made relating to explant handling: (i) 95-100% sterile cultures were achieved without removing the hairy epidermal tissue, (ii) after one week, the longitudinal cut face of explants (placed down on the MS media) turned black (probably due to polyphenols) and no callus/shoot developed along this face, and (iii) if the explant half was thin or was completely immersed in the media, callus and shoot development was either too slow or failed to develop. Considering all these observations, the hairy epidermal tissue on petiole explants was not removed in subsequent experiments. Petioles were also not cut longitudinally and care was taken not to immerse petioles deeply into the media. This method produced a consistently strong growth response with minimal steps.

9.3 Media development

9.3.1 Callus induction media (CIM): sterilised petiole explants

- (i) MS A: MS basal nutrients
- (ii) MS B: MS basal nutrients + 2% (w/v) sucrose
- (iii) MS C: MS B + BA (1 mg L^{-1})
- (iv) MS D: MS B + BA (1 mg L^{-1}) + IBA (0.01 mg L^{-1})
- (v) MS E: MS B + BA (1 mg L^{-1}) + IBA (0.1 mg L^{-1})
- (vi) MS F(0): MS B + BA (1 mg L^{-1}) + IBA (1 mg L^{-1})

Of the initial six MS-based media above [MS A to F(0)], MS F(0) was consistently the standout medium for callus generation from sand-grown petiole explants (grown from root cuttings). This medium comprised of MS basal nutrients, 2%

(w/v) sucrose and 1 mg L^{-1} each of BA and IBA. For all media tested, callus formed with polarity, at the petiole end closest to the root. The callus tissues were compact and had a light to dark green colour. Incidentally, it was also excellent for shoot induction from petiole explants (Plate 17A1-A2). After two weeks in each media (except for MS basal medium), shoots also started to develop at the opposing end to the callus tissue. The *in vitro* generated shoot tissues also had a crown segment (base of shoot emerging from petiole explant) (Plate 17A1). Shoot tissue developed from the vascular bundle of sterilised petiole explants (Plate 17B). Also worth noting is that unlike the sterilisation regime, petioles on the MS basal medium in this experiment did not produce callus or shoots. The different outcomes could be because the longitudinal cut petioles used in the sterilisation experiment absorbed nutrients (to support callus or shoot formation) more effectively from the basal medium. All callus, shoot, and root tissue are *de novo* as petiole explants were cut well above the meristematic crown of the sand-grown plants.

Concurrently, petiole explants from plants grown from *root divisions* were tested because of the number of available petioles and the good response from sterilisation trials. However from these trials, these petioles were far less consistent and vigorous in generating *in vitro* callus and shoot tissue. This is possibly because these petioles were older than those from root cuttings (Plate 17C1-C2).

The differing response of MS C to MS F(0) was in contrast to that reported by Huizing *et al.*, (1983) who ranked shoot and callus growth from these media as the same. This is possibly because of differences in petiole explant origin, age or sterilisation regime.

9.4 Callus growth media (CGM): *in vitro* petiole tissue

- (i) MS F(0): MS B + BA (1 mg L^{-1}) + IBA (1 mg L^{-1})
- (ii) MS F(1): MS B + BA (2.5 mg L^{-1}) + IBA (2.5 mg L^{-1})
- (iii) MS F(2): MS B + BA (3.5 mg L^{-1}) + IBA (3.5 mg L^{-1})
- (iv) MS F(3): MS B + BA (5.5 mg L^{-1}) + IBA (5.5 mg L^{-1})

Unlike petiole explants on MS F(0), callus formation in *in vitro* explants occurred along the edge of the petiole *and* at the cut end. Some parts of these calli, differentiated into root and shoots (Plate 18A). Since undifferentiated callus tissue was initially the target tissue type for somaclonal variation studies, other plant growth regulator-based media were tested to determine if only callus tissue could be generated from petioles. It has

been documented that increasing concentrations of plant growth regulators such as IBA and BA may inhibit shoot and root development from the callus tissue (Haaß *et al.*, 1991). Thus, using *in vitro* grown petiole segments, three variations to the MS F(0) media for generating undifferentiated callus from petioles were tested. These were MS F(1,2,3) (listed above). On these three media, callus tissue appeared larger than on the MS F(0) medium and root generation was perturbed. However, shoot production was not inhibited (Plate 18B).

Consequently, the focus was on inhibiting differentiation of callus after formation. Calli generated from petiole on MS F(0) medium were transferred to fresh MS F(0) medium to continue callus growth. However, it still could not maintain its undifferentiated callus state. After four weeks, shoot tissue clearly developed from the callus tissue (Plate 19A). Using *in vitro* callus tissue and petiole segments, two other callus growth media were tested:

- (i) MS J [MS B + Kinetin (0.3 mg L^{-1}) + NAA (4 mg L^{-1})]
- (ii) MS G [MS B + 2,4 D (0.5 mg L^{-1}) + BA (2 mg L^{-1})]

The MS J medium (from Abou-Mandour, 1983) successfully inhibited both shoot and root organogenesis from the callus tissue (Plate 19B). On the MS G medium (from Huizing *et al.*, 1987) the callus tissue formed was similar in appearance to those formed on MS J medium, except that only shoot development was inhibited. Thick, stunted root tissue still developed after four weeks on MS G medium (not shown). However, the callus morphology generated from both these media indicated that the callus tissues were unsuitable for subculture as they were very soft, friable and died upon subculture to the same media (not shown).

Given the lack of undifferentiated callus tissue that could be subcultured, the callus approach was abandoned in favour of *de novo* shoot formation from Pb-treated petioles (see results section 10.1 below).

Plate 17. MS-based callus induction media (CIM) trials after four weeks (petiole explants from sand-grown plants).

A1. (left to right) MS A, B, C (petiole explants from root cutting plants)

A2. (left to right) MS D, E, F(0) (petiole explants from root cutting plants)

B. *De novo* shoot developing from vascular bundle of petiole explant.

C1. (left to right) MS A, B, C (petiole explants from root division plants)

C2. (left to right) MS D, E, F(0) (petiole explants from root division plants)

Composition of media:

MS A: MS basal nutrients

MS B: MS basal nutrients + 2% (w/v) sucrose

MS C: MS B + BA (1 mg L^{-1})

MS D: MS B + BA (1 mg L^{-1}) + IBA (0.01 mg L^{-1})

MS E: MS B + BA (1 mg L^{-1}) + IBA (0.1 mg L^{-1})

MS F(0): MS B + BA (1 mg L^{-1}) + IBA (1 mg L^{-1})

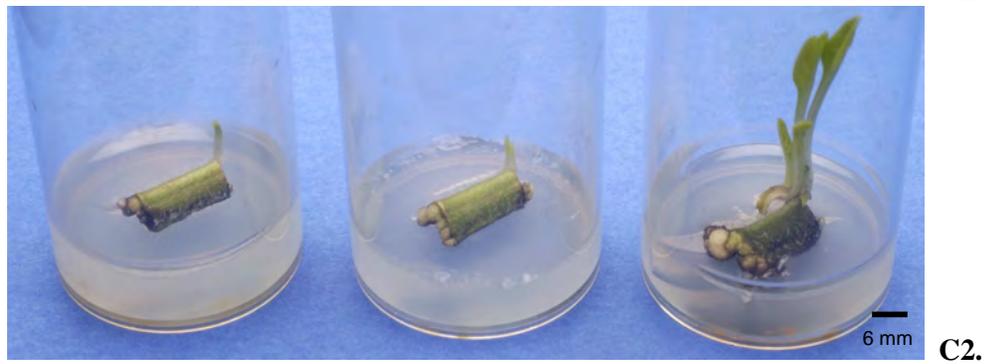
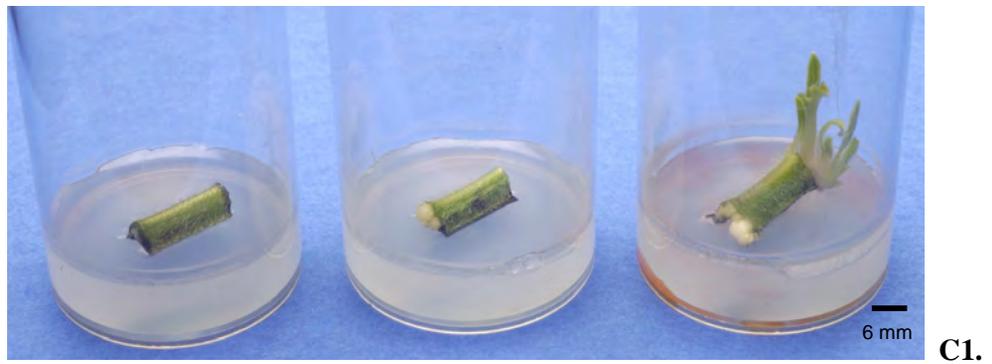
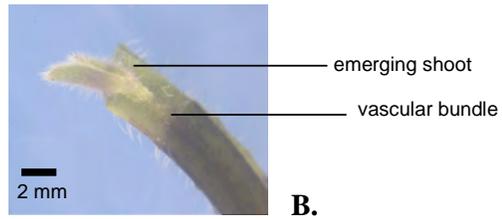
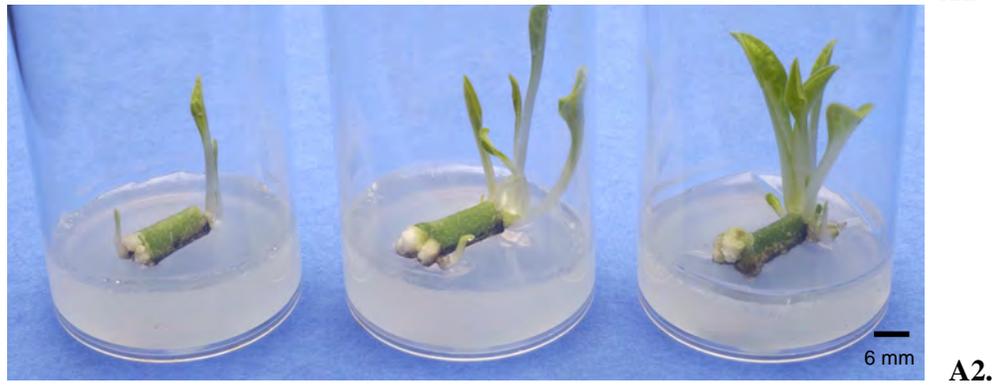
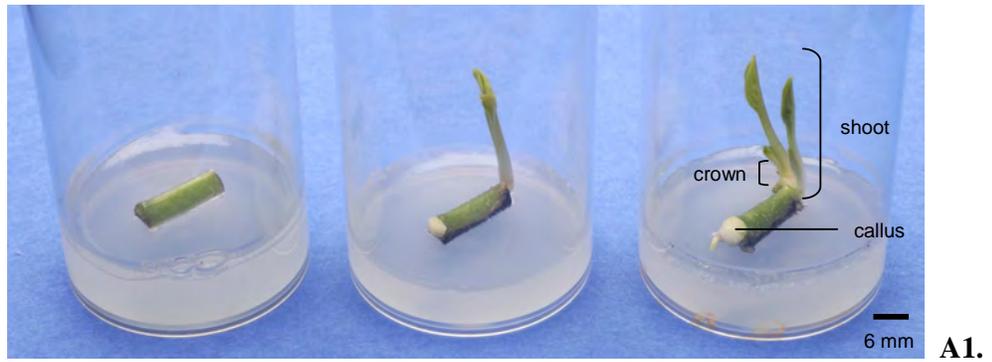


Plate 18. Callus and shoot growth (from *in vitro* petiole tissue) after four weeks.

Callus growth media (CGM) were:

A. MS F(0)

(red arrow: white roots emerging from petiole and callus)

B. (left to right) MS F(1), MS F(2), MS F(3)

Composition of media:

MS F(0): MS B + BA (1 mg L^{-1}) + IBA (1 mg L^{-1})

MS F(1): MS B + BA (2.5 mg L^{-1}) + IBA (2.5 mg L^{-1})

MS F(2): MS B + BA (3.5 mg L^{-1}) + IBA (3.5 mg L^{-1})

MS F(3): MS B + BA (5.5 mg L^{-1}) + IBA (5.5 mg L^{-1})

(MS B: MS basal nutrients + 2% (w/v) sucrose)

Plate 19. Callus growth (from *in vitro* callus tissue) after four weeks.

Callus growth media (CGM) were:

A. MS F(0)

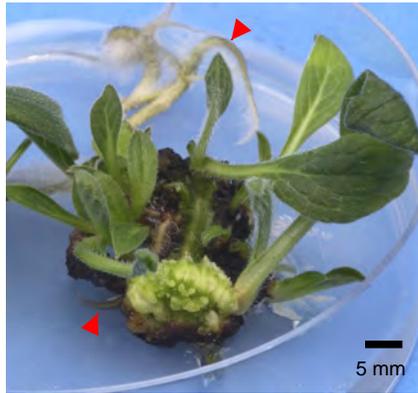
B. MS J

Composition of media:

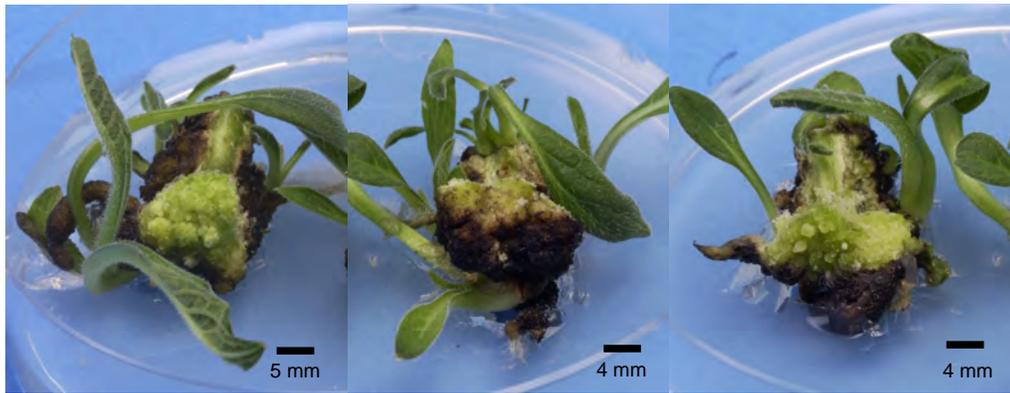
MS F(0): MS B + BA (1 mg L^{-1}) + IBA (1 mg L^{-1})

MS J: MS B + Kinetin (0.3 mg L^{-1}) + NAA (4 mg L^{-1})

(MS B: MS basal nutrients + 2% (w/v) sucrose)



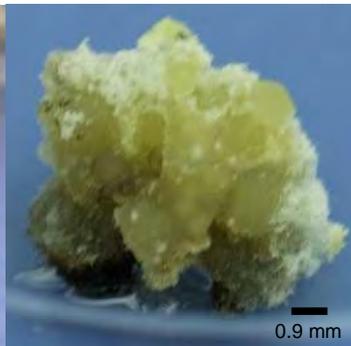
A.



B.



A.



B.

9.5 Root and whole plant induction media (RIM, WPIM)

9.5.1 Root induction media (RIM): *in vitro* shoots

- (i) MS A: MS basal nutrients
- (ii) MS B: MS basal nutrients + 2% (w/v) sucrose
- (iii) MS H: MS A + IBA (1 mg L⁻¹)
- (iv) MS L: MS B + IBA (1 mg L⁻¹)

Since there was not enough crown tissue at this stage of experimentation, shoots (leaf blade attached to petiole) were used instead to test the RIM media. They were immersed approximately 0.5 cm into four types of media; MS A, B, H and L (listed above). Roots readily formed in all media, but were the best on MS L (not shown).

9.5.2 Whole plant induction media (WPIM): *in vitro* crown tissue

- (i) MS L: MS B + IBA (1 mg L⁻¹)
- (ii) MS M: MS B + IBA (0.1 mg L⁻¹)
- (iii) MS N(0): MS B + IBA (1 mg L⁻¹) + BA (0.1 mg L⁻¹)
- (iv) MS N(1): MS B + IBA (1 mg L⁻¹) + BA (0.2 mg L⁻¹)
- (v) MS N(2): MS B + IBA (1 mg L⁻¹) + BA (0.4 mg L⁻¹)
- (vi) MS E: MS B + IBA (0.1 mg L⁻¹) + BA (1 mg L⁻¹)

Both roots and shoots were induced from *in vitro* crown tissue on the MS L medium. Thus for the crown tissue, this RIM medium was also a good whole plant induction medium (WPIM). However, petioles generated were very long. Modifications to the MS L medium were performed to determine if the abnormally long *in vitro* petioles could be shortened to better reflect non-tissue cultured plant structure and possibly the shoot:root distribution of Pb. In order to decrease the petiole length, the plant growth regulator composition of the MS L medium was modified and *in vitro* crown tissue on six different media were tested (listed above). Auxins (e.g. IBA) cause developmental changes such as cell elongation and formation of roots. Cytokinins (e.g. BA), the ‘opposite’ plant growth regulator to auxins, promotes opening of existing or generating new buds (Gamborg and Phillips, 1995). Decreasing IBA levels or increasing the cytokinin (BA) level was tested to reduce the petiole/cell elongation effect of IBA.

Lowering the IBA concentration from 1 mg L⁻¹ to 0.1 mg L⁻¹ in the MS M medium did not appear to affect the petiole length of *in vitro* *S. officinale* plants. From the medium set, MS L, M, N(0,1,2,3) and E, only the MS E medium (containing 1 mg L⁻¹ BA and 0.1 mg L⁻¹ IBA) decreased the petiole length (Plate 20A, B and C). However this medium could not be used to generate plants because it severely inhibited root growth, which would be important for Pb accumulation experiments (Plate 21). Consequently, the idea of attaining shorter petioles to reflect ‘normal’ *in vitro* plant structure was not further pursued. Plants could resume ‘normal’ structure once planted out to the environment.

It was interesting to note that relative to the MS L medium, MS N(0,1,2) and MS E media all produced much more shoots from the crown. As early as two weeks into subculture, the addition of the BA plant growth regulator to these media appeared to generate hydrated tissue from the base of the crown, from which adventitious shoots emerged (Plate 22A). When crowns were cut and examined under a stereomicroscope, addition of the BA brought about the development of at least one additional crown from the original one (Plate 22B and C). In future subcultures the hydrated tissue subsided, but additional crown formation still occurred.

The readiness of shoot and root development from the crown tissue on MS N(0) made this medium an excellent whole plant growth medium. When sufficient number of plants were generated from the crowns of petioles, whole plants were propagated by directly subdividing the crowns (when large enough) from *in vitro* whole plants (onto fresh MS N(0) medium) every 4-5 weeks. This removed the need for a separate root induction step. The overall steps involved in the generation of *in vitro* *S. officinale* plants are outlined in Plate 23.

9.6 MS N(0)/ F(0) liquid media

In Pb pre-treatment regimes it would be more practical to use liquid media to treat plant and tissues with Pb. However, callus and shoot production from *in vitro* petioles in MS F(0) liquid medium were not uniform or as responsive as the previous solid medium (agar) responses (Plate 24A). On MS N(0) liquid medium (without agar), crowns produced poorly structured whole plants compared to those on the solid medium (Plate 24B). This was also true for half strength MS N(0) and after addition of PVPP to reduce polyphenol toxicity (not shown). Thus, all propagations used solid media.

Plate 20. Shoot induction media (SIM) trials after four weeks
(from *in vitro* crown tissue).

- A. (left to right) MS L, MS M
- B. (left to right) MS N(0), MS E
- C. (left to right) MS N(1), MS N(2)

Composition of media:

MS L: MS B + IBA (1 mg L^{-1})

MS M: MS B + IBA (0.1 mg L^{-1})

MS N(0): MS B + IBA (1 mg L^{-1}) + BA (0.1 mg L^{-1})

MS E: MS B + IBA (0.1 mg L^{-1}) + BA (1 mg L^{-1})

MS N(1): MS B + IBA (1 mg L^{-1}) + BA (0.2 mg L^{-1})

MS N(2): MS B + IBA (1 mg L^{-1}) + BA (0.4 mg L^{-1})

(MS B: MS basal nutrients + 2% (w/v) sucrose)



A.



B.



C.

Plate 21. Root growth from shoot induction media (SIM) trials after four weeks
(from *in vitro* crown tissue).

- A. MS L
- B. MS M
- C. MS N(0)
- D. MS E

Composition of media:

MS L: MS B + IBA (1 mg L^{-1})

MS M: MS B + IBA (0.1 mg L^{-1})

MS N(0): MS B + IBA (1 mg L^{-1}) + BA (0.1 mg L^{-1})

MS E: MS B + IBA (0.1 mg L^{-1}) + BA (1 mg L^{-1})

(MS B: MS basal nutrients + 2% (w/v) sucrose)



A.



B.



C.



D.

7 mm

- Plate 22.** Shoot and crown growth (from *in vitro* crown tissue).
- A. Adventitious shoot growth after two weeks on MS N(0)
 - B. Crown development on MS L medium after four weeks
 - C. Crown development on MS N(0) medium after four weeks

Composition of media:

MS L: MS B + IBA (1 mg L^{-1})

MS N(0): MS B + IBA (1 mg L^{-1}) + BA (0.1 mg L^{-1})

(MS B: MS basal nutrients + 2% (w/v) sucrose)



A.



B.



C.

Plate 23. Overall steps in the generation of *in vitro* *Symphytum officinale* plants.

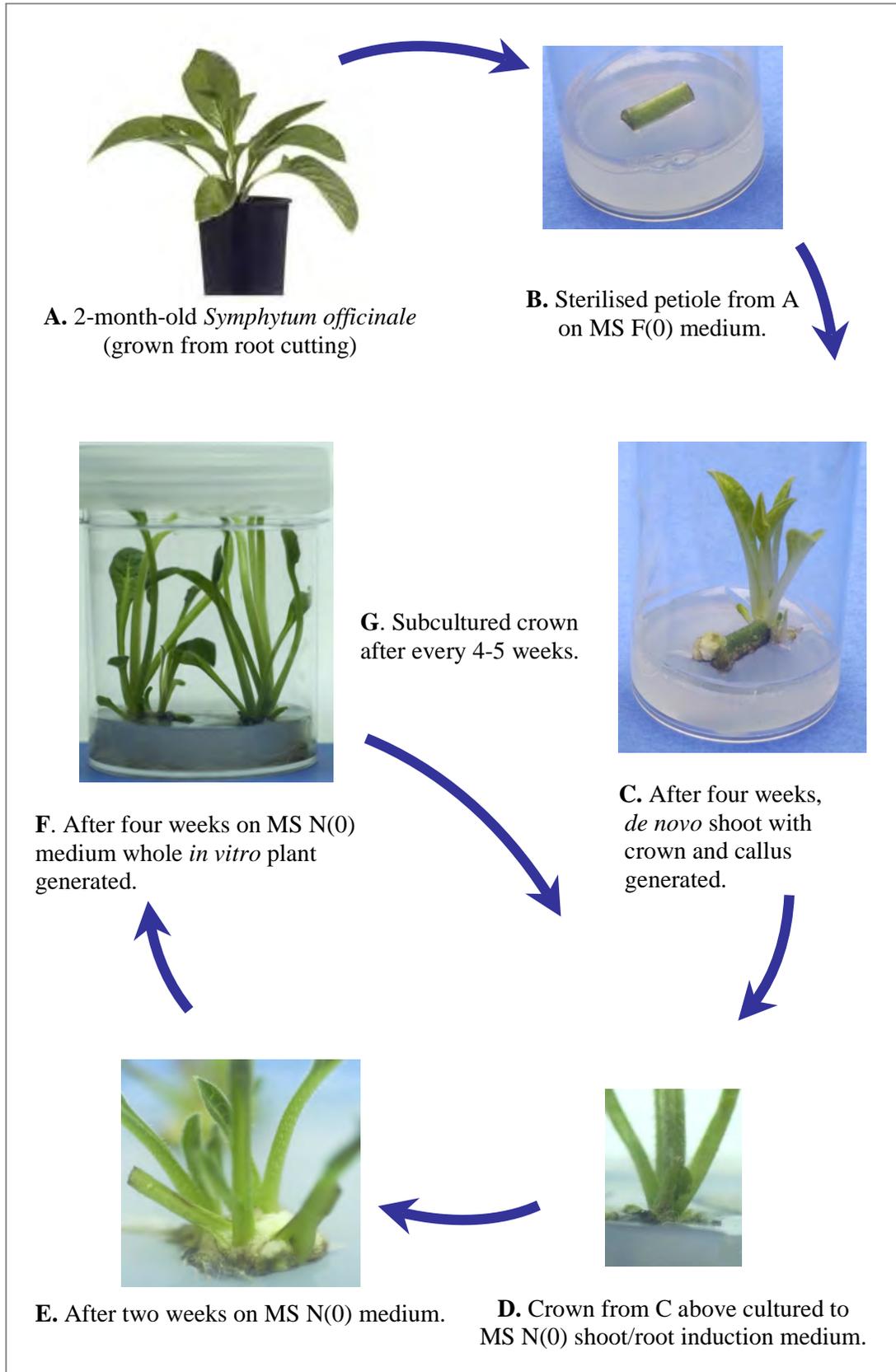


Plate 24. Liquid media trials.

- A. Differing responses of *in vitro* *Symphytum officinale* petioles (from one plant line) to MS F(0) liquid medium.
- B. Shoot and root growth from *in vitro* *Symphytum officinale* crown tissue in liquid (left) and solid agar-based (right) MS N(0) medium.



A.



B.

10 DEVELOPING *SYMPHYTUM OFFICINALE* LEAD MUTANTS

In this section the somaclonal variation approach was to directly generate *de novo* shoot tissue from Pb-treated petioles (from whole plants or shoot cuttings). The ideal outcome of this ‘pulse/gradual treatment’ was to generate plants with higher Pb accumulation ability through increased Pb tolerance. The Pb accumulation level, polyphenol level and superoxide dismutase activity of *S. officinale* plants (generated from Pb pre-treated petioles) after re-treatment with Pb were assessed to determine if and how mutation(s) occurred. Histochemical localisation for superoxide radicals and indirect assay of SOD activity (using NBT tissue staining) was also envisaged.

10.1 Lead treatment of whole *in vitro* plants

Petioles from whole plants pre-treated with chelated Pb (500 μ M PbEDTA and PbADA) were initially chosen for culture to closely resemble normal Pb accumulation and translocation conditions. No sucrose was initially added to the media to maximise stress. Considering that Pb toxicity in plants occurs in a range of 30-300 mg kg⁻¹ (d.w.) Pb (Zheljazkov and Fair, 1996) to 500 to 1000 mg kg⁻¹ Pb (d.w.) (Wierzbicka, 1995) (tissue unspecified), the levels of Pb in *in vitro* petioles and roots from PbEDTA or PbADA treatments (see 10.1.3 below) may be sufficiently toxic to generate mutants.

10.1.1 Pre-treatment # 1: 0, 500 μ M PbEDTA (no sucrose, pH 4.5)

Only one of the four lines (Line green) developed shoots from both control and PbEDTA treatments. These shoots developed very slowly compared to petioles from plants without any pre-treatment (i.e. directly from MS N(0)). Two months (compared to one month) was required to develop shoots sufficient for culture. In other lines, petiole contamination was evident at the surface and cut ends of the petiole explant, possibly due to HC treatment stress (both control and Pb) (Plate 25A).

10.1.2 Pre-treatment # 2: 0, 500 μ M PbEDTA (no sucrose, pH 7.0)

A similar experiment was performed, but in this case petioles were re-sterilised after pre-treatment. Surface contamination was still evident after one week (both control and Pb treatment), but in this case, it did not preclude the development of shoots in any of

the lines. Similar to pre-treatment #1, shoots still developed, though much slower than petioles directly from MS N(0) media.

10.1.3 Pre-treatment # 3: 0, 500 M PbEDTA and 500 M PbADA, (2% (w/v) sucrose, pH 7.0)

Pre-treated petioles were visibly healthy for up to three weeks, indicating that the nutritional stress (due to the omission of sucrose in the previous pre-treatments) may be partly responsible for the lower regeneration capacity of the petiole. However, no shoots developed. Instead, roots developed along the petiole length but mainly towards the root-facing end of the petiole. Very small or no callus formed in the cultures.

Unexpectedly after three weeks, the healthy-looking petiole and roots appeared to release white exudates from the cut ends and roots. These are likely to be endogenous microbes because it appeared to engulf and contaminate the culture over time (Plate 25B).

In terms of Pb accumulation from pre-treatments, plants exposed to PbADA appeared to accumulate more Pb in roots than those exposed to PbEDTA treatment (although not significantly different), supporting the findings in objective 2 (8.3.2). The levels of Pb in the shoots from both treatments were about 100-200 mg kg⁻¹ (d.w.). The lack of statistical significance between these treatments may be due to lower transpiration rates in the enclosed tissue culture system. Both PbEDTA and PbADA had significantly higher levels of Pb than control plants (Figure 42A, B and C)

10.2 Regeneration ability of stock plant tissues

Petioles from pre-treated whole plants did not develop shoots adequately (even in control treatments). Consequently, the shoot regeneration ability of stock plants (after ten subcultures) was re-checked to confirm that the treatment regime (and not the stock culture) caused the lack of shoot regeneration. Shoot generation from petioles of stock plants were re-tested and were still excellent after ten subcultures (Plate 26). Thus, the petiole contamination and lack of shoot regeneration appears to be due to the stress of the treatment regime on whole plants. The omission of sucrose (initially) and the response of roots to the liquid medium used may be responsible. At the completion of the pre-treatment regime, the liquid medium had started to turn slightly brown (possibly due to polyphenols).

Plate 25. Shoot generation from *in vitro* petioles (from Pb pre-treated whole plants). HC = Huang and Cunningham.

A. Pre-treatment # 1

Shoot regeneration from *in vitro* petioles after two months on MS F(0) medium. Petioles were from 4-week-old *in vitro* *Symphytum officinale* plants pre-treated with modified HC nutrient solution (pH 4.5) containing 0 (left) or 500 μ M PbEDTA (no sucrose) for one week.

B. Pre-treatment # 3

Progression of contamination of *in vitro* petioles on MS F(0) medium after three weeks in culture. Petioles were from 3-week-old *in vitro* *Symphytum officinale* plants pre-treated with modified HC nutrient solution (pH 7.0) with PbADA (2% (w/v) sucrose) for two weeks.

Plate 26. Regeneration ability of petioles from stock plants.

Test for shoot regeneration ability of *in vitro* *Symphytum officinale* petioles from stock plants (untreated, directly from MS N(0) medium). Shoot growth after six weeks on MS F(0) medium.



A.



B.



A.

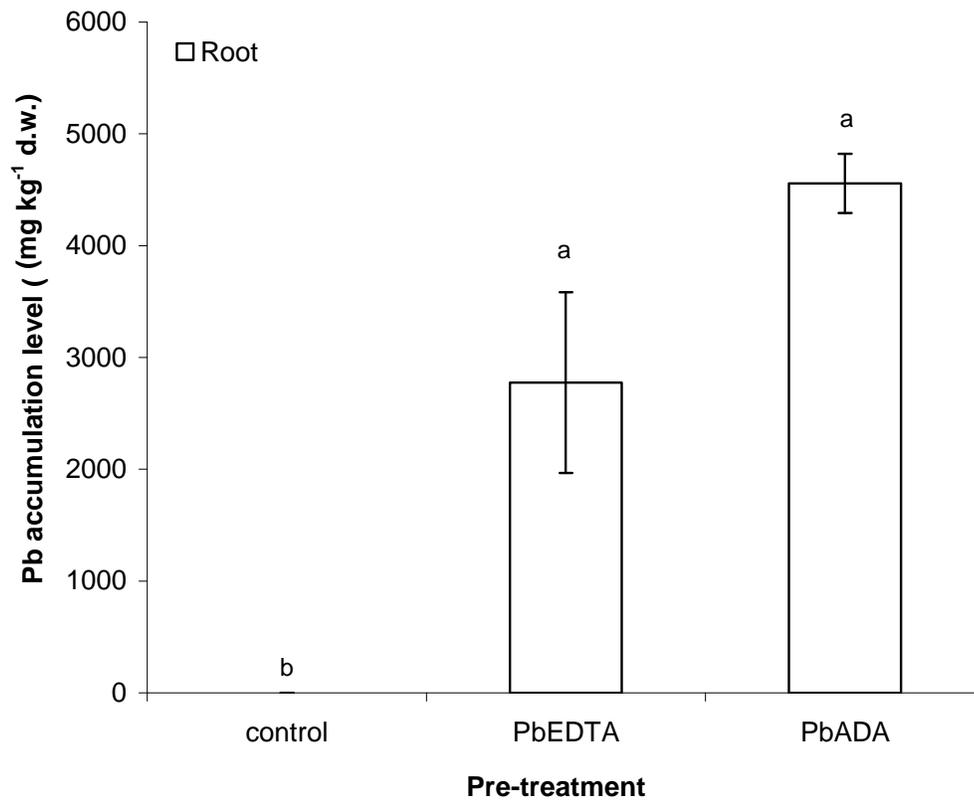


Figure 42A. Pb accumulation level in 3-week-old *Symphytum officinale* roots of whole plants pre-treated for 14 days in modified HC nutrient solution (pH 7.0) containing 2% (w/v) sucrose with 0 (control), or 500 μM $\text{Pb}(\text{NO}_3)_2$ with or without 500 μM EDTA or ADA. All plants were grown and pre-treated under *in vitro* conditions. Values are mean \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). HC = Huang and Cunningham.

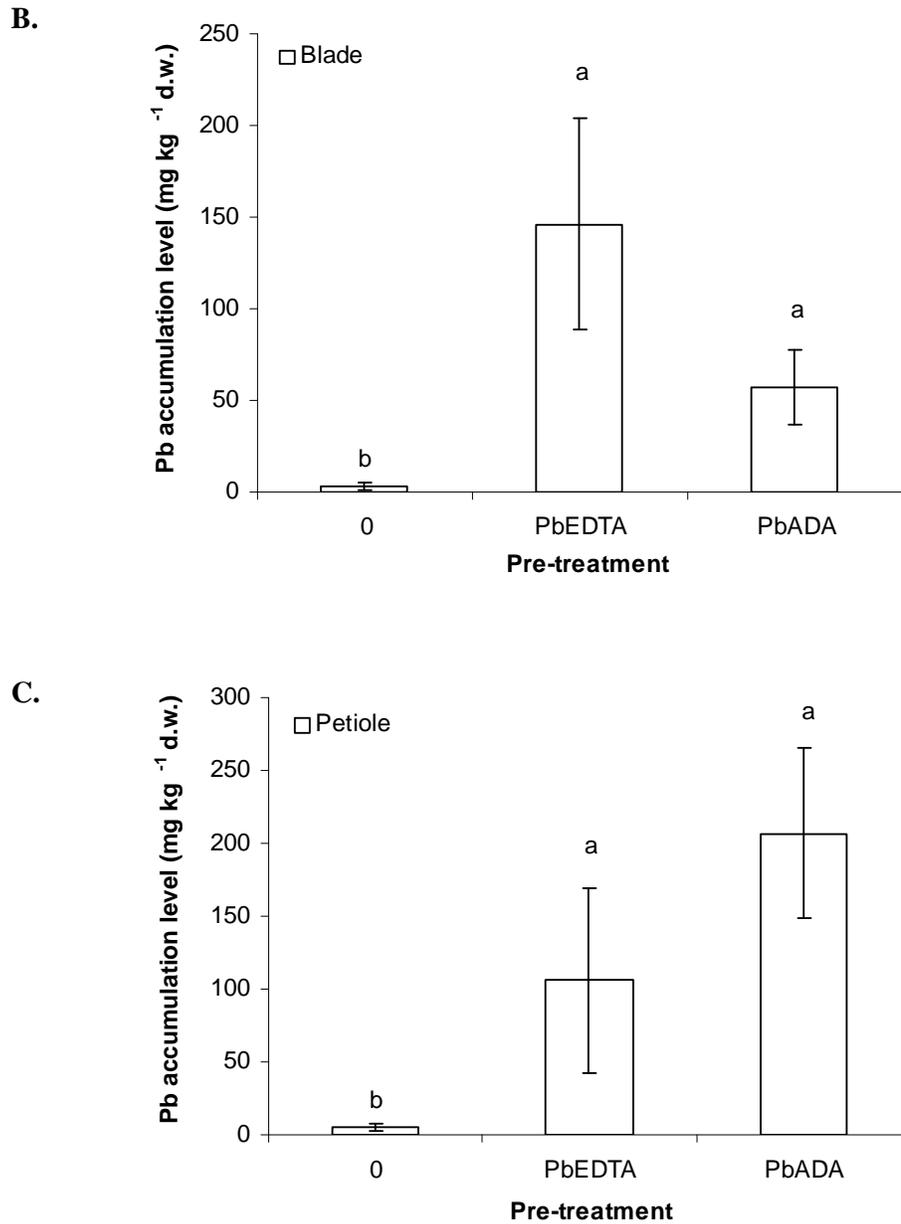


Figure 42B and C. Pb accumulation level in 3-week-old *Symphytum officinale* (B) blade and (C) petiole tissue of whole plants pre-treated for 14 days in modified HC nutrient solution (pH 7.0) containing 2% (w/v) sucrose with 0 (control), or 500 μM $\text{Pb}(\text{NO}_3)_2$ with or without 500 μM EDTA or ADA. All plants were grown and pre-treated under *in vitro* conditions. Values are mean \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). HC = Huang and Cunningham.

10.3 Lead pre-treatment of shoot cuttings

Petioles from Pb pre-treated whole plants (in the previous section) did not develop shoots probably due to stress-induced contamination. Consequently, several changes were made to the pre-treatment regime.

- (i) shoot cuttings (blade plus petiole), not whole plants were used
- (ii) direct Pb treatment for one week
- (iii) exposure to chelated and unchelated Pb (see results section 10.3.3 for rationale)

10.3.1 Pre-treatment of sand-grown (non-sterile) petioles

Due to the length of time involved before the results of 10.3 above could be analysed, the following investigation was carried out simultaneously to the experiment in 10.3. Petioles from shoot cuttings of sand-grown plants (non-sterile) were treated with modified HC nutrient solution (pH 7.0) with and without 500 M PbADA (2% (w/v) sucrose). However, these petioles became contaminated after the sterilisation step, and even on shoot generation media containing 0.05% (v/v) PPM (an anti-microbial agent called Plant Preservative Mixture) (not shown). The use of non-sterile shoots and nutrient media containing Pb may have facilitated microbial growth through the petiole cut end and/or increased contamination on the petiole surface, making it difficult to achieve surface sterilisation. This justifies the use of *in vitro* plant materials and sterile conditions for Pb pre-treatment regimes (10.3.2 to 10.3.3).

10.3.2 Pre-treatment # 4: 0, 100, 250 and 500 M Pb(NO₃)₂ (pH 4.5) (*in vitro* petioles)

Unlike control treatments, the cut face of the petiole base blackened with Pb treatment. Apart from a few minor black spots on the surface of the 4.5-week-old petiole, the one cm base of these petioles were not as severely damaged as the 3-week-old petioles used in the next treatment (not shown).

After four weeks, formation of callus and shoots were clearly visible in all petioles regardless of pre-treatment (Plate 27A). A set of petioles also grew much slower (Plate 27B).

Plate 27. Pre-treatment # 4: Shoot generation from *in vitro* petioles (from Pb pre-treated shoots). HC = Huang and Cunningham.

- A. Shoot regeneration from *in vitro* *Symphytum officinale* petioles after six weeks on MS F(0) media. Petioles were from *in vitro* *Symphytum officinale* shoot cuttings (4.5-week-old plants) pre-treated with modified HC nutrient solution (pH 4.5) containing 2% (w/v) sucrose with (from left to right) 0, 100, 250 or 500 μ M Pb(NO₃)₂ for one week. All petioles shown were from line red.

- B. Same as 26A above, except that growth of this set was noticeably slower.



A.



B.

10.3.3 Pre-treatment # 5: 0, 500 M Pb(NO₃)₂ and 500 M PbADA (pH 4.5) (*in vitro* petioles)

Two forms of Pb (unchelated and chelated with ADA) were used as the stress factors for somaclonal variation. The unchelated form was used because metals in this form, such as Al and Fe, are more toxic in culture than its chelated counterpart (Conner and Meredith, 1985a and b). The likely reason is that unchelated metals generate more free radicals that can increase the chances of mutations. According to Levine (2000), ROS can cause DNA breaks and increase mutation rates, a major driving force in plant evolution. Additionally, it would be of interest to test the recognition of Pb pre-treatment form (chelated vs. unchelated) upon re-treatment.

After one week of pre-treatment, Pb stress was observed at the petiole base of the shoot tissue (Plate 28A). In control solution, the one cm petiole base of shoots rooted and turned red, possibly reflecting active antioxidative defence activity at the cut end. Shoots in all Pb solutions did not root. In unchelated Pb treatments, two out of the three petiole bases blackened severely. Based on the Pb levels in the petiole (see results section 12.1.1), only the first three pre-treatments were chosen for shoot regeneration: 0 (control), 500 M Pb(NO₃)₂, 500 M Pb(NO₃)₂ with 500 M ADA (Pb:ADA, 1:1 molar ratio).

Within two weeks all petioles, regardless of treatment, developed callus of varying sizes at the root pole end. After six weeks, shoots developed from the callus ends, along the petiole length and sometimes at the shoot end. The development of a callus largely depended on the size of the petiole cut end; the larger the petiole width, the larger the callus. Examples of the best growth responses are shown in Plates 28B and C; the photos shown are *not* representative of the trend observed across the treatments. Across treatments, there appeared to be no consistent visible differences in shoot size, callus size or morphology. Two months on the MS F(0) medium were required to obtain a sufficient number of shoots in all treatments, before transferring to MS N(0) medium. This took twice as long than anticipated. Despite this, more importantly no contamination such as those observed in pre-treated whole plants (results section 10.1), occurred. Shoot and callus growth were successfully generated from Pb-pre-treated petioles of shoot cuttings. The shoot cuttings also successfully developed into whole plants on the MS N(0) medium (Plate 29). The question of whether there was enough Pb in the cultured petiole to induce somaclonal variation is discussed in Chapter 4.

10.4 Lead levels in pre-treated *in vitro* shoot cuttings

10.4.1 Pre-treatment # 4: 0, 100, 250 and 500 M Pb(NO₃)₂, pH 4.5

In unchelated Pb treatments it was initially, in error, thought that the 500 M Pb(NO₃)₂ pre-treated petioles used for culture contained up to 4642 mg kg⁻¹ (d.w.) Pb (Figure 43A). However, the rapid (within four weeks) development of shoots from these petioles suggested otherwise. Whilst the Pb level was accurately measured, it did not reflect the level of Pb in the petioles cultured. Upon investigation, Pb was unevenly distributed in the petiole tissue; most of the Pb was in the base of the petiole. Since more petiole base was discarded prior to tissue culture than for Pb analysis, the Pb level in the 4.5-week-old petioles were actually not significantly different compared to the control.

However, the Pb level found in the blade was significant (Figure 43B). The blade tissue contained 50 mg kg⁻¹ Pb, four times higher ($P \leq 0.05$) than the control. Although the cultured petiole did not contain significant levels of Pb, it is possible that it may have been indirectly affected by the high concentration of Pb at the base of the petiole and by the Pb that pass through the petiole to the blade. Additionally, these Pb levels meet the plant toxicity range of Pb (30-300 mg kg⁻¹), but whether this amount is large enough to indirectly affect the parameters measured is discussed below.

10.4.2 Pre-treatment # 5: 0, 500 M Pb(NO₃)₂ and 500 M PbADA, pH 4.5

As with the above experiment, the Pb level in the petiole of 500 M Pb(NO₃)₂ (unchelated Pb) pre-treated shoot cuttings were not significantly higher than the control plants. However unlike the above experiment (10.4.1), no Pb accumulated in the blade of the shoot cuttings. This may be because the shoot petioles were thinner than the 4.5-week-old shoots used above, making them more susceptible to Pb toxicity. Unlike the above experiment, the petiole bases of these shoot cuttings were severely damaged after one week of exposure to unchelated Pb (pre-treatment). This would likely hinder transport of Pb and possibly other nutrients to the remaining shoot tissue (Plate 28A). This was the only sign of toxicity compared to the control treatment.

The 500 M PbADA pre-treatment (1:1 molar ratio) may have contained sufficiently toxic levels of Pb to increase the chances of inducing mutation(s). The petiole used for culture (with the petiole base discarded), contained 393 mg kg⁻¹ Pb

(d.w.) whilst the blade contained an average of 317 mg kg⁻¹ Pb (d.w.) The Pb level in 500 M PbADA (1:2 molar ratio) pre-treated shoots were slightly higher, but not significantly different. The 1:1 molar ratio of PbADA treatment was chosen for culture because there was more Pb in the petiole due to less Pb translocation to the blade (compared to the 1:2 molar ratio pre-treatment) (Figure 44).

Plate 28. Pre-treatment # 5: Effect of Pb exposure on petioles and shoot regeneration from petioles. HC = Huang and Cunningham.

A. Petiole base from 3-week-old *in vitro* shoots treated for one week with modified HC nutrient solution (pH 4.5) containing 2% (w/v) sucrose with (from top to bottom):

0 (control)

500 M $\text{Pb}(\text{NO}_3)_2$ (Pb)

500 M $\text{Pb}(\text{NO}_3)_2$ and 500 M ADA (PbADA 1:1)

500 M $\text{Pb}(\text{NO}_3)_2$ and 1000 M ADA (PbADA 1:2)

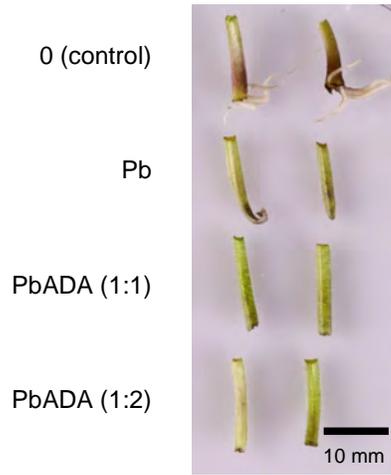
B. Shoot regeneration from *in vitro* petioles after two weeks on MS F(0). Petioles were from *in vitro* *Symphytum officinale* shoot cuttings (from 3-week-old plants) pre-treated for one week with modified HC nutrient solution (pH 4.5) containing 2% (w/v) sucrose with (from left to right):

0 (control)

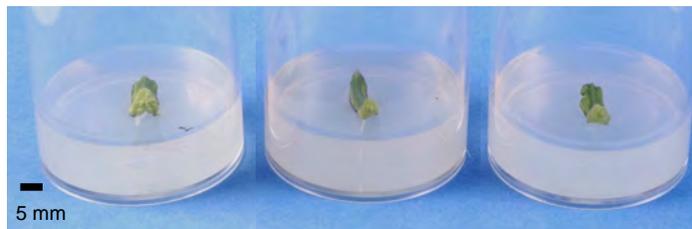
500 M $\text{Pb}(\text{NO}_3)_2$ (Pb)

500 M $\text{Pb}(\text{NO}_3)_2$ and 500 M ADA (PbADA 1:1)

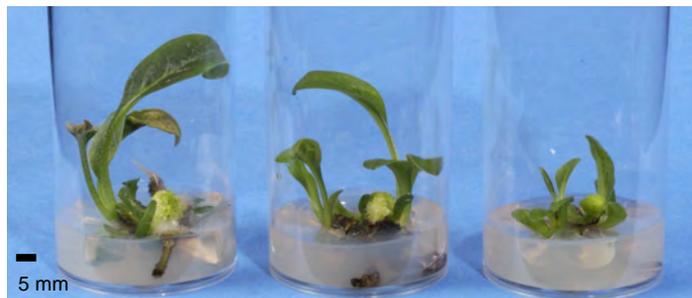
C. Same as 28A, except at six weeks on MS F(0).



A.



B.



C.

Plate 29. Pre-treatment # 5: Whole plant generation.

In vitro *Symphytum officinale* plants generated on MS N(0) after one month. Plants were developed from petioles of shoot cuttings pre-treated with modified HC (pH 4.5) containing 2% (w/v) sucrose only (left) or containing 500 μ M PbADA (right). Plants pre-treated with 500 μ M Pb(NO₃)₂ was analogous the plants shown here.

HC = Huang and Cunningham.



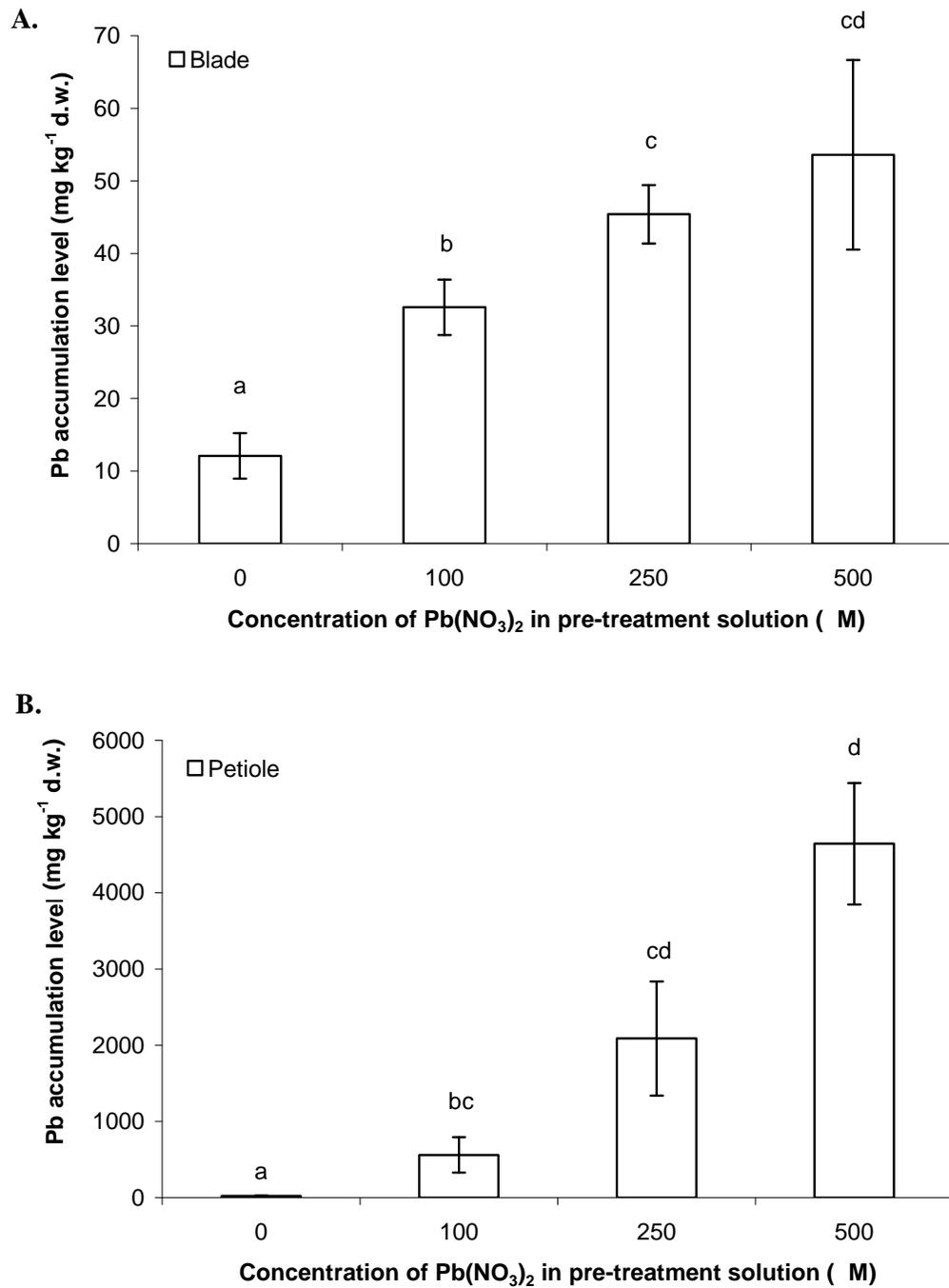


Figure 43. Pb accumulation level in 4-week-old *Symphytum officinale* (A) petiole and (B) blade tissue of shoot cuttings after pre-treatment for seven days in modified HC nutrient solution (pH 4.5) containing 2% (w/v) sucrose with 0 (control) to 500 M $Pb(NO_3)_2$. All tissues were grown (from plants) and pre-treated under *in vitro* conditions. Values are mean \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). HC = Huang and Cunningham.

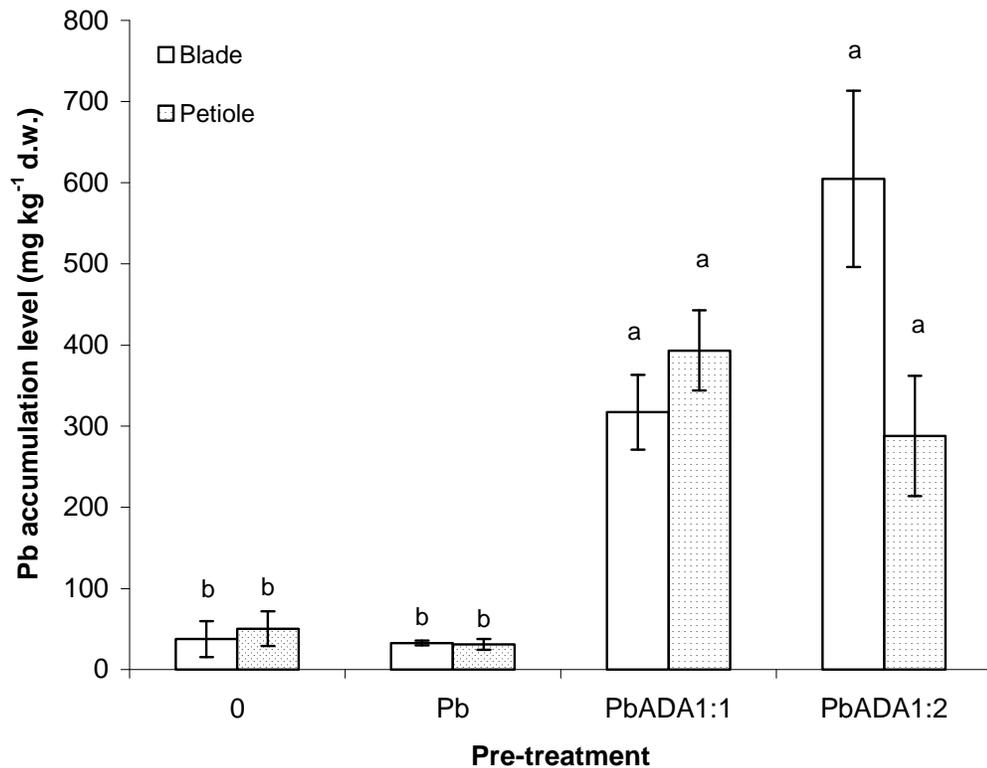


Figure 44. Pb accumulation level in 3-week-old *Symphytum officinale* shoot cuttings (blade and petiole) after pre-treatment for seven days in modified HC nutrient solution (pH 4.5) containing 2% (w/v) sucrose with 0 (control), 500 M $\text{Pb}(\text{NO}_3)_2$ (Pb), 500 M $\text{Pb}(\text{NO}_3)_2$ and 500 M ADA (PbADA 1:1), or 500 M $\text{Pb}(\text{NO}_3)_2$ and 1000 M ADA (PbADA 1:2). All tissues were grown and pre-treated under *in vitro* conditions. Values are mean \pm S.E. of four replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). HC = Huang and Cunningham.

11 INDICATOR TESTS FOR POTENTIAL MUTANTS

11.1 Histochemical detection of superoxide anions

11.1.1 Plant segments (preliminary NBT protocol design)

The aim was to determine the effect of treatment solutions (dH₂O, 500 μ M and 1000 μ M of Pb(NO₃)₂, H₂O₂, EDTA and PbEDTA) on *in vivo* superoxide anion generation (an indirect indicator of SOD levels) in petiole sections and leaf discs (using the NBT reaction) because these are the target tissues of chelate-assisted Pb phytoextraction.

The main difficulty encountered in this experiment was attaining consistent results. This was due to several factors. Firstly, the petiole and leaf sections were covered in trichomes and a cuticle, making it difficult for the treatment solution and subsequent NBT to enter the tissue, even with lengthy vacuum infiltration. Secondly, it appeared that wounding of the tissue (by cutting and immediate infiltration of the tissue in the treatment solution) produced superoxide anions which masked any effects of the treatment solution. Thirdly, the chlorophyll removal methods affected visual staining.

To overcome these difficulties, petiole sections were incubated in dH₂O for one hour after sectioning and before exposure to treatment solution, to allow any wound response to subside. Secondly, petiole sections were not infiltrated. Thirdly, uncut root tips (using whole tissue cultured plants) were examined. Finally, the chlorophyll removal method was altered to boiling petiole sections in 70% (v/v) ethanol for 5-10 minutes, as opposed to heating at 40°C for up to one hour in 96% (v/v) ethanol.

In both petiole sections and leaf discs, only high concentrations of Pb(NO₃)₂, EDTA and PbEDTA (500 μ M and higher) appeared to decrease superoxide anion levels after 24 hours; this was reflected by a decrease in purple staining relative to tissues without Pb treatment (dH₂O). The decrease in superoxide anion levels may be due to an elevated ROS-induced antioxidative response, or more likely, cell death because the leaf discs (in particular) changed from a healthy green to an olive colour after Pb treatment. The most visible difference in purple staining was observed in the vascular bundle of petioles. However the main control for this experiment, H₂O₂, was unreliable. The concentration used (500 μ M) was too low to reduce superoxide anion levels, so these preliminary findings in the end were not meaningful. However, this preliminary experiment did help develop the more reliable NBT staining method below.

11.1.2 Whole plants: root tissue staining patterns

The effects of various treatments on roots of whole *in vitro* *S. officinale* plants are summarised in Table 8 (below) and illustrated in Plate 30. Treatments appeared to affect three zones in the root tip, the meristematic zone (MZ), distal-transition zone (DTZ), and the elongation zone (EZ). Maltais and Houde (2002) identified these zones in studies with maize and aluminum (Al) (Figure 45A).

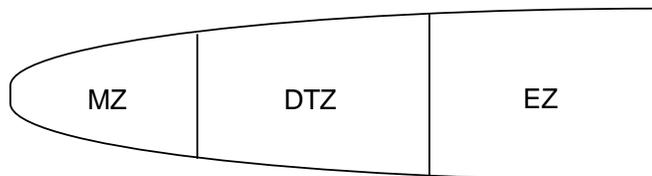


Figure 45A. Root tip zones affected by treatments in Table 8: meristematic zone (MZ), distal-transition zone (DTZ) and elongation zone (EZ).

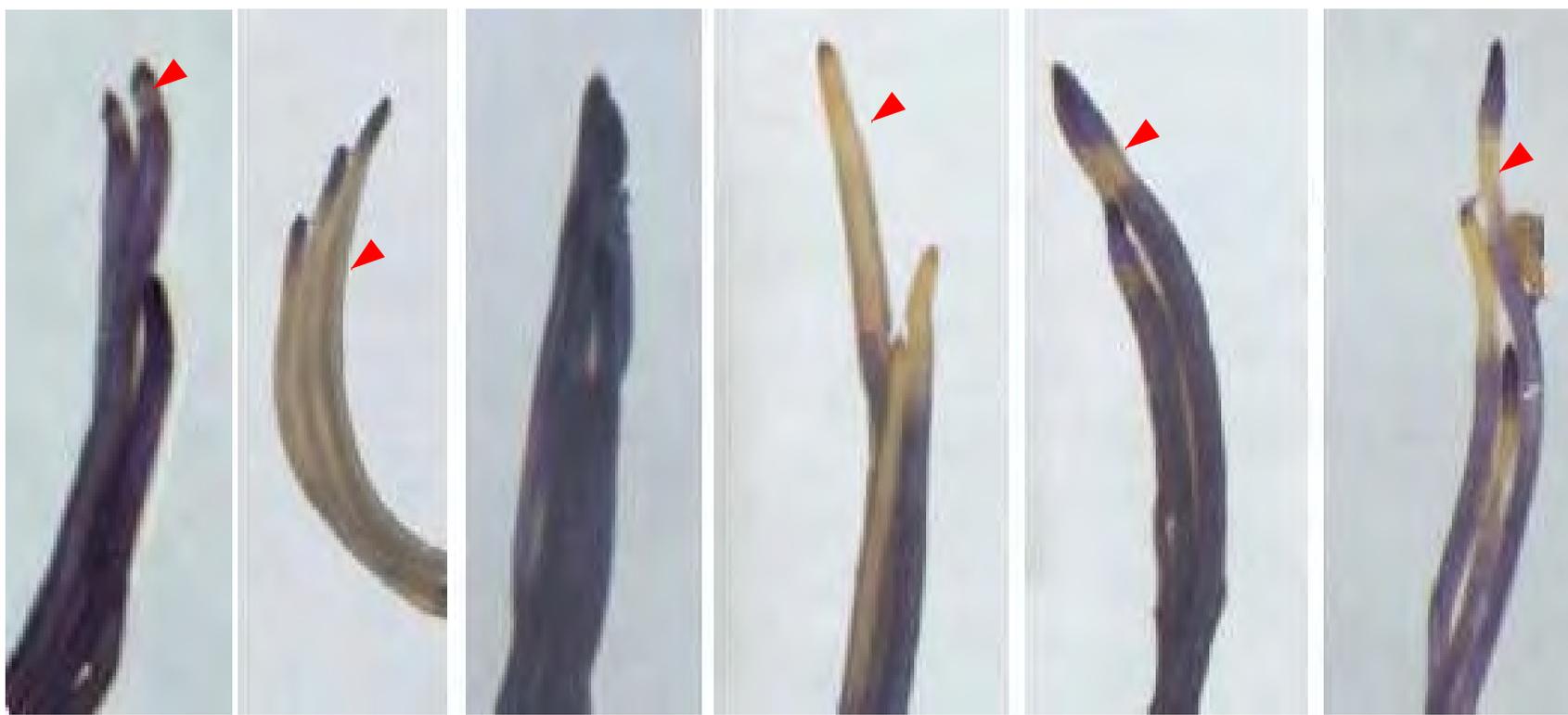
11.1.3 Overall interpretations

Overall, the decrease in endogenous root tip staining may reflect a decrease in superoxide anion levels (Figure 45B). In the case of H_2O_2 , this decrease is likely due to oxidative stress/cell death (a stage where superoxide anion production by ROS-generating enzymes, such as NADPH oxidases, cease) because the purple staining on the entire root was reduced. In Pb treatments (unchelated and chelated), the decrease in purple staining could also be due to oxidative stress or cell death. However, it is more likely due to an increase in ROS destruction (i.e. activation of the antioxidative defense systems such as superoxide dismutase or antioxidants) because reduction in staining was only localised to the root tip. Unchelated Pb treatments resulted in a greater loss of purple staining from root tips than chelated Pb treatments. As observed in objective 2, this possibly reflects the higher Pb concentration in the root tissue of unchelated treatments.

Table 8. Effect of various treatments on superoxide anion levels in roots of *in vitro* *Symphytum officinale* plants (also see Plate 30). All tissues were from *S. officinale* plants grown under *in vitro* conditions and treated for 24 hours under non-sterile conditions.

Treatment	Observation	Possible interpretation
Control 1: dH ₂ O at pH 3.5 and 6.0	Most stained purple throughout root tissue. In some roots a small region of the DTZ was not stained and appeared constricted	Endogenous superoxide anion level present in tissue.
Control 2: 5 mM H ₂ O ₂	Significantly reduced purple staining throughout root.	Reduction in overall superoxide anion level: oxidative stress/ cell death
250 M EDTA	All roots stained purple throughout root tissue.	Possible increase in superoxide anion free radicals level as no clearing observed in DTZ compared with control, but insufficient to activate antioxidative defense response.
250 M Pb(NO ₃) ₂	No purple staining at root tips only.	Loss in staining at only the root tips indicates that Pb reduced superoxide anion production at the MZ and DTZ region only: activated antioxidative defense response or caused cell death.
PbEDTA (1:1 ratio) 250 M Pb(NO ₃) ₂ , 250 M EDTA	No purple staining just behind the root tip, in the DTZ. Root also appears to be constricted in this zone.	As above for Pb, except that with EDTA there appears to some alleviation to the toxic effect of just Pb (as staining now observed just behind in the DTZ).
PbEDTA (1:2 ratio) 250 M Pb(NO ₃) ₂ , 500 M EDTA	As above, except lack of staining in the DTZ appears to have lengthened.	As above for PbEDTA, except more superoxide anions and toxicity at higher EDTA concentration; stronger antioxidative response than at 1:1 ratio.

0.5 mm



dH₂O
pH 6.0

H₂O₂
5 mM

EDTA
250 μM

Pb(NO₃)₂
250 μM

PbEDTA (1:1)
250 μM Pb(NO₃)₂
250 μM EDTA

PbEDTA (1:2)
250 μM Pb(NO₃)₂
500 μM EDTA

Plate 30. NBT localisation of superoxide anions (purple staining) in roots. Red arrows indicate region devoid of purple NBT stain (reflecting a decrease in superoxide anion levels relative to the dH₂O control).

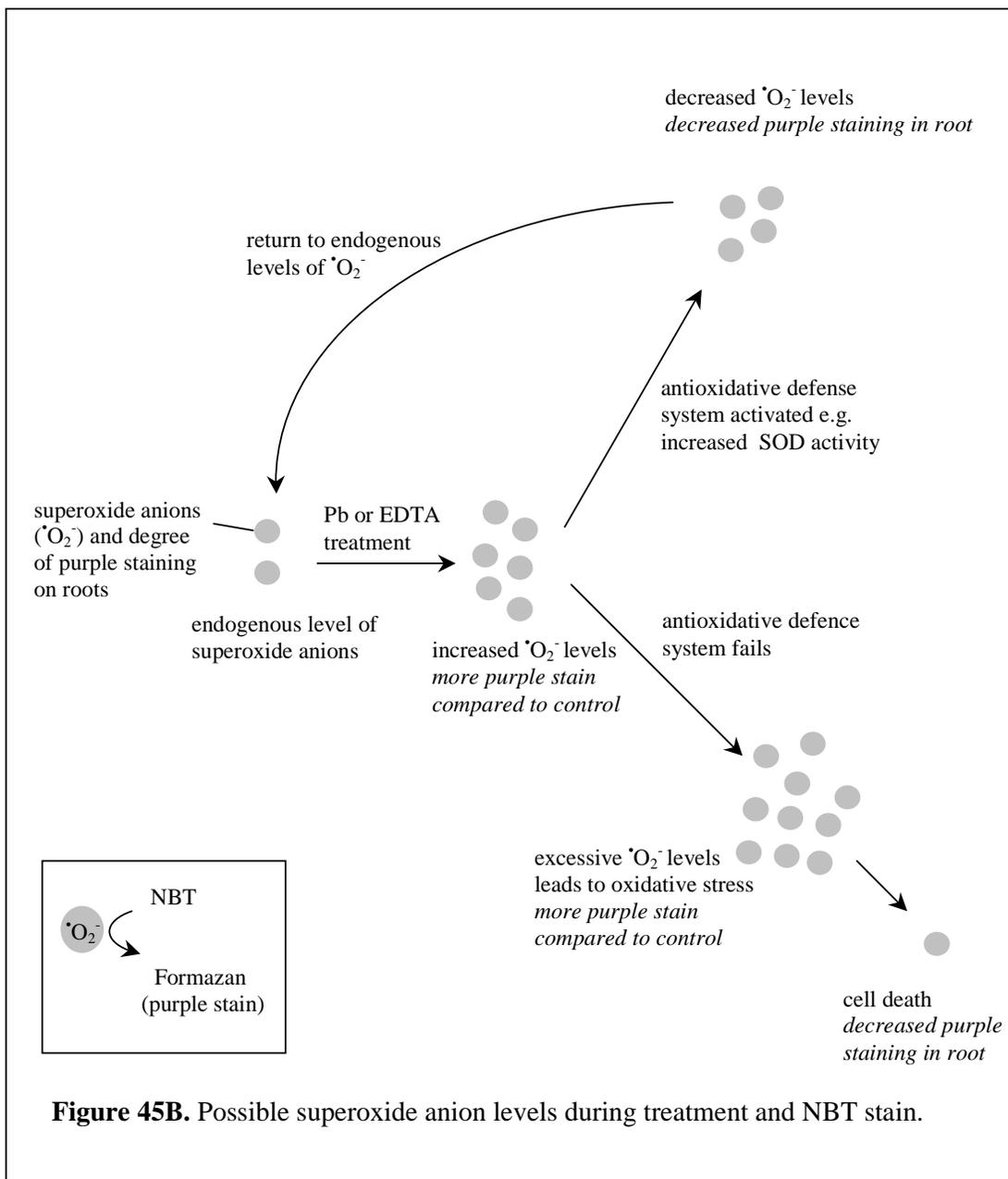


Figure 45B. Possible superoxide anion levels during treatment and NBT stain.

Interestingly however, this does not apply when the two chelated Pb treatments are compared to each other. EDTA at twice the molar ratio of $\text{Pb}(\text{NO}_3)_2$ appeared to induce a stronger antioxidative defence response (more loss in purple staining) than at the 1:1 molar ratio of these reagents. This was unexpected given that less Pb in the root tissue (due to the higher EDTA concentration, see objective 2) should in theory lead to less superoxide anion production and therefore less antioxidative defence activity. The observed results may be because 500 μM EDTA (alone or in synergy with Pb ions) actually increased superoxide anions and consequently a stronger antioxidative

response. This increased superoxide anion production may be supported by the root response to the lower EDTA concentration. At 250 μ M, EDTA treatment appeared to increase the superoxide anion levels (more purple staining).

Overall, the root staining patterns were consistently replicable and more reliable than shoot tissue (petiole section or leaf discs). They were also much simpler to handle than shoot tissue, as they did not require cutting or vacuum infiltration (to allow the stain to pass through the leaf cuticle) or chlorophyll removal (for the purple stain to be visible). Moreover, Pb appears to accumulate mostly in the root tissue and is the first site of Pb contact with the plant. Considering all these factors, intact roots of whole plants for localisation of superoxide anions (via the NBT method) of any mutants found in the next section was chosen (despite the difference in treatments, due to the temporal differences in carrying out this and the next experiment).

11.2 Analysis of superoxide dismutase (SOD) activity

11.2.1 Extract stability

Superoxide dismutase activity in fresh blade tissue extracts were compared with those stored for one month at -80°C (Figure 46). No difference in activity was observed ($P \geq 0.05$), suggesting that the extracts were stable and could be stored for at least this length of time prior to analysis. This was critical given the number of samples to be analysed.

11.2.2 Extraction buffer

Three extraction buffers based around 100 mM potassium phosphate (pH 7.8) were tested to determine which was the best buffer to extract and retain SOD activity in blade tissue extracts:

- Buffer A: 100 mM potassium phosphate + 1% (w/v) PVPP, pH 7.8
- Buffer B: Buffer A + 0.25% (v/v) Triton X-100
- Buffer C: Buffer B + 10% (v/v) glycerol + 0.2 mM ADA + 2 mM DTT

The simplest buffer, Buffer A, resulted in the highest SOD activity. Triton X-100 is commonly added to extraction buffers (at 0.5-1% v/v) to help extract membrane-bound SOD enzymes, such as those in the mitochondria (Neil *et al.*, 2002; Bollag *et al.*, 1996; Polle *et al.*, 1989). However in this study, addition of 0.25% (v/v) Triton X-100 to

Buffer A (forming Buffer B) drastically reduced SOD activity; a 68% drop in activity was observed ($P \leq 0.05$, Figure 47A). This value is higher than in Figure 47B, perhaps due to experimental error in buffer preparation. However, the decreasing trend due to Triton X-100 remained. This was also true for 0.1% (v/v) Triton X-100 (Appendix H).

Addition of other additives to Buffer A (glycerol for stabilising enzyme under freezing conditions, DTT to minimise enzyme oxidation and 0.2 mM ADA to chelate potential metal-ion inhibitors) did not improve SOD activity in extracts from untreated blade tissue (Figure 47B). Relative to Buffer A, all these additives tended to reduce SOD activity. The precise reason for this is unknown.

As suggested by Fisher *et al.*, (2004), the addition of EDTA to buffers for SOD extraction of tissues containing high amounts of metals should be avoided. This is because metal-chelator complexes can form in the crude extracts and mimic SOD activity. This could be problematic, even if boiled controls were used. For example, if mimics were present, crude extracts from metal-treated tissues would require more dilution to achieve linear absorbencies for SOD activity, whereas crude extracts from untreated tissues (control) samples would not. This would lead to a discrepancy between treatments, even with boiled extracts, because such controls only correct within treatments not between. However, fortunately the addition of 0.625 mM EDTA to extraction Buffer A (referred to as Buffer D) did not appear to elevate SOD activity in the Pb-treated root extracts from objective 2 (Appendix I). Of the spikes tested, it appears that only FeEDTA and not PbEDTA can mimic SOD activity (Appendix J).

11.2.3 Distribution of SOD in *Symphytum officinale*

The distribution of SOD in *in vitro* *S. officinale* plants was in the following order, from highest to lowest: shoot blade (52%) > root (30%) > petiole (18%) (Figure 48). More SOD was found in the shoots because chloroplasts of shoot cells are one of the key sites for this enzyme (Greene, 2002). Blade extracts required a 10-fold dilution to achieve linear SOD activity, whilst roots and petioles only required a 2-fold dilution. Although SOD activity was lower in roots than the shoot blade, root SOD levels were measured in the following section (section 12) as this tissue contained most polyphenols and Pb in the plant. Shoot SOD activity was also to be analysed if mutants could be developed.

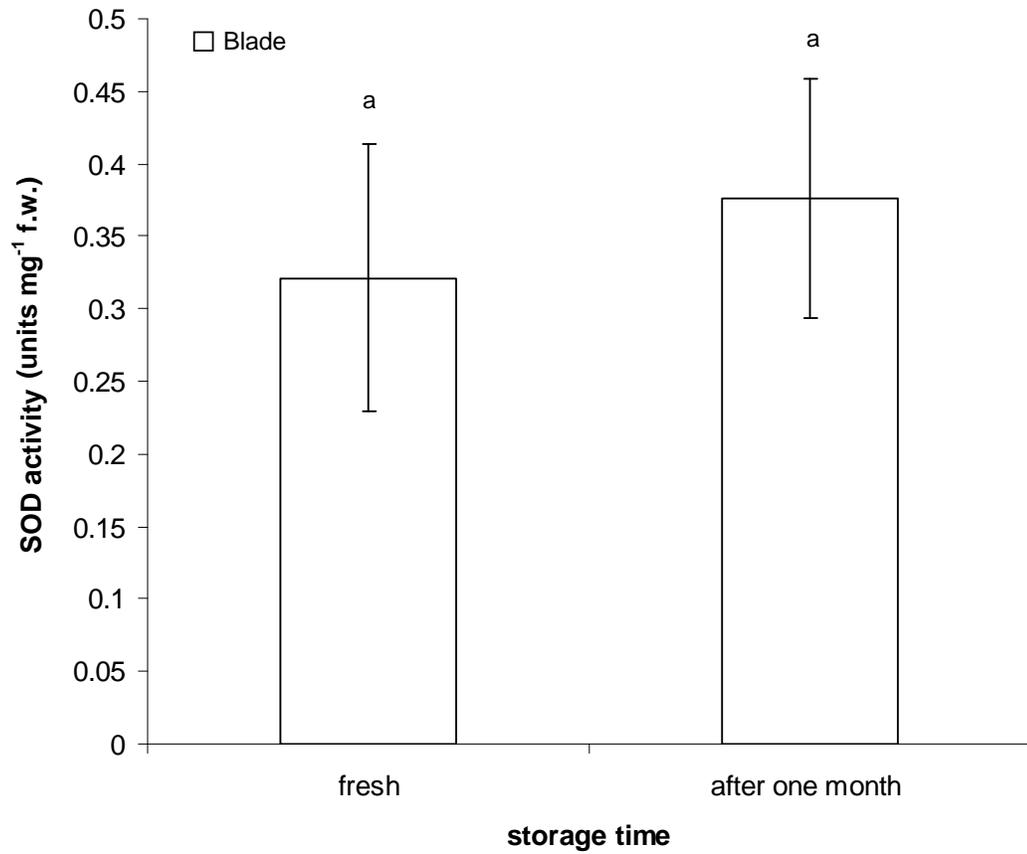


Figure 46. Stability of SOD extracts from blade tissue after fresh extraction and storage at -80°C . Values are means \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$).

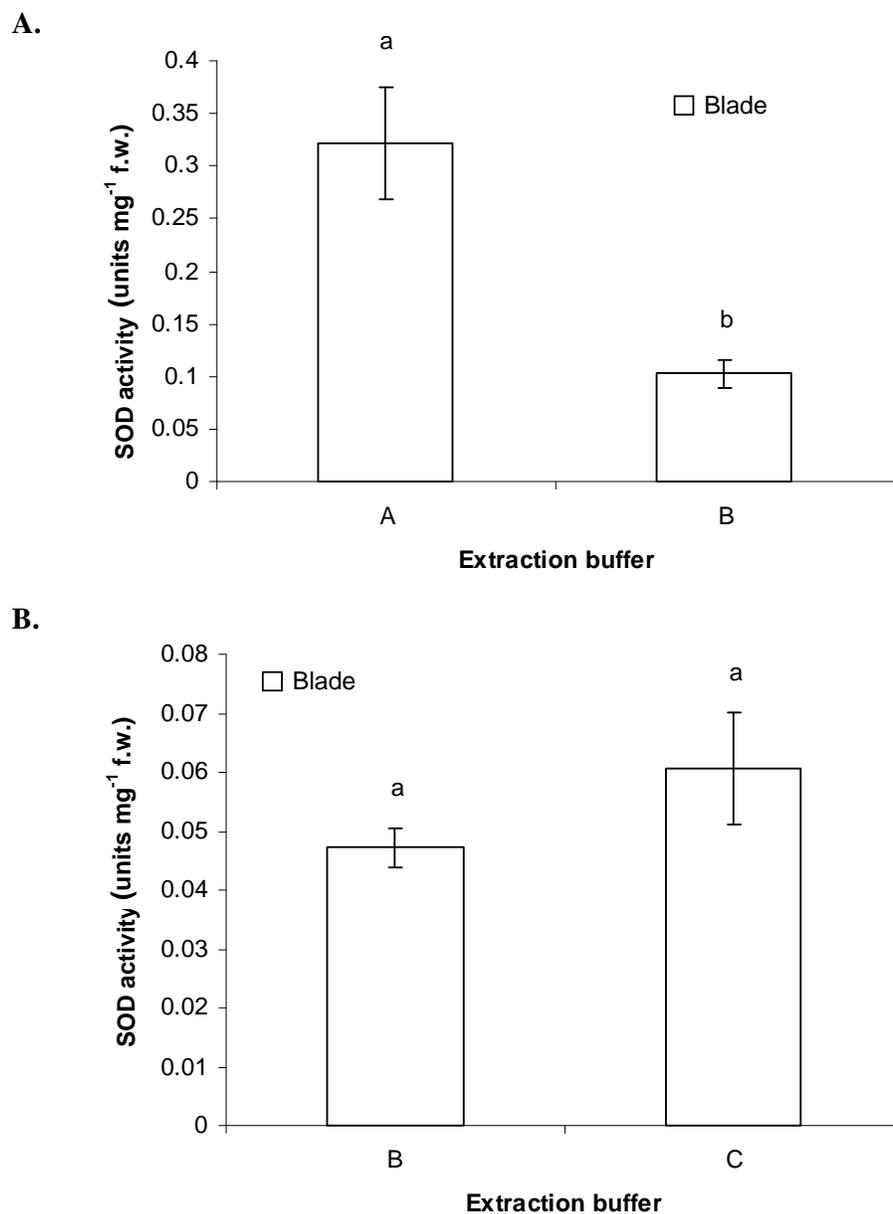


Figure 47. Comparison of different extraction buffers on SOD activity in blade tissue of *in vitro* grown *Symphytum officinale*. Values are means \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$).

Composition of buffers:

Buffer A: 100 mM potassium phosphate + 1% (w/v) PVPP, pH 7.8

Buffer B: Buffer A + 0.25% (v/v) Triton X-100

Buffer C: Buffer B + 10% (v/v) glycerol + 0.2 mM ADA + 2 mM DTT

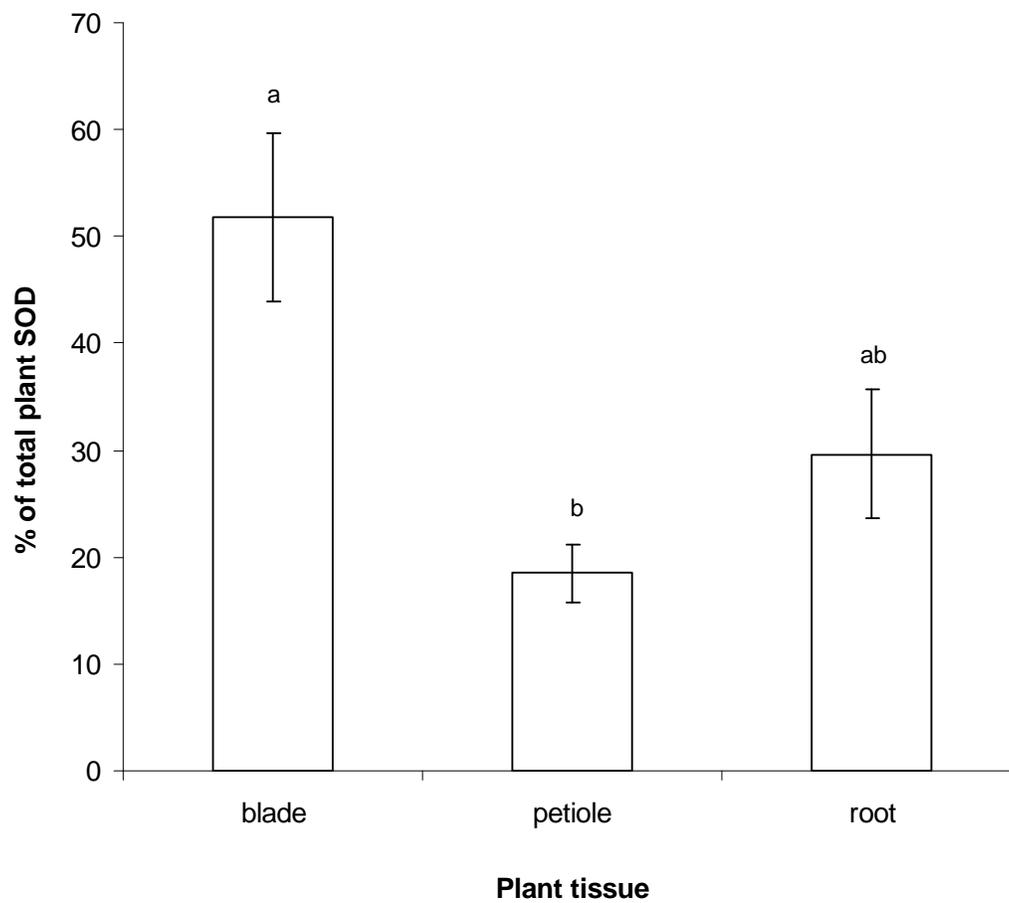


Figure 48. Distribution of SOD in *in vitro* grown *Symphytum officinale* plant tissues. Values are means \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$).

12 LEAD RE-TREATMENT

Significant differences due to Pb pre-treatment were observed in the morphology of plants pre-treated with unchelated Pb (12.1.3). Pb pre-treatments did not significantly affect other parameters (Pb, SOD and polyphenol levels) measured ($P \geq 0.05$). However, some promising trends were observed. These are discussed below.

(Note that all pre-treatments on *in vitro* plant material were performed under sterile conditions, whilst all re-treatments on *in vitro* plant material were performed in non-sterile conditions. All re-treatments used the modified HC nutrient solution).

12.1 Pre-treatment # 4

In this section, plants from petioles pre-treated with 0 or 500 μM $\text{Pb}(\text{NO}_3)_2$ (pH 4.5) (pre-treatment # 4), were re-treated with 0, 500 μM PbADA (pH 7.0) or 0, 500 μM $\text{Pb}(\text{NO}_3)_2$ (pH 4.5).

12.1.1 Effect of pre-treatment on lead, polyphenol and SOD levels upon re-treatment with 500 μM PbADA

In order to compare with 12.2 below, only trends in the form of percentages are discussed here rather than the actual Pb accumulation values because, unexpectedly, Pb was lost (via volatilisation) due to the furnace over heating (above 550°C).

In terms of trends, the levels (expressed as percentage) of the Pb, polyphenol and SOD parameters in Pb pre-treated plants were close to 100% of those without pre-treatment (Table 9). Hence pre-treatment appeared to have no effect on these parameters. In contrast to 12.2, the lack of a pre-treatment effect could be due to a lack of direct or indirect Pb stress, strong recovery from Pb stress or perhaps a shorter culture time (one month in this case compared to two months in the other re-treatments). The plants used were generated from those illustrated in Plate 27A. A lack of recognition to PbADA also cannot be ruled out because upon 500 μM $\text{Pb}(\text{NO}_3)_2$ (unchelated Pb) re-treatment (12.1.3), more defined trends were apparent.

Table 9. Pb, polyphenol and SOD activity trends in Pb pre-treated plants compared to plants without Pb pre-treatment. All plants were exposed (in re-treatment) to modified HC nutrient solution (pH 7.0) containing 500 μ M PbADA. Values are means \pm S.E. of three or four replicates. HC = Huang and Cunningham.

Parameter measured	% Change upon re-treatment (100% = no change)
Pb level (roots)	90.410 \pm 13.296
Pb level (shoots)	91.951 \pm 22.423
Polyphenol level	111.785 \pm 24.413
SOD activity	92.719 \pm 14.791

12.1.2 Effect of pre-treatment on lead, polyphenol and SOD levels upon re-treatment with 500 μ M Pb(NO₃)₂

Pb pre-treatment (500 μ M Pb(NO₃)₂) did not appear to significantly affect the plant's ability to cope with 500 μ M PbADA re-treatment, but how would it cope if the re-treatment solution (500 μ M Pb(NO₃)₂) was the same as the pre-treatment solution?

Unchelated Pb pre-treatment visibly affected the morphological appearance of the roots and shoots. Before re-treatment with unchelated Pb, and after three weeks in unleaded nutrient solution (longer than the normal one week time due to change in equipment availability), the number of lateral roots on the longest root of the plant was reduced by 63% ($P \leq 0.05$) (Figure 49A). At this stage the shoot morphology was not noticeably different. Only after re-treatment with unchelated Pb did this occur. After re-treatment, the blade and petiole length of the largest shoots on unchelated Pb-pre-treated plants were noticeably shorter than non-pretreated plants: by 26% and 20% on average respectively ($P \leq 0.05$) (Figure 49B). The general health of these plants appeared to be more stressed than non-Pb treated plants; their growth vigour was markedly reduced and leaves were necrotic (Plate 31). Roots of all plants were markedly brown by day seven of unchelated Pb treatment.

In terms of Pb accumulation, polyphenol and SOD levels in the roots, the same trends as the following experiment were observed (though smaller in magnitude). Pb levels appeared to decrease slightly (17% on average) (Figure 50A), some of which may

be due to a small increase in shoot Pb levels (Figure 50B). Similarly, polyphenol levels appeared to decrease (35%) and there was an increase in SOD activity (56%) (Figure 51A and B). No control (without Pb) was performed here as previous experiments showed that the Pb levels in re-treatment controls were the same, regardless of pre-treatment.

Clearly although there was not a significant amount of Pb in the petiole segment cultured, Pb, whether in the discarded petiole base, in the blade tissue or in the pre-treatment solution did *indirectly* affect plant growth. At a biochemical level, oxidative stress may have also occurred, considering the trends observed in the polyphenol and SOD levels (see Chapter 4). The indirect effects could be due to one or more of the other numerous documented effects of Pb on plant tissues. Examples include, reduced photosynthesis and transpiration rates due to inhibition of the electron transport chain in mitochondria and chloroplasts (Bazzaz *et al.*, 1974; Carlson *et al.*, 1975; Koeppe, 1981) and reduction of uptake and accumulation of ions that are critical to the plant health like calcium, phosphorous (Huang and Cunningham 1996) and nitrogen (Huang *et al.*, 1974; Lee *et al.*, 1976; Xiong *et al.*, 2006). Recognition of the form of Pb used in the pre-treatment upon re-treatment and longer culture time (two months in this case, because of the slower growth response of on MS F(0) media), could also be contributing factors. The plants used in this section were generated from those illustrated in Plate 27B (page 202).

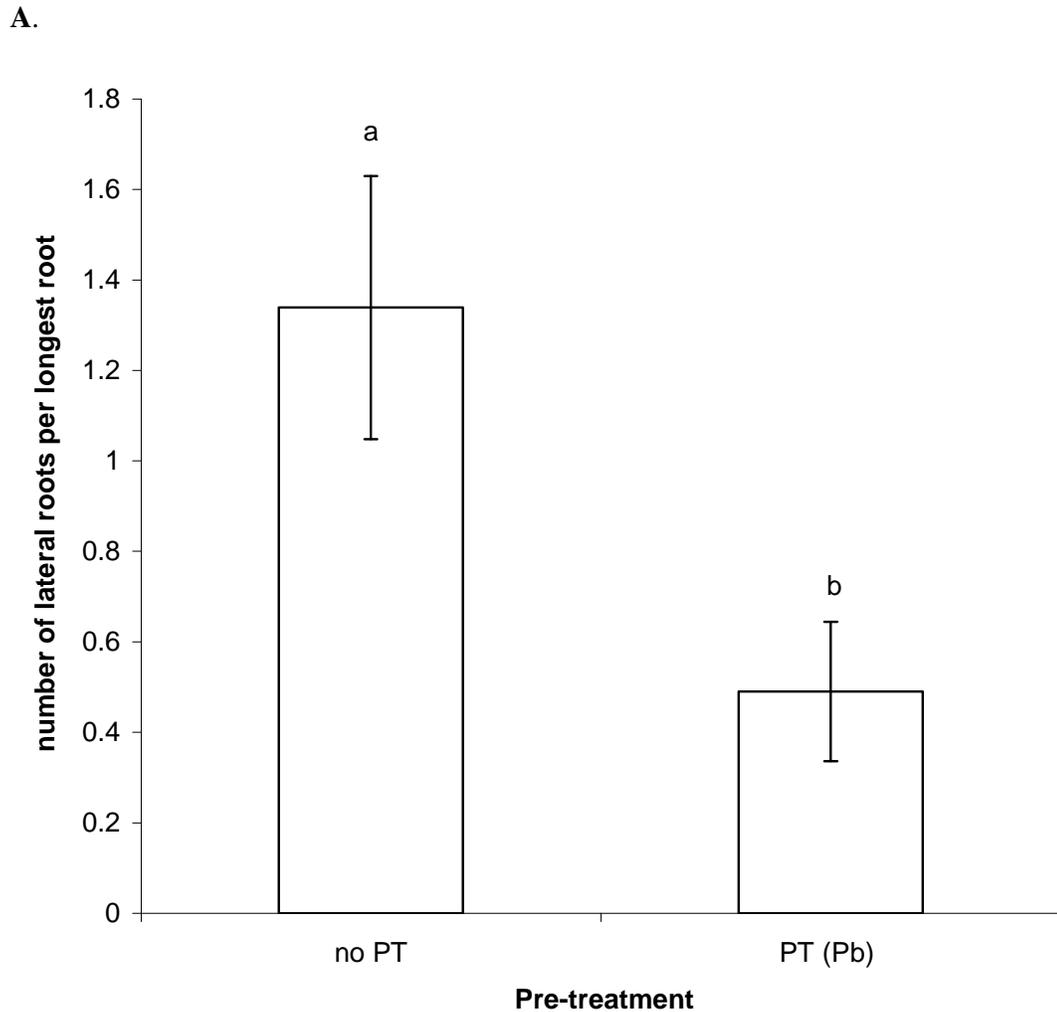


Figure 49A. Morphological measurements of visibly different 4-week-old *Symphytum officinale* roots of **prior** to Pb re-treatment (under non-sterile conditions). Plants were developed from *in vitro* petioles from shoot cuttings pre-treated with (PT) or without (no PT) 500 μ M Pb(NO₃)₂ (under *in vitro* conditions). Values are mean \pm S.E. of four replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). (PT = Pre-treatment).

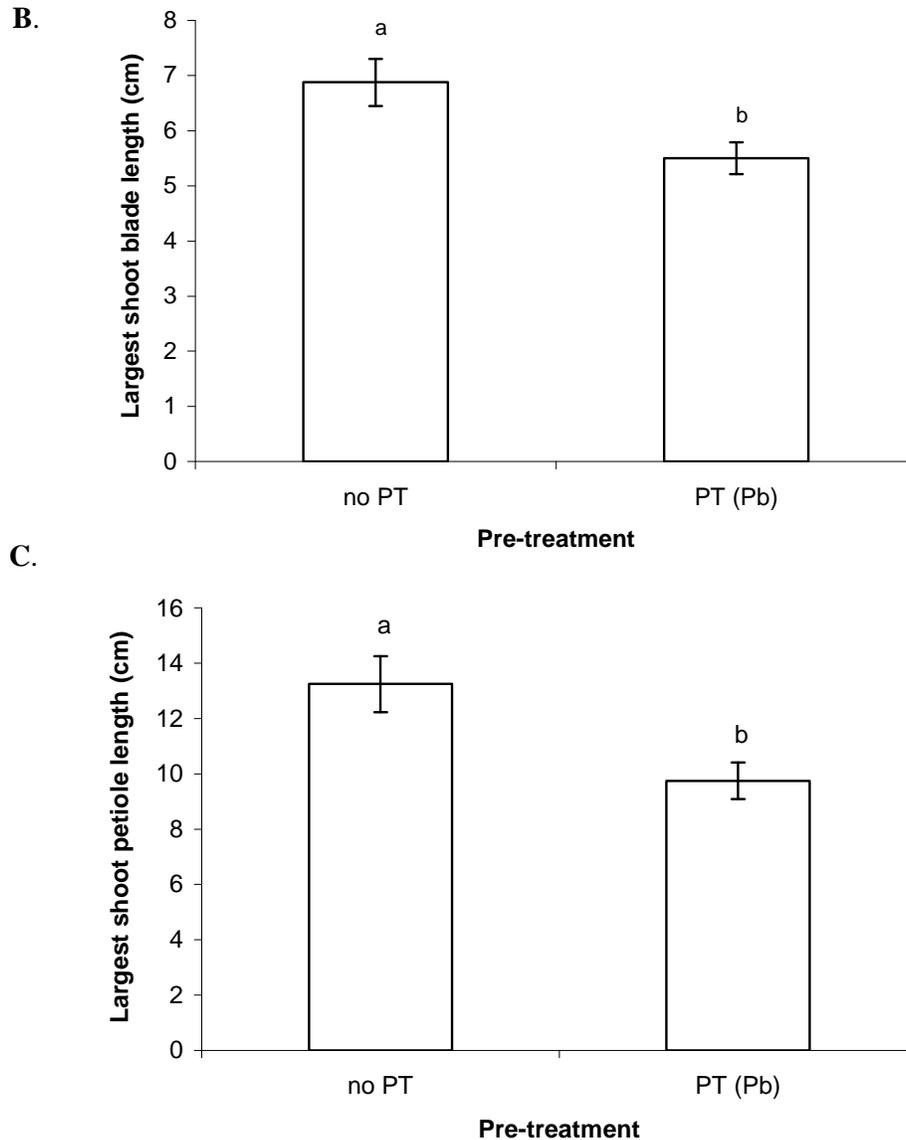


Figure 49B and C. Morphological measurements of visibly different 4-week-old *Symphytum officinale* shoot (B) blade and (C) petiole **after** Pb re-treatment of plants. Plants were developed from *in vitro* petioles from shoot cuttings pre-treated with (PT) or without (no PT) 500 μ M Pb(NO₃)₂ (under *in vitro* conditions). All plants were re-treated with modified HC nutrient solution (pH 4.5) containing 500 μ M Pb(NO₃)₂ for seven days (under non-sterile conditions). Values are mean \pm S.E. of four replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). HC = Huang and Cunningham. PT = Pre-treatment.



Plate 31. *In vitro* *Symphytum officinale* plants that were all re-treated with modified HC nutrient solution (pH 4.5) containing 500 μ M $\text{Pb}(\text{NO}_3)_2$, for seven days (under non-sterile conditions).

Plants were developed from *in vitro* petioles from shoot cuttings pre-treated with (PT) or without (no PT) 500 μ M $\text{Pb}(\text{NO}_3)_2$ (under *in vitro* conditions).

- A. Four plant lines without Pb pre-treatment
- B. Four plant lines with Pb pre-treatment

HC = Huang and Cunningham, PT = Pre-treatment.

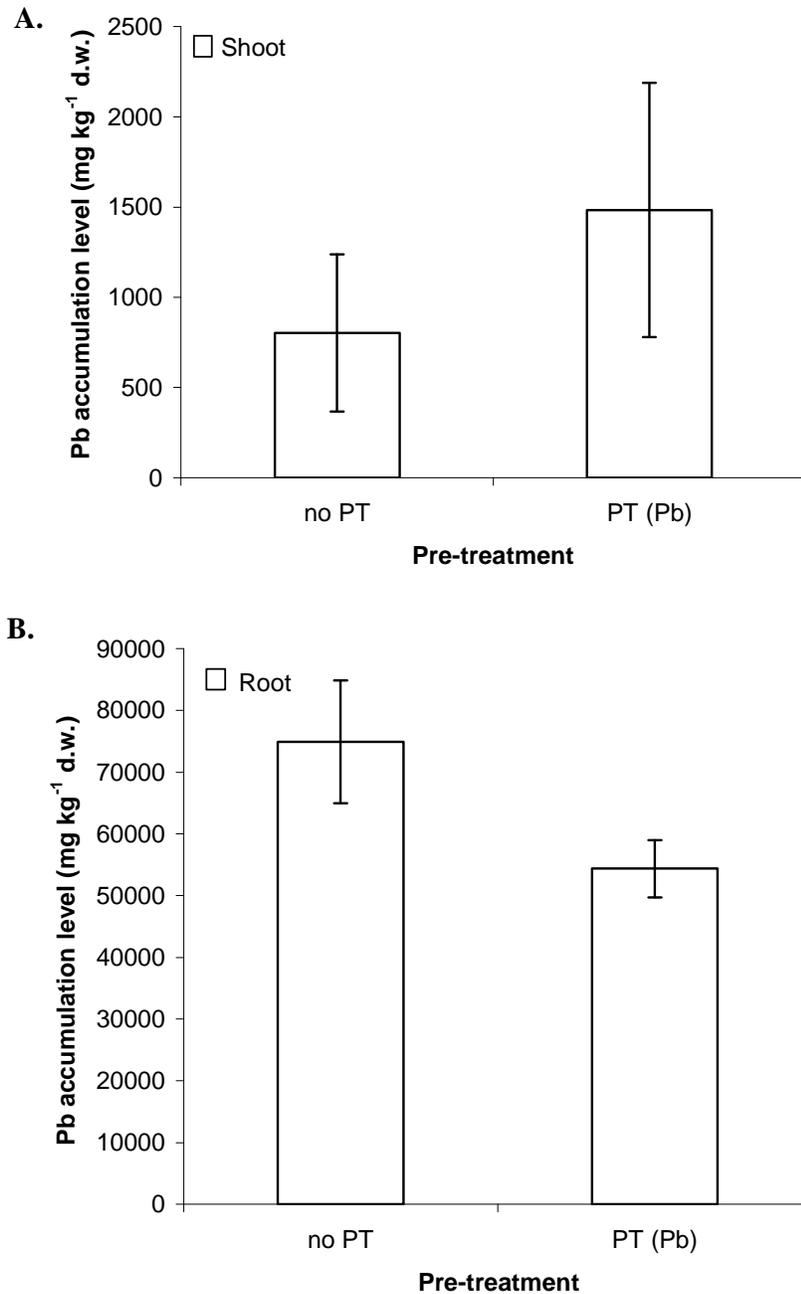


Figure 50. Pb accumulation levels in (A) shoots and (B) roots of 4-week-old *Symphytum officinale*. Plants were developed from *in vitro* petioles from shoot cuttings pre-treated with (PT) or without (no PT) 500 μ M Pb(NO₃)₂ (under *in vitro* conditions). All plants were re-treated with modified HC nutrient solution (pH 4.5) containing 500 μ M Pb(NO₃)₂ for seven days (under non-sterile conditions). Values are mean \pm S.E. of three replicates. All values are not significantly different ($P \geq 0.05$). HC = Huang and Cunningham. PT = Pre-treatment.

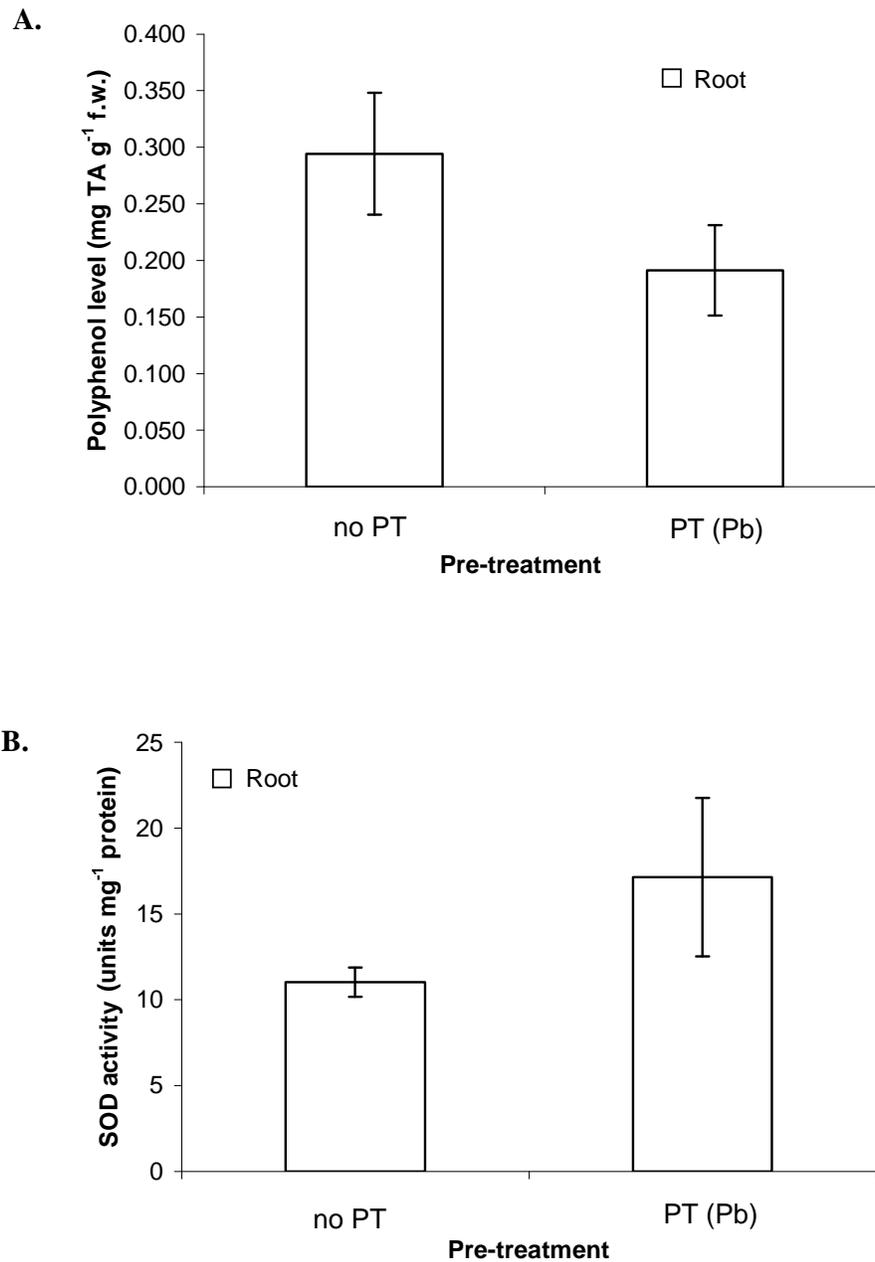


Figure 51. Polyphenol levels (A) and SOD activity (B) in roots of 4-week-old *Symphytum officinale*. Plants were developed from *in vitro* petioles from shoot cuttings pre-treated with (PT) or without (No PT) 500 μ M Pb(NO₃)₂ (under *in vitro* conditions). All plants were re-treated with modified HC nutrient solution (pH 4.5) containing 500 μ M Pb(NO₃)₂ for seven days (under non-sterile conditions). Values are mean \pm S.E. of three replicates. All values are not significantly different ($P \geq 0.05$). HC = Huang and Cunningham. PT = Pre-treatment.

12.2 Pre-treatment # 5

In this section, plants developed from petioles pre-treated with 0, 500 M $\text{Pb}(\text{NO}_3)_2$ or PbADA (pH 4.5) (pre-treatment # 5), were then re-treated with 0 or 500 M PbADA (pH 7.0).

12.2.1 Effect of pre-treatment on lead, polyphenol and SOD levels

At root level, pre-treatment appeared to decrease Pb accumulation in roots; Pb levels appeared to decrease by 64% and 58% (average d.w.) basis for unchelated Pb and PbADA pre-treated plants (Figure 52A). The Pb root levels were much higher here (over 25,000 mg kg^{-1} d.w. roots) than in 10.1.3 (only about 5,000 mg kg^{-1} d.w. roots). This may be because whole plants were exposed to PbADA in a semi-enclosed system with higher transpiration rates. Shoot levels did not exhibit noticeable trends (Figure 52B).

A noticeable decreasing trend was also observed in root polyphenol levels of pre-treated plants in unleaded re-treatment solutions; an average drop of 21% and 46% was observed with unchelated Pb and PbADA pre-treatments respectively. Re-treatment with PbADA further reduced polyphenol levels of unchelated Pb and PbADA pre-treated plants, but the variation in polyphenol levels were too large to state a trend (Figure 53A). This decrease in polyphenol levels is more likely to reflect a decrease in *extractable* polyphenol levels due to polymerisation (as opposed to a loss in polyphenol production). This is because the levels of superoxide dismutase (SOD), one of the enzymes involved in its polymerisation, appeared to be higher in plants pre-treated with unchelated Pb or PbADA (by 87% and 48% on average respectively) than untreated. PbADA re-treatment appeared to reduce SOD levels in all plants by around 32-36% except for unchelated Pb pre-treated plants. These appeared to have a constant SOD levels from control to PbADA re-treatments – however, this is likely due to higher variation in PbADA re-treatments (Figure 53B).

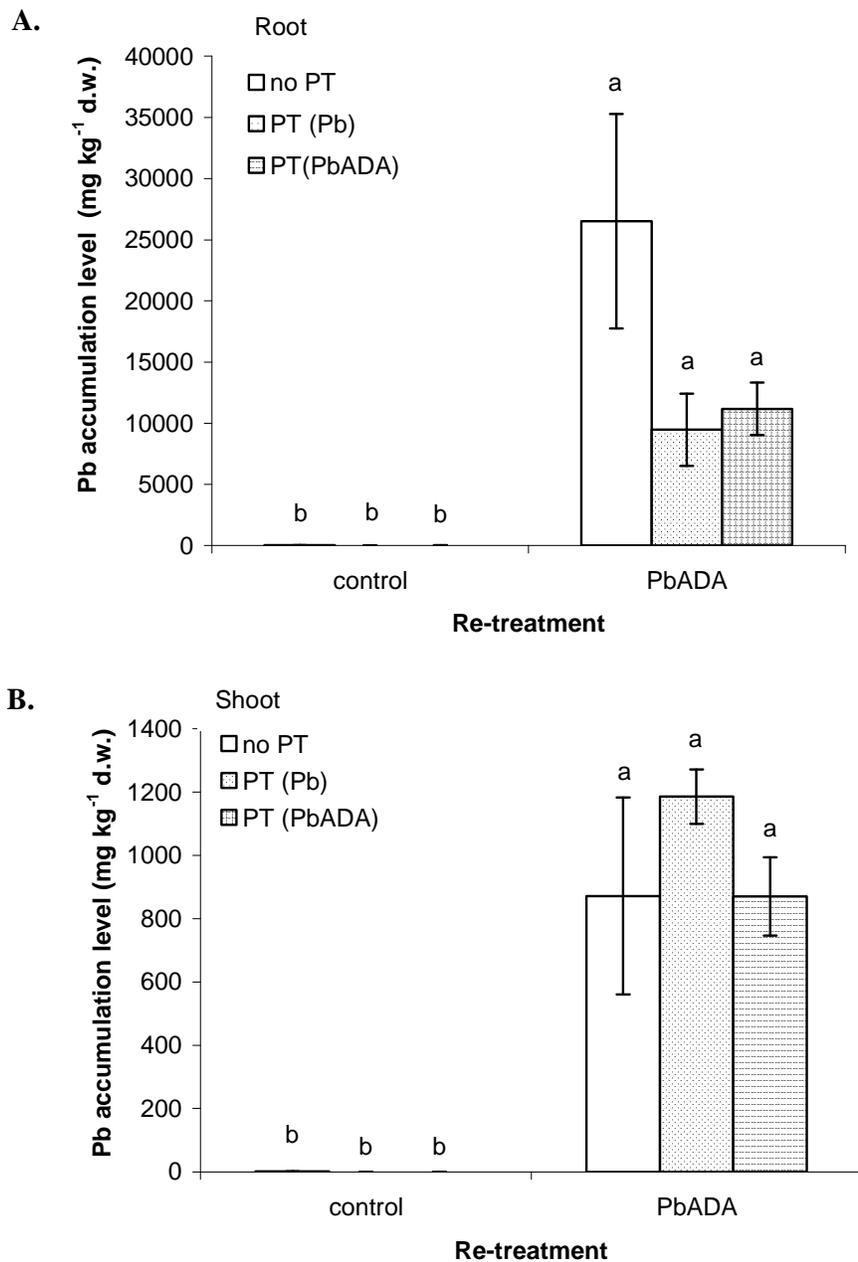


Figure 52. Pb accumulation levels in (A) roots and (B) shoots of 4-week-old *Symphytum officinale*. Plants were developed from *in vitro* petioles from shoot cuttings pre-treated with (PT) or without (no PT) 500 μM $\text{Pb}(\text{NO}_3)_2$ or 500 μM PbADA (under *in vitro* conditions). All plants were re-treated with modified HC nutrient solution (pH 4.5) containing 500 μM PbADA for seven days (under non-sterile conditions). Values are mean \pm S.E. of four replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$). HC = Huang and Cunningham. PT = Pre-treatment.

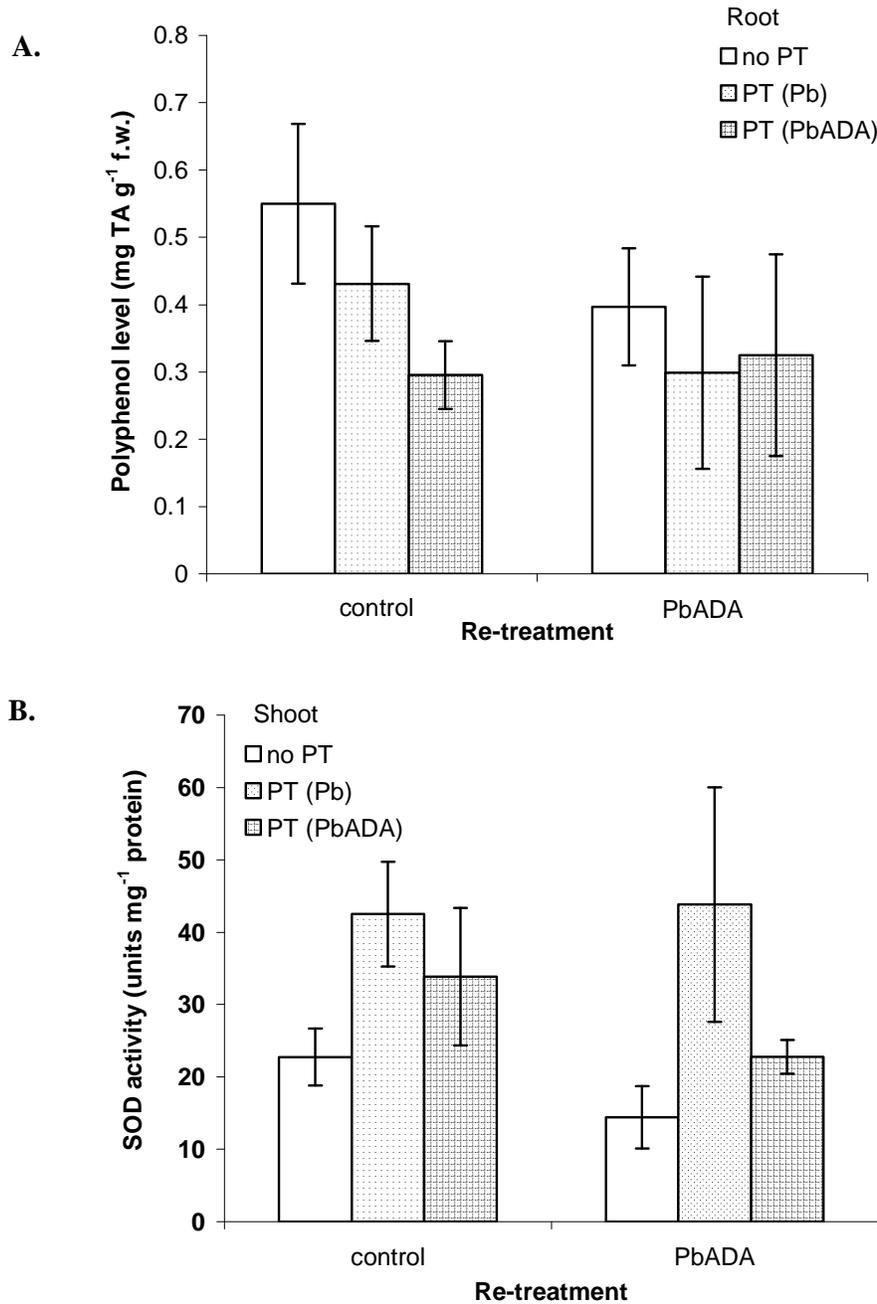


Figure 53. Polyphenol levels (A) and SOD activity (B) in roots of *Symphytum officinale*. Plants were developed from *in vitro* petioles from shoot cuttings pre-treated with (PT) or without (no PT) 500 μ M Pb(NO₃)₂ or 500 μ M PbADA (under *in vitro* conditions). All plants were re-treated with modified HC nutrient solution (pH 4.5) containing 500 μ M PbADA for seven days (under non-sterile conditions). Values are mean \pm S.E. of four replicates. All values are not significantly different ($P \geq 0.05$). HC = Huang and Cunningham. PT = Pre-treatment.

Considering that there was very little Pb in the actual petiole of plants generated from the unchelated Pb-treatment, the decreasing trends observed with Pb and polyphenol levels may instead be due to stress (oxidative stress) imposed by the Pb treatment (indirect Pb effect) rather than Pb itself (direct Pb effect). This was supported by two observations (i) the same pre-treatment and re-treatment (12.1.1) did not affect trends, possibly because the older and larger (4.5-week-old) shoots used was noticeably less stressed (especially at the petiole base) than the younger and thinner (3-week-old) shoots used here and (ii) shoot development (on MS F(0) media) from the thinner petioles of these younger shoots was also twice as long, for the same reasons as (i) above. Conversely, the trends from plants developed from PbADA pre-treatment could be due to a direct effect of Pb because a significant amount of Pb ($393 \text{ mg kg}^{-1} \text{ d.w.}$) was present in the petiole used to generate *in vitro* plants.

CHAPTER 4

FURTHER DISCUSSION AND CONCLUSIONS

1 Objective 1

Increased metal accumulation ability does not necessarily require tolerance but it is a desirable feature for phytoremediation purposes, in terms of improved vegetation on contaminated soils (Hall, 2002; Huang *et al.*, 1997b).

In this objective the focus was how the tolerance mechanism of chelation, particularly by tannin-type polyphenols, may play a role in Pb accumulation and thus the phytoremediation potential of plants. At the start of this study, very few papers suggest the biological role of tannin-metal chelation in metal tolerance or accumulation in plants. The exception to this includes studies on the copper hyperaccumulation in *Armeria maritima* ssp *halleri* (Lichtenberger and Neumann, 1997), and Al tolerance in *Lotus pedunculatus* (Stoutjesdijk *et al.*, 2001). Chelation studies to date also mostly appear to focus on cadmium followed by zinc and copper; lead (Pb) studies on the other hand have been comparatively rare. Moreover, there were no direct studies relating to Pb-tannin chelation in high biomass plants with phytoremediation potential.

1.1 Plant selection

With respect to *Brassica juncea* and *Pisum sativa*, one potential criticism is that the area of remediation can be limited. Whilst they have a high shoot biomass and have been shown to accumulate high levels of Pb in shoots, their actual rooting system is comparatively small. This was one reason why *Symphytum officinale* was so attractive from the onset of this experiment; *S. officinale* was high in shoot and root biomass. Additionally, reasonable amounts of the metabolites of interest (polyphenols) were present in the roots and shoots of this plant.

In choosing this plant, months were invested in establishing it from rootstocks because at the time no retailers in New Zealand sold comfrey in this form. Plants were

grown to at least 3-4 month-old before Pb treatment. This was to obtain a sufficient amount of plant material for analysis and to give a more accurate representation of Pb accumulation and tolerance in mature plants used in field studies. Seeds were also not available. Another plant with seedlings would have increased the number of initial experiments such as fresh weight changes relative to Pb concentrations and would have made the overall handling of plant material easier.

1.2 Polyphenols in *Symphytum officinale*

After developing a specific extraction and analysis regime for polyphenol and tannins in *S. officinale*, the distribution and level of these metabolites were determined to help decide which tissues should be targeted for analysis after Pb treatment. The most important conclusion was that the content of polyphenols, particularly tannins, in 3-month-old *S. officinale*, was the highest in root tissue. Moreover, the higher tannin content of lateral roots (compared to main roots) formed the basis of comparing the role of polyphenols and tannins in Pb accumulation and tolerance at an *in vivo* and *in vitro* level.

1.3 Determining tolerance and accumulation for objective 1

At an anatomical level, tolerance was based on *S. officinale* remaining relatively alive after Pb treatment (indicative at shoot level) and initial toxicity (root growth inhibition). Essentially this is **adaptive tolerance** as it explains how plants cope/survive the toxic effects from heavy metals like Pb (e.g. root growth inhibition and lipid peroxidation) and why no symptoms of Pb stress at shoot level is apparent, despite the high Pb levels in roots (Wierzbicka, 1995; Verma and Dubey, 2003). Heavy metal tolerance associated with a lack of visible root and/or shoot stress was not realistic because this form of tolerance is a feature that normally occurs in natural low-biomass hyperaccumulators or engineered mutants, not high biomass plants such as *S. officinale*.

Tolerance (at a biochemical level) was tested against whether or not tannins from *S. officinale* roots had the ability to chelate Pb (tolerance mechanism 1). This required determining if there was an *in vivo* correlation between Pb accumulation and tannin levels within *S. officinale*, and if *S. officinale* tannin extracts had an ability to chelate meaningful amounts of Pb under *in vitro* conditions.

1.3.1. Evidence for role of tannins in lead accumulation and adaptive tolerance in *Symphytum officinale*

At an anatomical level, shoots of plants exposed to 250 μM $\text{Pb}(\text{NO}_3)_2$ appeared just as healthy as the control plants because there were no observable signs of Pb stress in *S. officinale* shoots (e.g. necrosis). Thus, despite the high concentrations of Pb in roots and typical root growth inhibition, overall the plant appeared to cope with Pb stress. The restriction of Pb to roots, a tolerance mechanism of some plants to heavy metals (Thurman, 1981), may in part be due to Pb-tannin chelation at root level. Moreover at root level, there may be a positive and important attribute for the predominant Pb accumulation in the lateral roots – it may allow the polysaccharide (energy) reserves in the main roots to aid shoot growth when the overall root capacity was reduced due to Pb stress.

At a biochemical level, the results demonstrate that *S. officinale* root tannins have the ability to chelate Pb. This may not only be an adaptive tolerance mechanism under Pb stress, but also how plants accumulate Pb. There was no large increase in polyphenol levels in the lateral or main roots of *S. officinale* upon treatment with modified HC nutrient solution containing 250 μM $\text{Pb}(\text{NO}_3)_2$. This suggests that the pre-existing levels of polyphenols might be involved in adaptive tolerance of Pb. This supports other findings where only sensitive plants show large increases in polyphenol levels (and enzymes related in its production) under heavy metal stress (Lavid *et al.*, 2001a; Constabel and Ryan, 1998). The most significant finding however, was that a strong positive correlation between pre-existing root polyphenols (e.g. tannins) and Pb accumulation in both *in vivo* (hydroponic studies) and *in vitro* (tannin extracts) studies was observed. Firstly, the AAS and TEM results showed that higher-tannin lateral roots accumulated more Pb than lower-tannin main roots. Secondly, large Pb deposits were found in known tannin deposition sites of plant cells (i.e. the primary cell wall and suberised region). Finally, *in vitro* studies showed that crude immobilised *S. officinale* root tannins and the purified tannic acid (Sigma) had the ability chelate and remove meaningful amounts of Pb. Under the conditions of the study, up to 74 mg L^{-1} Pb (element) could be removed from the $\text{Pb}(\text{NO}_3)_2$ solution.

It is imperative to note that it would be impossible to test every potential Pb chelating compound from *S. officinale* due to the time and resources required to identify

them. Consequently, a limitation of this study was evaluating *exactly* how meaningful a role tannins may play in plants with respect to other known potential Pb-binding compounds (including phosphates (Cotter-Howells *et al.*, 1999; Eltrop *et al.*, 1991), pectin (Lane *et al.*, 1978; Moreno *et al.*, 2003), hemicellulose (Lane *et al.*, 1978) and lignin (Marmioli *et al.*, 2005)) in Pb accumulation and tolerance. However, its function is likely to be important if the following are considered: (i) their chemical structure has an abundant amount of metal-chelating hydroxyl groups, (ii) tannins are the fourth most abundant metabolite in vascular plants after cellulose, hemicellulose and lignin (Kraus *et al.*, 2003), (iii) they are precursors to a Pb-binding compound lignin, and (iv) polyphenols in general are known to protect the living plant against environmental stress (Chalker-Scott and Kramer, 1989).

To the best of my knowledge, this is the first report demonstrating Pb-tannin chelation and its possible role in Pb accumulation, adaptive tolerance and Pb phytoremediation. These results corroborate recent studies suggesting the role of polyphenols in tolerance and accumulation to heavy metals and in non-hyperaccumulating plants. For example, Lavid *et al.*, (2001b) showed that *Nymphaea* spp (waterlily) polyphenols and their polymerisation maybe involved in Cd and Cr tolerance because these metabolites were associated with metal-ion chelation and accumulation. Other examples include a correlation between Pb accumulation and total soluble phenolics in sunflower and tobacco plants (Ruso *et al.*, 2001) and the involvement of condensed tannins in Pb accumulation in *Athyrium yokoscense* (fern) gametophytes (Kamachi *et al.*, 2005). Using SEM/EDX and EXAFS spectroscopy, evidence of Pb-O bindings in Pb-exposed *Juglans regia* (European walnut) root powder and Pb-impregnated cellulose and lignin (whose precursor are tannins) also suggested that polyphenol compounds (in this case the cell wall) chelate Pb. No observations of Pb stress at root or shoot level was conducted (Marmioli *et al.*, 2005).

1.3.2. Significance of lead-tannin chelation to phytoremediation

Chelation of Pb by tannins would be most important for chelate-assisted phytoextraction, rhizofiltration and phytostabilisation.

In chelate-assisted phytoextraction, 40% of the PbEDTA complex formed dissociates (Geebelen *et al.*, 2002), producing free toxic Pb (at which stage is unknown).

In this situation, Pb-tannin chelation in shoots would provide an important Pb tolerance and accumulation mechanism for the following reasons: (i) increased detoxification of free Pb ions, (ii) Pb can be removed from the more susceptible cytoplasm if Pb binds to the cell wall, and (iii) compared to the synthesis of specific bio-molecules for chelation (e.g. phytochelatins), it may be more energy efficient form of tolerance as it uses a more permanent and pre-made bio-molecules for chelation (Marmiroli *et al.*, 2005). Taking these reasons into consideration, choosing plants with high tannin content in shoots may be more beneficial for this form of phytoremediation, providing that can be transported there in the first instance. This chelation may be particularly beneficial for recently improved phytoextraction methods where EDTA is added in increments rather than in a single dosage (Barcosi *et al.*, 2003; Grčman 2003). Such EDTA application would require the plant to tolerate increasing amounts of PbEDTA in the shoot over a longer phytoextraction period. However, there may be a “Catch-22” situation here if the complex splits at root level (or if unchelated Pb phytoextraction is used). Roots with high tannin levels may immobilise Pb at root level (i.e. create a sink for Pb in roots), which is not ideal for chelate-assisted phytoextraction. Based on PbEDTA studies however, there is every indication that the whole complex is intact at root level for Pb transport to the shoots (Vassil *et al.*, 1998; Jarvis and Leung, 2001) and therefore this may not be an issue.

In phytostabilisation, tannins could act as a matrix for binding the Pb in the root or contribute to the Pb-binding matrix in the soil after roots die. In rhizofiltration, plant roots are used to remove metals from polluted waterways. Evidence of tannin involvement in Pb chelation would be beneficial because: (i) of the same reasons stated in the above paragraph (at shoot level), and (ii) enhanced rhizofiltration (i.e. increase Pb accumulation at root level) by specifically selecting plants with roots that are high in biomass and tannins. According to dietary studies by Jackson *et al.*, (1996), low and high tannin levels is considered to be 2.2% (d.w.) and 6.3% (d.w.) of the tissue respectively.

Thus overall, when plants are selected for screening a particular type of phytoremediation, the presence, levels and tissue distribution of phytochemicals (e.g. tannins) should be considered, in addition to other factors such as biomass.

1.3.3. The immobilised tannin protocol

The Lavid dialysis method could be a more reliable *in vitro* Pb-tannin chelation method if only tannins from one root type or purified tannins were used. For the objectives of this study, where the different levels of tannins between lateral and main roots were being compared, the Lavid dialysis method was not suitable. The method was susceptible to interferences (i.e. non-polyphenol high molecular weight Pb-chelating compounds) because the dialysis tubes did not specifically trap high molecular weight polyphenol compounds. Fructans appear to be an interference compound. Thus the contrary trends of the Lavid dialysis method (compared to the hydroponic studies) appeared to be because the main roots had more fructans (but lower tannin levels) than lateral roots.

This research developed a new, simple and most importantly a more specific *in vitro* Pb-tannin chelation method (using PVPP-immobilised tannins) to show that crude *S. officinale* tannins chelate Pb ions under *in vitro* conditions. The idea was inspired by the use of PVPP to bind tannins in the Folin-Ciocalteu assay for tannins. It also showed that tannins bound to PVPP still had enough functional groups to bind meaningful amounts of Pb. This could also reflect tannins bound in the cell wall. Whilst fructan spiking (with inulin from chicory) showed that this method was not completely void of fructan interferences, it did allow the *in vitro* trend between tannin level and Pb chelation to be observed; higher tannin lateral roots chelated/removed more Pb from solution than lower tannin main roots. This method was validated using solely lateral root extracts at increasing levels and purified tannins (tannic acid from Sigma).

The use of immobilised tannins for binding pollutants is also not new. Numerous papers have documented tannin immobilisation to various matrices including aldehyde gels (Nakajima and Sakaguchi 2000), collagen fibres (Wang *et al.*, 2005; Liao *et al.*, 2004), and matrices with amine groups - aminopolystyrene and poly VT (Nakajima and Sakaguchi, 1990). In all the above papers, tannin immobilisation involved making the tannin-binding matrix *de novo*, which is a very complex process. For example aldehyde gels are formed from mixing tannins (e.g. from persimmons) with formaldehyde and potassium peroxodisulfate. The resulting solid gel is then crushed into small pieces (Nakajima and Sakaguchi, 2000). However, to the best of my knowledge none have been immobilised in one simple step with PVPP for metal chelation studies, and none in

particular for examining Pb-tannin chelation as a tolerance and accumulation mechanism in plants.

2 OBJECTIVE 2

2.1 Phytoremediation potential

To address the issue of EDTA-induced metal-leaching from soils and to enhance phytoremediation, studies into using transplanted plants (Wu *et al.*, 1999), Pb concentration (Kumar *et al.*, 1995), conserving EDTA application dose (Barcosi *et al.*, 2003; Wenzel *et al.*, 2003), chelating agent type (Wu *et al.*, 1999; Grêman 2003) and pH of soil solution (Blaylock *et al.*, 1997; Schor-Fumbarov *et al.*, 2003; Li *et al.*, 2003) have been performed.

Following these chemical-based initiatives to enhance phytoremediation, this objective set out to study the effects of Pb concentration, and the type and concentration of chelating agents. With respect to chelating agent and pH, it was particularly interesting to notice that in all the papers mentioned above, there was little or no regard/attention to the effect of competing soil ions, particularly Fe, on Pb uptake and accumulation. In soil extraction studies, a pH greater than 7.0 is strongly recommended for soil extraction of Pb, especially when Fe levels are high (Kim *et al.*, 2003; Kim and Ong, 2000; Chen and Hong, 1995). However, in most EDTA-based phytoextraction studies this is largely ignored because a pH < 5.0 is considered important for reducing root adhesion and increasing translocation of Pb to shoots (Blaylock *et al.*, 1997).

2.1.1. Phytoextraction potential of *Symphytum officinale*

From the phytoextraction studies performed, increased unchelated Pb concentrations resulted in an increase in Pb accumulation at the shoot and root level. Thus Pb accumulation took place in a concentration dependent manner.

In terms of the effect of chelating agents (EDTA), an expected increase in accumulation of Pb in shoots (compared to Pb treatments without chelating agents) was observed. Of all the chelated Pb phytoextraction studies performed at pH 4.5, the

highest average concentration of Pb in *S. officinale* shoots was 558 mg kg⁻¹ (d.w.) (after treatment with modified HC nutrient solution containing 500 μM PbEDTA). Although this was seven times higher than without EDTA, it only equates to an average of 0.058% Pb (d.w.) in shoots.

The use of EDTA is controversial. EDTA can spread Pb contamination, through leaching (Wenzel *et al.*, 2003; Kos and Lestan, 2003). From an environmental stance, this is particularly concerning considering phytoremediation is at least three years long. Moreover, EDTA is already discharged from the wastewater of industries such as textile and paper manufacturing, reaching rivers and groundwater. Consequently, it is already under scrutiny because of concerns on their influence on metal availability, mobility and persistent in the environment (Nowack and Sigg, 1996; Nowack, 2002). Despite this, EDTA is still considered the chelating agent benchmark (Meers *et al.*, 2004). Two alternative main chelating agents to EDTA were considered to determine if the phytoextraction potential could be improved: EDDS and ADA. EDDS was initially chosen because it was as effective as EDTA and yet biodegradable (Vandervivere *et al.*, 2001; Grčman 2003; Kos and Lestan, 2003;). But unlike ADA, its Pb complex was negatively charged and not suitable for assessing the repulsion hypothesis in results section 2.2. However, despite the change in chelating agents and factors that may increase its phytoremediation potential (e.g. a regenerable shoot system and a high shoot and root biomass), the Pb levels in the shoot fell well short of the phytoextraction target of 1% Pb (d.w.) in *S. officinale* shoots. At pH 7.0, Pb levels in shoots of plants treated with modified HC nutrient solution containing 500 μM PbADA were not substantially higher compared to PbEDTA at pH 4.5, reaching on average only 628 mg kg⁻¹ (d.w.) Pb (0.068% Pb d.w. in shoots). However, the level of Pb accumulated in the roots was 2.4 times higher than PbEDTA. Thus environmentally, at pH 7.0 ADA is better than EDTA as more Pb uptake into the roots would lead to less Pb leaching through the soil.

At a laboratory scale, *B. juncea* (Blaylock *et al.*, 1997; Vassil *et al.*, 1998) and *Pisum sativa* (Huang and Cunningham, 1996) plants were able to reach the Pb phytoextraction target (1% Pb d.w. in shoots). In this study, the levels of Pb in the *S. officinale* shoots were more comparable to phytoextraction studies conducted with *Brassica rapa* (Grčman *et al.*, 2003) and *B. napus* (Wenzel *et al.*, 2003) in the field. The Pb levels in shoots were similarly between 600-800 mg kg⁻¹ d.w (0.06-0.08% d.w).

The discrepancy in trends between these studies and in those reaching the 1% threshold may be due to plant type and the very favourable conditions that were used. These conditions include using: (i) immature plants or seedlings, (ii) continuous flow of nutrient solution (to maintain balance of nutrients) and minimise a build up of exudates, and (iii) very high concentrations of Pb or low concentrations but high total amount of Pb (if the sum of Pb used over the duration of the experiment was calculated).

2.1.2. Rhizofiltration and phytostabilisation potential

Phytostabilisation is starting to gain more attention because of the environmental concerns of chelating agents and length of time that may be necessary for phytoextraction (Nowack, 2002; Marmiroli *et al.*, 2005).

S. officinale is better suited for rhizofiltration and phytostabilisation because it appears to be a root accumulator of Pb. Most of the Pb was trapped in the roots. The highest Pb accumulation in the root was achieved after seven days in modified HC nutrient solution containing 500 M $\text{Pb}(\text{NO}_3)_2$ (about 21,000 mg kg^{-1} or 2% Pb d.w.), which is average compared to other roots of plants exposed to $\text{Pb}(\text{NO}_3)_2$ (Table 10).

Table 10. Pb accumulation levels in roots of plants exposed to $\text{Pb}(\text{NO}_3)_2$ in hydroponic solution.

Plant species*	Pb accumulation level in roots mg kg^{-1} (d.w.)	Reference
<i>Brassica juncea</i> L.	103,500	Kumar <i>et al.</i> , (1995)
<i>Brassica juncea</i> L.	136,000	Dushenkov <i>et al.</i> , (1995)
<i>Sesbania drummondii</i>	60,000	Sahi <i>et al.</i> , (2002)
<i>Ambrosia artemisiifolia</i> L.	24,000	Huang and Cunningham, (1996)
<i>Nicotiana tabacum</i>	24,900	Kumar <i>et al.</i> , (1995)
<i>Zea mays</i> L.	4900	Huang and Cunningham, (1996)
<i>Chamaecytisus palmensis</i>	2500	Jarvis and Leung, (2001)

*Duration of treatments, $\text{Pb}(\text{NO}_3)_2$ concentration and age of plants are variable.

2.2 Why did EDTA reduce lead accumulation in roots?

In this study, EDTA increased shoot levels of Pb, which supports its role in aiding Pb translocation. Interestingly however, Pb accumulation at root level drastically decreased; the level of Pb transported to shoots was only a fraction of the level of Pb lost from the roots. Another fraction was due to EDTA desorption. However, much of the Pb (compared to the unchelated Pb treatments) was still unaccounted for. Since the root and shoot biomass are approximately the same, shoot translocation could not have accounted for this reduction. This has important implications for phytoextraction because, perceivably, if the concentration of Pb in the roots of chelated Pb-treated plants were higher, more Pb would accumulate in the shoots.

When EDTA was added to the Pb-containing matrix in other studies, this trend has never been noted. This may be because some articles only publish the Pb levels in the shoot (Vassil *et al.*, 1998; Grêman 2003; Geeblen *et al.*, 2003; Huang *et al.*, 1997) or show that EDTA in fact increased root accumulation of Pb (Barcosi *et al.*, 2003; Wenzel *et al.*, 2003; Wu *et al.*, 1999). Slight decreases in root Pb accumulation were also present in experimental data by Blaylock *et al.*, (1997) and Jarvis and Leung (2001) that was not accounted for by translocation to shoot. However given the amount lost, this is likely to be due to the root washing step (with dH₂O or EDTA prior to Pb analysis) in their experiments.

Clearly the results in this study were an exception to one of the main functions of EDTA – to increase Pb bioavailability and thus the uptake and accumulation of Pb. Although charge repulsion of the PbEDTA complex and Pb specificity (factors investigated in this study) may play a role towards reducing the Pb accumulation level in PbEDTA treatments, none of these factors appeared to play a large role because much of the Pb loss (unchelated vs. chelated) remained unaccounted for. Thus, the main reasons for the reduction are still unclear. Moreover the mechanism underpinning this reduction, whether it is entirely due to effects at root level or due to effects at shoot level, is unknown.

2.2.1. Other possible factors: root exodermis

After combining these experimental findings with previous papers documenting a decrease in metal uptake when metals were in chelated form (see below for references), perhaps another factor not tested here – formation of the root exodermis – may play a larger role.

The exodermis is the layer of cells behind the epidermis and is particularly a feature of wetland plants. It is made of a mixture of suberin (wax-like substance) and lignin (cross-linked or polymerised polyphenols). It helps conserve oxygen supplies in roots of wetland plants grown hydroponically or on dry land (e.g. *Oryza sativa*). The extent to which this layer is formed depends on the environment; stressed environments such as drought, salinity, heavy metals or nutrient stress can increase its formation, repelling the uptake of water and hydrophilic substances (Hose *et al.*, 2001). In terms of speed of synthesis, it appears that only a few days in hydroponics is sufficient for its formation – exodermis analysis has been conducted on 8-10-day old monocot and dicot seedlings grown in hydroponics (Schreiber *et al.*, 1999).

S. officinale thrives in moist, marshy places (Chevalier, 1996) as well as in the terrestrial environment. It is conceivable that such an exodermis layer may also be present in *S. officinale* (although no attempt was made to determine this), particularly under the 14-day hydroponic system used. Perhaps some indication of its presence include: (i) the drop in polyphenol levels (due to polymerisation; see 3.3.4 below for more details), and (ii) increased whitening of the distal zone in the root tip when concentrations of EDTA were in excess of Pb during NBT localisation of superoxide anions (which is also a potential indicator of increased SOD activity, one of the enzymes involved in lignin and exodermis formation).

The stress of unchelated Pb and PbEDTA may increase the formation of the exodermis. Pre-treatment with EDTA could exacerbate the formation of this layer by destabilising ions in the cell wall (as discussed in Chapter 3). Ultimately, increased formation of the exodermis may reduce ion uptake (e.g. Pb ions). Increased polymerisation may also reduce the number of Pb binding sites. Most significantly, increased formation of the exodermis layer may increase the hydrophobicity of the root surface due to the suberin content of this layer. Suberin blocks hydrophilic compounds (Degenhardt and Gimmler, 2000). Thus, since EDTA (and to a lesser extent ADA) is

very hydrophilic (Wu *et al.*, 1999), this could explain why there is a drastic decrease in chelated Pb accumulation (whether in ADA or EDTA chelated form) compared to the unchelated ion. Perhaps the most telling piece of evidence may be why all the papers that documented a decrease in metal (Cd, Zn) uptake upon addition of EDTA, all happened to be aquatic plants. In *Nymphaea aurora*, EDTA reduced Cd accumulation in all the plant tissues; in lamina and petioles Cd decreased by 80%, whilst in roots a 50% reduction was observed (Schor-Fumbarov *et al.*, 2003). The lamina and petioles are not known to contain an exodermis, but do have a similar layer to the roots: the lipophilic cuticle (Schreiber *et al.*, 1999). Huebert and Shay (1992) demonstrated that when EDTA was in excess of Cd or Zn, metal accumulation in *Lemna trisula* (duckweed) was drastically reduced by at least 80%. In the case of *Lemna aequinoctialis*, 8.9 M CdCl₂ with 10 M EDTA reduced Cd accumulation by 18%, but at 100 M EDTA, Cd was reduced by 95% (Srivastava and Appenroth, 1995). Clearly in these examples, excess concentrations of EDTA reduced Cd and Zn accumulation. In the case of *Nymphaea* the authors suggested that Cd binding groups may compete with EDTA whereas in the *Lemna* species, a preference for free Cd²⁺ ions as opposed to chelated forms was suggested. Exactly why *Lemna* prefers this form of Cd was unknown.

In terms of phytoremediation, the exodermis feature of semi-aquatic plants could limit the benefit of tannins in *chelate-assisted* phytoextraction. Choosing a high tannin plant that does not inhabit semi-aquatic environments (i.e. does not form a significant exodermis) may be a very important feature for plant selection for this type of phytoextraction. Alternatively, using a Pb-specific chelating agent that is less hydrophilic may be important for its success. For phytostabilisation however, the exodermis may be beneficial to help the plant survive; the exodermis may not only reduce potential number of Pb binding sites (exclusion) but also trap Pb in the surface root tissue.

3 OBJECTIVE 3

3.1 Development of *in vitro* *Symphytum officinale* lines

There are only a few reports on the tissue culture of *S. officinale*. Of the *in vitro* *S. officinale* studies to date, all have been for investigating secondary metabolite production, namely alkaloids, fructans (Abou-Mandour *et al.*, 1987; Huizing *et al.*, 1983; Haa *et al.*, 1991) and glutamine (Tanaka *et al.*, 1974). In these studies cell suspension, callus cultures and whole plants were developed from shoot tissue, and all subcultures were performed with callus or cell suspension cultures.

In this study, *in vitro* crown division was used to propagate and establish *in vitro* *S. officinale* plants. The process developed was much simpler than those previously documented. In short, the process involved:

- (i) Attention to petiole size, placement on media and the type and number of incisions all influenced the success of the culture. The hairy petiole epidermis did not need to be removed to generate a sterile culture. A ten minute, 10% (v/v) bleach sterilisation regime achieved a 100% sterile culture.
- (ii) The best medium for shoot and callus induction from petiole explants was the MS F(0) medium. This consisted of a MS basal nutrient medium supplemented with 2% (w/v) sucrose and 1 mg L⁻¹ each of IBA and BA.
- (iii) The shoot/crown from the MS F(0) medium developed into a whole plant (root and shoot) on the MS N(0) medium (MS basal medium supplemented with 2% (w/v) sucrose and 1 mg L⁻¹ IBA and 0.1 mg L⁻¹ BA).
- (iv) Plants were subcultured by subdividing the crown tissue from whole plants grown on MS N(0), in a similar manner to sand-grown root divisions. Whole plants were developed and subcultured via the shoot/crown every 4-5 weeks onto fresh MS N(0).

3.2 Somaclonal variation and phytoremediation

There appears to be no reports on the tissue culture-oriented manipulation of *S. officinale* or any other plants for improved Pb phytoremediation (without the use of genetic engineering). Moreover, there are few reports using tissue culture to study the

effects of Pb itself; two examples include *Populus* cell cultures (Ksiazek *et al.*, 1984) and *Sesbania drummondii* (Sharma *et al.*, 2005).

Tissue culture has been used more commonly for phytoremediation-related studies of other heavy metals. It has been used to: (i) propagate heavy metal tolerant plants such as the Ni-tolerant *Stackhousia tryonii* Bailey, which is difficult to establish from seed (Bhatia *et al.*, 2002) and (ii) screen for heavy metal tolerant callus or cell lines (somaclonal-based studies) and determine their effect during treatment or upon re-exposure to the heavy metal. Recent examples of this include Cd tolerance in cell suspension cultures of *Cucumis sativa* L. (cucumber) (Gzyl and Gwozdz, 2005) and Cd stress in sugar cane callus cultures (Fornazier *et al.*, 2002), and Cr and Ni tolerance in *Echinochloa colona* L. callus cultures (Samantaray *et al.*, 2001). Tissue culture is also used for establishing *in vitro* plant lines for manipulation by genetic engineering.

In this objective, tissue culture was used to induce somaclonal variation in *in vitro* *S. officinale* cultures to improve or at least understand certain processes behind Pb tolerance and accumulation. The conventional ‘shock’ treatment approach was used whereby the petiole was exposed to a high concentration of Pb for a short period and the tissue used for the regeneration of shoot tissue with the stress factor. This was because there are higher chances of genetic variations with the ‘shock’ treatment approach (Tal, 1990; Remotti, 1998). However, the method used for somaclonal variation was not conventional in terms of the tissue used and method of Pb exposure (the stress agent). Firstly, somaclonal variation occurring in callus tissue was not used because mutations observed in this tissue do not necessarily occur in differentiated tissues (Tal, 1990; Remotti, 1998). Secondly, it was novel to try to have Pb *within* the tissue (rather than in the conventional external medium) to induce somaclonal variation. Additionally, there was uncertainty surrounding the bioavailability of Pb in the solid agar medium and the degree of effect it would have on petiole tissues covered by an epidermis with trichomes.

To determine if somaclonal variation occurred in plants generated from Pb pre-treated *in vitro* petioles the activity of the superoxide dismutase (SOD) and level of polyphenols were assessed. These particular parts of the antioxidative defence system were assessed because of their role associated with polyphenol polymerisation and Pb chelation (respectively). Effects on Pb accumulation were also assessed.

3.3 Plants grown from lead pre-treated petioles

3.3.1. Developing plants from lead pre-treated petioles

After a number of lengthy attempts, petioles of *in vitro* shoot cuttings pre-treated with unchelated and chelated Pb, successfully developed *de novo* shoots on MS F(0). However, *de novo* shoot production from pre-treated petioles (including control) was slower than anticipated; 6-8 weeks was required as opposed to 4-5 weeks directly from MS N(0). The pre-treated whole plant regime failed to produce any shoots because the treatment was too severe to allow *de novo* shoot production.

3.3.2. Effect of lead treatment on *in vitro* plants

In recent years there have been numerous studies on the effect of heavy metals (mostly unchelated) on the antioxidative defense system. Though the direction of SOD activity is variable with different stress conditions (Gratao *et al.*, 2005), most papers report an increase in activity upon heavy metal exposure at or above concentrations used in this study (Malecka *et al.*, 2001; Ali *et al.*, 2003; Ruley *et al.*, 2004; Reddy *et al.*, 2005). Studies on the effect of heavy metals on polyphenol levels have been comparatively few. Generally, heavy metals also lead to an increase in soluble polyphenol levels (Schützendübel *et al.*, 2001; Tripathi and Tripathi 1999; Ruso *et al.*, 2001). In these reports the increase in antioxidative defense activity was a reflection of adaptation and ultimate survival during periods of oxidative stress.

In contrast to these reports, in this study 500 M Pb or PbADA appeared to reduce polyphenol levels and SOD activity relative to the control plants. This indicates oxidative stress and a weakened antioxidative defense system. This contrast is possibly because of the plant type, concentration of Pb and most probably the duration of the treatment (seven days).

3.3.3. Effect of lead pre-treatment and re-treatment with lead

For phytoextraction, ideally pre-treatment would increase Pb accumulation by increasing its tolerance threshold to Pb. The SOD activity and polyphenol trends under such circumstances are unknown. However, according to hyperaccumulators this may

involve higher polyphenol levels in pre-treated plants prior to re-treatment and unchanged SOD activity; these factors would represent a plant that can accumulate more metals by having intrinsically more chelating compounds to detoxify metal-ions or control the metal-ion concentrations without activating its second line of defense: SOD and the antioxidative defense system. Of course such large positive improvements are not realistic, but trends indicating this would be of interest.

In this objective, Pb pre-treatment (either direct or indirectly) clearly did not produce somaclonal variants with better Pb tolerance and accumulation abilities, reflecting the difficult nature of such experiments. Instead, the opposite may have occurred – pre-treatment appeared to produce *S. officinale* somaclonal variants with reduced phytoremediation potential (i.e. more stressed plants may have been developed).

When compared to the control (no Pb pre-treatment), this was particularly apparent in plants that were generated from Pb pre-treated petioles with a significant concentration of Pb (direct Pb stress with PbADA), or petioles with little Pb but with significant observable stress (indirect Pb stress with unchelated Pb). In particular, the morphology of plants developed from unchelated Pb pre-treated petioles appeared more stressed upon re-exposure to unchelated Pb. Shoots were stunted and more necrotic. At root level, root growth was also significantly more inhibited, as the number of lateral roots of pre-treated plants was noticeably reduced, even prior to Pb re-treatment. Pb tolerant plants should not have such observable signs of stress.

In terms of Pb, polyphenol levels and SOD activity, plants developed from all Pb-based pre-treatments showed no statistically significant trends. However, some discernible trends were present. In general, Pb accumulation in Pb pre-treated plant roots was reduced upon re-exposed to Pb, compared with non-Pb pre-treated plants. Shoot levels did not substantially increase. This decrease in Pb root accumulation may be linked to a stress-related *increase* in root exodermis formation (as discussed in 2.2.1 above). The apparent decrease in polyphenol levels and an increase in SOD activity in Pb pre-treated plants may be indicators of this formation*. Formation of the root

*However this trend only holds *within* re-treatment type, not between treatments (i.e. '0' (control) and 'PbADA' re-treatments) because the degree of oxidative stress/cell death *between* the treatments is too great after seven days in lead solution.

exodermis, particularly under stress (e.g. heavy metal-induced oxidative stress), involves increased lignin biosynthesis. Since lignin biosynthesis is a peroxidase-mediated polyphenol polymerisation process (that uses polyphenols as precursors) a reduction in polyphenol levels is expected and was observed here (Chalker-Scott and Krahmer, 1989; Strycharz and Shetty 2002a and b). Superoxide dismutase (SOD) enzyme is the first enzyme in the cascade involved in lignin biosynthesis – it is one of the main producers of the peroxidase substrate, H_2O_2 (Wojtaszek, 1997; Lyons *et al.*, 2000; Schützendübel and Polle, 2002). Thus conceivably, an increase in SOD activity is expected with increased lignin biosynthesis during increased exodermis formation. Ultimately the increased formation of the exodermis would explain and support the reduction in Pb accumulation in this study and in the EDTA studies in 2.2 above.

Despite the statistically non-significance data, the trends and effects of pre-treatment on the parameters measured (Pb, polyphenol, SOD levels and plant growth) do give some insights into possible events in Pb tolerance/stress. Additionally, trends were still apparent after several subcultures, suggesting that some degree of genetic variation as opposed to adaptive variation may have occurred.

4 FUTURE STUDIES

4.1 Objective 1

4.1.1. Microscopy

Up to now, transmission electron microscopy has been used to determine the cellular distribution of Pb because of the high resolution and relative ease of detecting electron dense Pb particles. Early ultrastructural studies of Pb in tissues (especially at cell wall level) include *Allium cepa* seedlings (Wierzbicka, 1987), *Populus* callus cell cultures (Ksiazek *et al.*, 1984), *Pistum sativa* (Malone *et al.*, 1974), and *Raphanus sativus* seeds (Lane and Martin, 1982). More recently, transmission electron microscopes have EDX (Energy Dispersive Analysis of X-rays) or X-ray microanalysis instrumentation attached to confirm that the observations are Pb or other heavy metals (Sahi *et al.*, 2002; Neumann *et al.*, 1995). In future studies, it would be of interest to use such

instrumentation to confirm if the dark vesicles observed in this study are Pb or other electron dense materials such as tannins. However it cannot exclude the possibility that they are tannin-Pb complexes. To help determine this perhaps the TEM observations should be paired with confocal microscopy. Polyphenols in the cell walls and vacuoles can be viewed with confocal microscopy due to their autofluorescence (Hutzler *et al.*, 1998). It would be interesting to track if there is a ‘movement’ of tannins from the vacuole to the cell wall, in response to Pb stress, with confocal microscopy. A similar ‘movement’ of tannins from the vacuole to the cell wall has been reported in histochemical and ultrastructural studies of beech trees leaves (*Fagus sylvatica*) in response to increasing ultra-violet radiation stress (Bussotti *et al.*, 1998).

Other interesting microscopy studies could also include TEM of plant shoots (from Pb treatments), in particular the crown section in between these tissues. More work also needs to be conducted on how plants transport unchelated Pb or other heavy metals. Although the plant used in this study was effective in retaining Pb in the roots, other plants such as *B. juncea* and hyperaccumulators are capable of transporting unchelated ions to the shoot. How some non-hyperaccumulating plants, without the aid of chelating agents, can stop retention in the roots cell walls and aid translocation (Blaylock *et al.*, 1997) is of interest.

Maize plants can translocate endophytic bacteria via the xylem and colonise maize stem intercellular spaces (Sattelmacher, 2001). It is conceivable that Pb-laden vesicles, (observed in this experiment and which appear very similar in size to such endophytic bacteria) may be another mechanism for unchelated Pb transport to shoots. Moreover, if the Pb in the vesicles were bound to tannins, and this would support the suggestion by Dahmani-Muller *et al.*, (2000) of phenolic-metal transport to the shoots. The question then is how is it transported at root level to reach the xylem?. It could be possible that like the endophytes, such vesicles could enter the xylem via the numerous natural openings in the root such as lateral root formation points. It is also possible that they can flow through openings made by endophytes (which use cellulytic and pectinolytic cell wall degradation enzymes to aid movement through the cell wall) (Sattelmacher, 2001) and into then xylem. TEM and confocal studies of the cell wall, particularly at or along the sites of lateral root initiation (pericycle of primary root) or at endophyte opening points (Raven *et al.*, 1992), would be of interest. It would be especially interesting to

determine if Pb is seen in vesicle-like structures in the cell walls of shoots cells not exhibiting Pb stress early on in the treatment.

If this apoplastic vesicle-based route into the xylem is proven true, this could support the challenge made to the assumption that heavy metals, such as Zn, enter the xylem only via the symplastic route (White *et al.*, 2002). Although the method of apoplastic entry into the xylem was not identified, White *et al.*, (2002) suggested that some of the Zn reaching the xylem must be through the apoplastic route, especially at high concentrations. This was because the delivery of Zn into the xylem exceeded the influx of Zn into the root cell (symplastic route) and the Zn following this route could not supply sufficient amounts of Zn for hyperaccumulation.

4.1.2. *In vitro* lead-tannin chelation

There are several experiments using the *in vitro* chelation method that would be of interest for future studies:

- (i) Fructans in *S. officinale* roots could be isolated to determine the type of fructan in *S. officinale* roots and their *in vitro* Pb chelation ability. Additionally higher concentrations of purified chicory inulin or artichoke inulin (which has more units of fructose than chicory inulin) could also be tested. This would add credence to the findings in this study that suggest fructans interfered in our *in vitro* Pb-tannin chelation studies.
- (ii) Study of the tannin-Pb complex in more detail using electrospray ionisation mass spectroscopy (Ross *et al.*, 2000).

4.2 Objective 2

4.2.1. Pb accumulation studies

At the time, 250 M $\text{Pb}(\text{NO}_3)_2$ treatment was performed to simultaneously test the phytoremediation ability of *S. officinale* and chelation-based tolerance factors. Whilst this may be considered high, it is a more realistic concentration in order to achieve the time limits of phytoremediation. Numerous phytoremediation experiments have in fact used $\text{Pb}(\text{NO}_3)_2$ concentrations up to 1000 M (Dushenkov *et al.*, 1995; Kumar *et al.*,

1995; Blaylock *et al.*, 1997; Sahi *et al.*, 2002). Moreover it was necessary to test such concentrations because there may be a threshold level before Pb translocation occurs (Kumar *et al.*, 1995).

Although it would not reflect a real phytoremediation situation, it would have been interesting to test the tolerance and accumulation ability of this plant at lower Pb concentrations (that are less inhibitory to root growth), such as 50 and 100 μM Pb concentrations. The rationale for this is that the stress associated with high Pb concentrations may lead to tannin (or tannin-Pb) absorption to the cell wall, creating a more permanent sink for Pb in the roots. If less stress occurred, perhaps more tannin would remain in the root vacuole, creating a more mobile phase for Pb to move to the shoots. Martinez *et al.*, (2006) also raised this possibility. Although not related to Pb stress, similar examples of tannin retention in the vacuole or in the inner cell fraction includes the leaves of *Fagus sylvatica* L. (to less ultraviolet radiation stress) (Bussotti *et al.*, 1998), and *Vitis vinifera* L. (in unripe grape berries) (Gagne *et al.*, 2006). If this were possible, it would be important for tannin-Pb chelation in the root vacuole to be temporary for phytoextraction. According to Elbaz *et al.*, (2006) sequestration in the root vacuoles can inhibit xylem loading and consequently hyperaccumulation in the shoots of the zinc hyperaccumulator, *Thlaspi caerulescens*.

Apart from testing lower concentrations of Pb, it would be of interest to increase the duration of Pb exposure from 7 to 14 or 21 days (for unchelated Pb) to determine if time, similar to studies by Huang and Cunningham (1996), would also influence the amount of Pb translocated to the *S. officinale* shoots. If given the resources to safely address the Pb liquid waste disposal issue, it would also be of interest to have a continuous feeding solution set up for such longer durations. This is because longer hydroponic durations may lead to an accumulation of Pb chelating compounds in the solution over time, thus inhibiting the uptake and accumulation level of Pb. A similar finding was found with rice seedlings (Yang *et al.*, 2000).

In terms of chelating agents, ADA was able to match or supercede EDTA at lower and higher pH (pH 7.0). Since, ADA also has benefits in terms of specificity for Pb and ease of recycling and recovery, it should be tested in demonstrated phytoremediators such as *B. juncea* and *Sesbania drummondii*.

4.2.2. Polyphenol polymerisation

There are a number of experiments to prove if the formation (or increased formation) of the root exodermis and/or polyphenol polymerisation leads to reduced chelated-Pb accumulation.

- (i) Confocal microscopy of root tip to observe exodermis formation in real time.
- (ii) Study plants treated with only 500 M EDTA (in terms of root length, SOD/POD, exodermis formation etc)
- (iii) Measure the POD activity, the enzyme directly involved in polyphenol polymerisation (at low and high Pb concentrations).
- (iv) Add external IAA (plant growth regulator) to the medium with and without EDTA or PbEDTA, to determine if IAA can reduce polymerisation and/or root exodermis formation and increase Pb accumulation. This idea comes from studies on the involvement of IAA in polymerisation (or cross-linking). Firstly, Jansen *et al.*, (2001) found that UV tolerance in duckweed mutants was associated with *decreasing* IAA levels caused by the peroxidase-mediated breakdown of endogenous IAA and cross-linking of UV-B absorbing phenolics. Secondly, binding of Pb to the cell wall is thought to modify the normal cell wall cross-links into structures less susceptible to IAA breakage and therefore elongation (Lane *et al.*, 1978). Lane *et al.*, (1978) showed that elevated levels of IAA are thought to reverse this process. If IAA reduces polymerisation and increase Pb accumulation, this could explain why Lopez *et al.*, (2005) found that increasing applications of IAA to PbEDTA solutions increased shoot concentrations of Pb in alfalfa leaves by 2800%.

4.3 Objective 3

4.3.1. *Symphytum officinale* tissue culture

Pre-treatment did appear to reduce Pb accumulation, but in order to be more certain of the causes (relating to SOD activity and polyphenols), statistically significant data needs to be compiled. The main experiments would include:

- (i) Replicating pre-treatments on individual lines showing the greatest difference.

- (ii) More than three to four replications.
- (iii) If sufficient root tissue can be obtained, studying only the root tips (up to the elongation zone) as histochemical superoxide anion staining showed that most of the differences were located in this region.
- (iv) For publication purposes, quantitative analysis of $\cdot\text{O}_2^-$ production will be required. Re-treatment with lower Pb concentrations (e.g. 250 μM level) and earlier (e.g. after 24 hours).
- (v) Study plants developed from pre-treatment without re-treatment.

It was envisaged that (i) could be performed, but because the longer than envisaged time involved in generating shoots from Pb pre-treated shoot petioles and their subculture, this could not be achieved by the ‘one person’ research team. The efforts were also not helped by a lack of shoot generation from Pb pre-treated whole plants and contamination of established tissue culture stock plants (an unforeseen consequence of changing the size of stock plant containers). For time and practicality reasons, it would also be helpful to invest time in developing a liquid medium suitable to support consistent whole plant growth from the crown tissue.

4.3.2. Trends based on levels

While studying changes in levels or activity of Pb, polyphenols and SOD (in response to treatments) give some significant and important insights, there are inherent limitations; the significance of changes to the composition, structure, and location of target compounds under investigation may be missed. For example Konno *et al.*, (2005) demonstrated that copper affected polysaccharide synthesis in *Lycopodium japonicum* prothallium, resulting in changes to not only the levels but also the composition of pectins in the cell wall. In another example, Lojonen *et al.*, (2001) showed that there was no trend between the distance of a polluted site (Ni-Cu smelter) and the level of total polyphenols in white birch levels. However, when individual phenolic acids in the leaves were measured, one type of phenolic acid (gallic acid) did show a significant increase towards the smelter. Thus in this regard, future studies could include identifying and measuring specific types of tannins and distinguishing between tannins in the cell wall and vacuole. Other studies could include determining the degree of polymerisation of polyphenols in the cell wall, and similar to other studies (Fornazier *et*

al., 2002; Sandalio *et al.*, 2001), the relative ratios of SOD isozymes and their cellular location (e.g. apoplast vs. cytoplasm).

4.3.3. Engineered phytoremediators

Somaclonal variation is one approach towards improving the intrinsic heavy metal remediation ability of plants. Other approaches include screening for chemical-induced (e.g. EMS) mutants, cultivars of plants displaying remediation potential (Nehnevajova *et al.*, 2005; Kumar *et al.*, 1995) and using genetic engineering. At the time of this thesis, there was a moratorium (part of the Hazardous Substance and New Organism Act, 1996 and Biosecurity Act, 1993) on releasing GMOs in New Zealand (Ministry for the Environment – online, 2006). Hence, the genetic modification approach was not the focus in this study. Nonetheless in future, genetic engineering studies could include:

- (i) Overexpression of genes that produce enzymes (e.g. SOD in mitochondria or chloroplasts) or Pb-binding compounds (e.g. tannins, particularly in vacuoles of shoot tissue) related to the antioxidative defence system. This is because overproduction of SOD has (in some cases) been linked with reduced oxidative stress (Yu *et al.*, 1999; Apel and Hirt, 2004) and variety of transgenic plants with heightened phytochelatin levels have been shown to extract more Cd, Cu and Pb etc than wild plants (Eapen and D'souza, 2005; Martinez *et al.*, 2006). Minimising oxidative stress could be another avenue to keep metal transporters (e.g. ATPases) functional rather than adding new transporters (Xiong *et al.*, 2006). However while increased growth has been reported (Martinez *et al.*, 2006), others reports highlight that over-expressing genes may be very energy expensive. For example, over expressing metallothionein genes in *Nicotiana tabacum* lead to a lower plant yield compared to the untransformed plants (Tong *et al.*, 2004).
- (ii) If increased chelating agents or enzymes are required in the plant, increased nitrogen assimilation would be required to provide the nitrogen backbone of these substances or those that synthesise them. Thus this may require over-expression of the nitrate reductase (NR) gene.
- (iii) Genetic engineering could be a tool for understanding metal tolerance better. For example, tracking SOD expression/response to heavy metals using fluorescence

markers (e.g. GUS gene) in real time (and without wounding) in whole tissues using confocal microscopy may be of interest.

- (iv) If the root exodermis is an inhibiting factor for chelated Pb transport, perhaps underexpressing peroxidases in the root (to reduce polymerisation and/or exodermis formation) may be of interest for wetland plants.
- (v) Perhaps the chloroplast should not only be viewed as a site of Pb damage or site to insert genes but could also be targeted as an above ground sink/accumulation site for heavy metals, in addition to the vacuole. Evidence for this possibility includes Pb accumulation in the chloroplast of shoot cells of *Chamaecytisus proliferus* treated with PbEDTA (Jarvis and Leung, 2001) and the high accumulation (more than 60% of total) Cd in chloroplasts of *Euglena gracilis*, a unicellular photosynthetic organism lacking a vacuole (Mendoza-Cozatl and Moreno-Sanchez, 2005).

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APPENDIX A

Major Salt Stock (10 X)		1 L	2 L
KNO ₃		2.022 g	4.044 g
Ca(NO ₃) ₂ ·4H ₂ O		1.181 g	2.306 g
MgSO ₄ ·4H ₂ O		0.493 g	0.986 g
NH ₄ NO ₃		0.0808 g	0.1616 g
KH ₂ PO ₄		0.0068 g	0.0136 g
Minor Salts Stock (100 X)		500 mL	1 L
KCl		0.1864 g	0.3727 g
FeEDTA		0.36705 g	0.7314 g
H ₃ BO ₃		0.0371 g	0.07421 g
MnSO ₄ ·4H ₂ O		0.0223 g	0.0446 g
ZnSO ₄ ·7H ₂ O		0.0072 g	0.0144 g
CuSO ₄ ·5H ₂ O		0.00249 g	0.00499 g
Na ₂ MoO ₄ ·2H ₂ O		0.00121 g	0.00242 g
NiSO ₄ ·6H ₂ O		0.00078 g	0.00155 g
Nutrient Solution			
(Full Strength)	500 mL	1000 mL	2000 mL
Major Salts Stocks (10 X)	50 mL	100 mL	200 mL
Minor Salts Stocks (100 X)	5 mL	10 mL	20 mL
Nanopure distilled H ₂ O	to 500 mL	to 1 L	to 2 L
Adjust pH to 4.5-5.0			

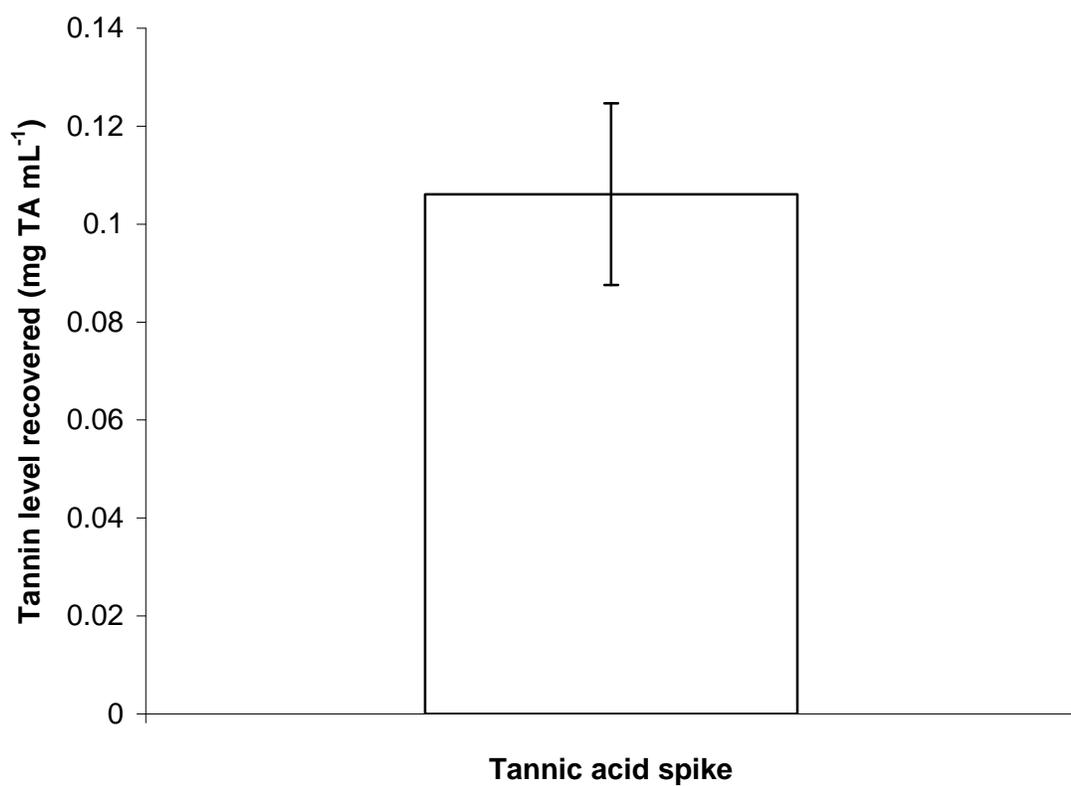
A1. Modified Huang and Cunningham (HC) nutrient solution.

Note: This nutrient solution has been formulated specifically for use in Pb accumulation experiments. It has a low pH and P concentrations (5 M in this version of the HC nutrient solution) to ensure maximum Pb solubility/availability in solution (Huang and Cunningham, 1996).

Major Salt Stock (10 X)	1 L	2 L	
NH ₄ NO ₃	16.5 g	33 g	
KNO ₃	19 g	38 g	
CaCl ₂ .2H ₂ O	4.4 g	8.8 g	
MgSO ₄ .7H ₂ O	3.7 g	7.4 g	
KH ₂ PO ₄	1.7 g	3.4 g	
Minor Salts Stock (100 X)	1 L	2 L	
KI	0.083 g	0.166 g	
H ₃ BO ₃	0.620 g	1.240 g	
MnSO ₄ .4H ₂ O	2.230 g	4.460 g	
ZnSO ₄ .7H ₂ O	0.860 g	1.720 g	
CuSO ₄ .5H ₂ O	0.0025 g	0.005 g	
CoCl ₂ .6H ₂ O	0.0025 g	0.005 g	
Na ₂ MoO ₄ .2H ₂ O	0.025 g	0.050 g	
Organic stock (100 X)	500 mL	1 L	
Myo-inositol	5 g	10 g	
Nicotinic acid	0.025 g	0.05 g	
Pyroxidine-HCl	0.025 g	0.05 g	
Thiamine-HCl	0.005 g	0.01 g	
Glycine	0.1 g	0.2 g	
Iron stock (100 X)	200 mL		
A: FeEDTA.7H ₂ O	1.39 g		
B: Na ₂ EDTA.2H ₂ O	1.865 g		
Nutrient Solution (Full Strength)	250 mL	500 mL	1 L
Major Salts Stocks (10 X)	25 mL	50 mL	100 mL
Minor Salts Stocks (100 X)	2.5 mL	5 mL	10 mL
Organics (100 X)	2.5 mL	5 mL	10 mL
Iron stock (100 X)	2.5 mL	5 mL	10 mL
Sucrose (2% w/v)	7.5 g	15 g	30 g
Adjust pH to 5.8			
Adjust to volume with dH ₂ O			
Agar (0.8% w/v)	2 g	4 g	8 g

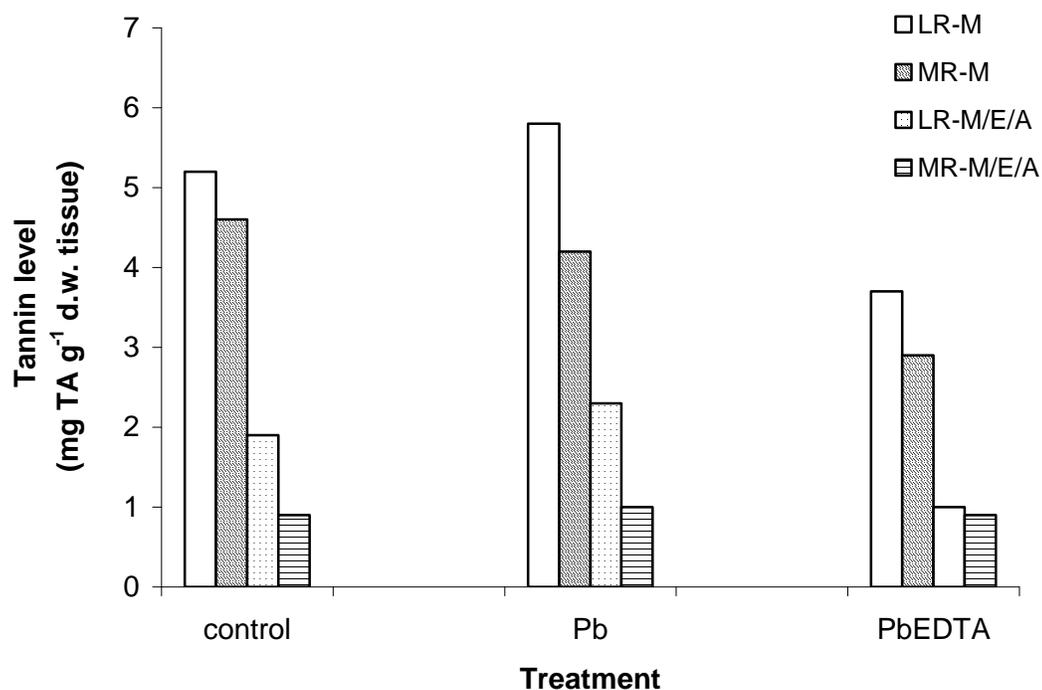
A2. Murashige and Skoog (MS) medium (1962)

APPENDIX B



Level of tannin recovered after *Symphytum officinale* lateral root powder was spiked with 0.1 mg mL⁻¹ tannic acid and freeze dried for five days. Values are mean \pm S.E. of three replicates. TA = tannic acid.

APPENDIX C



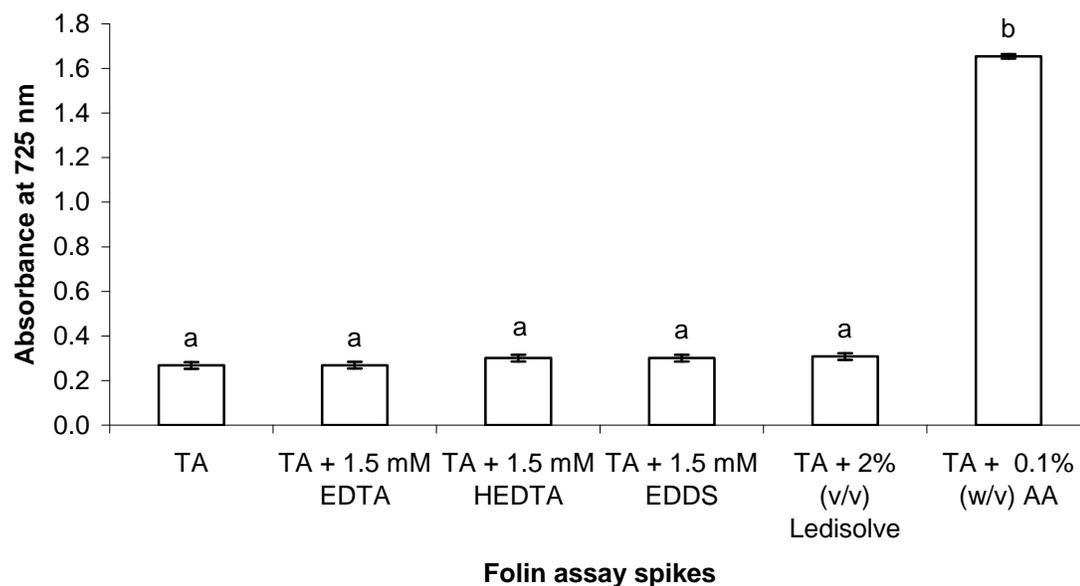
Effect of EDTA and ascorbic acid in 50% (v/v) methanol on tannin extraction from lateral and main roots (LR, MR) of *Symphytum officinale*. Values are from one replicate.

M: 50% (v/v) methanol

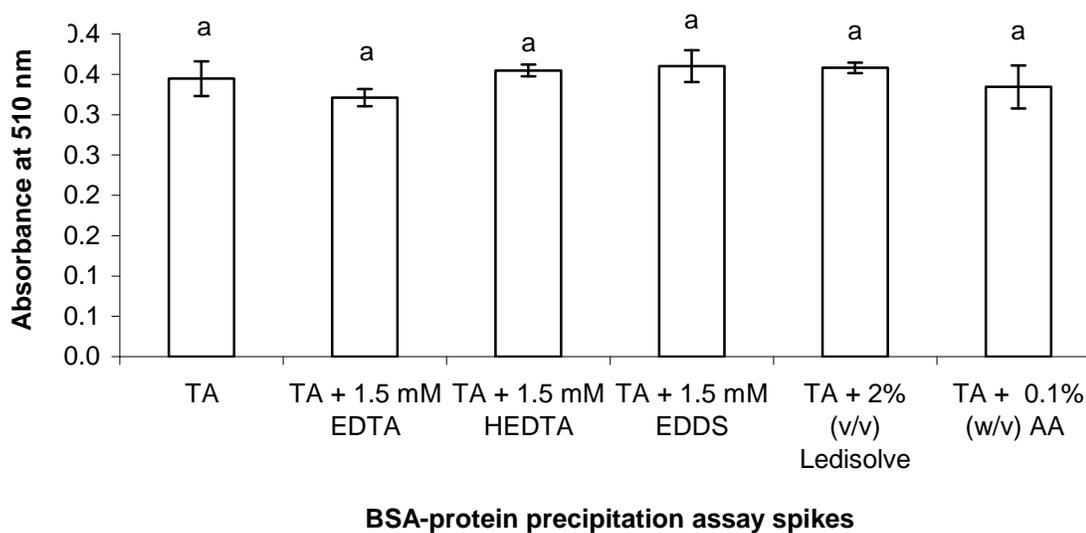
M/E/A: 50% (v/v) methanol, 1.5 mM EDTA, 0.1% (w/v) ascorbic acid

APPENDIX D

A.



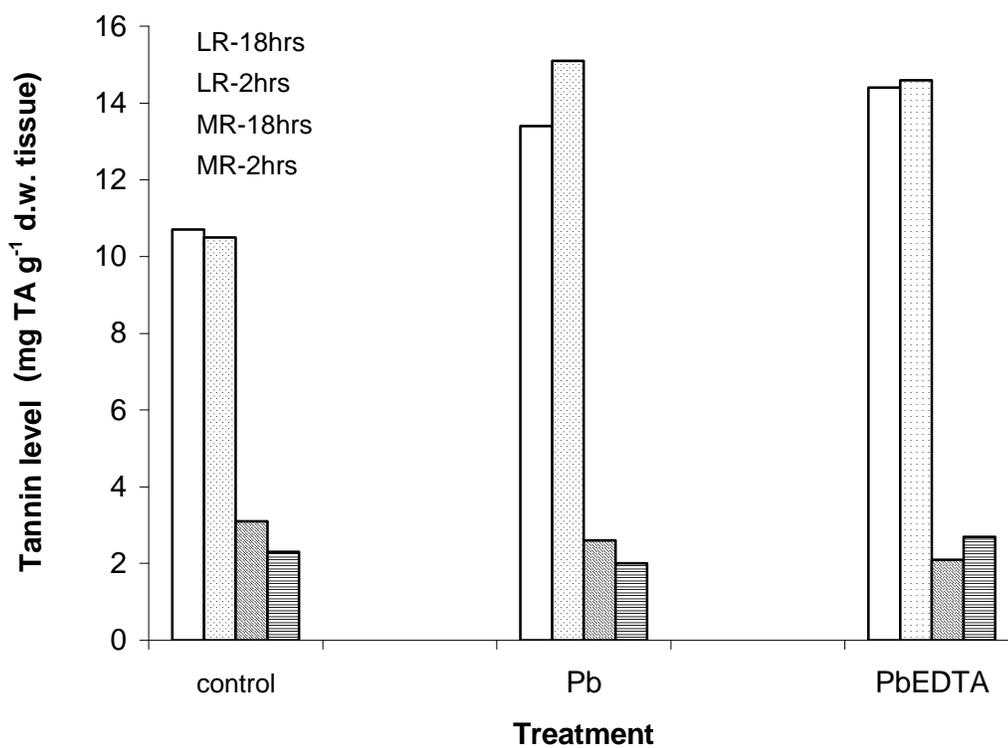
B.



Effect of chelating agents (EDTA, HEDTA, EDDS, Ledisolve) and polyphenol extraction additives (AA) on absorbance used for the: (A) Folin-Ciocalteu assay and (B) BSA-protein precipitation assay. Values are mean of three replicates \pm S.E. Values not sharing the same letter are significantly different ($P \leq 0.05$).

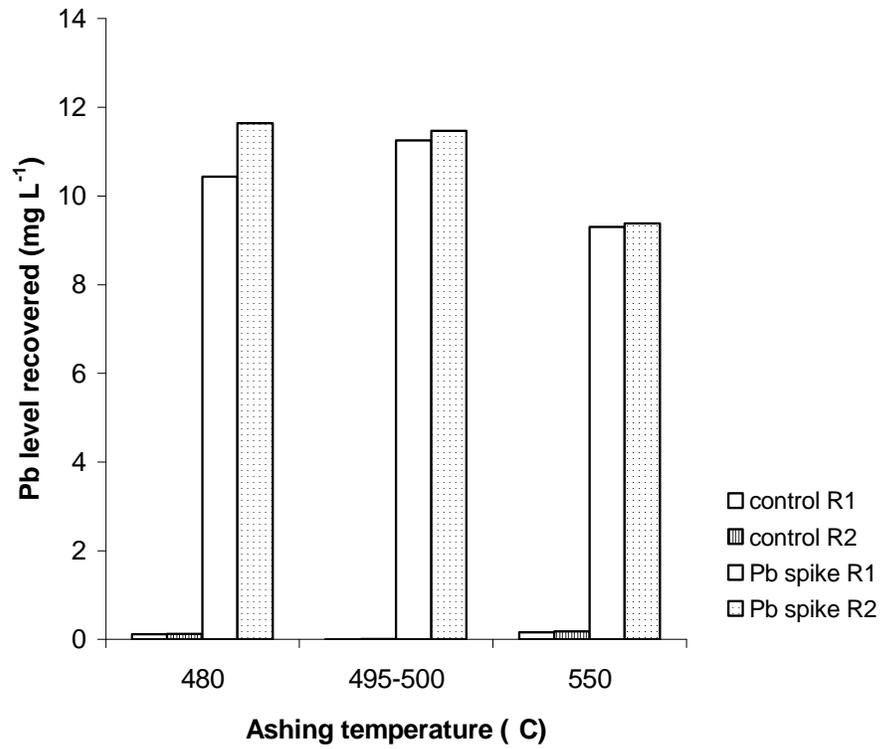
TA = tannic acid. AA = ascorbic acid.

APPENDIX E



Indication of effect of time on extraction (orbital shaking) of tannin levels from lateral and main roots (LR, MR) of *Symphytum officinale*. Values are from one replicate. TA = tannic acid.

APPENDIX F



Effect of ashing temperature on Pb (element) recovery. Values are mean absorbencies from the same sample.

Replicate 1 (R1), Replicate 2 (R2)

Spike level: 12 mg L⁻¹ Pb from 1000 mg L⁻¹ Pb (analytical grade BDH) standard.

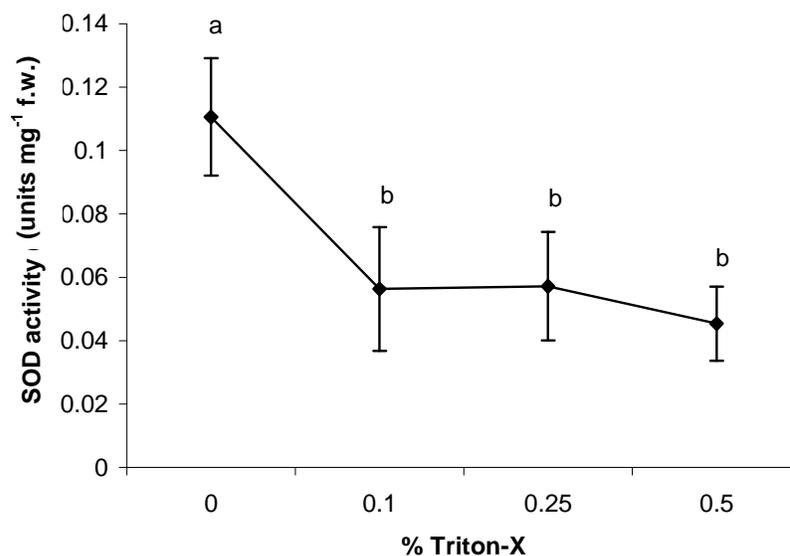
APPENDIX G

Treatment (no EDTA pre-treatment)	Total root weight (g)	Total shoot weight (g)
control	1.053 ± 0.416	0.977 ± 0.043
Pb(NO ₃) ₂	1.509 ± 0.202	0.820 ± 0.245
PbEDTA	1.559 ± 0.057	1.007 ± 0.154

Treatment (EDTA pre-treatment)	Total root weight (g)	Total shoot weight (g)
control	1.762 ± 0.829	1.287 ± 0.355
Pb(NO ₃) ₂	1.884 ± 0.256	1.070 ± 0.107
PbEDTA	1.516 ± 0.251	1.397 ± 0.169

Example of total weight of root and shoot from 3-4 month-old *Symphytum officinale* plants. These plants (results section 8.3) were grown for three days in modified HC nutrient solution (pH 4.5), with or without 500 M EDTA, followed by four days in modified HC nutrient solution (pH 4.5) containing 0 (control), or 500 M Pb(NO₃)₂ with or without 500 M EDTA. Values are mean ± S.E. of three replicates. All values are not significantly different (P≥0.05). HC = Huang and Cunningham.

APPENDIX H

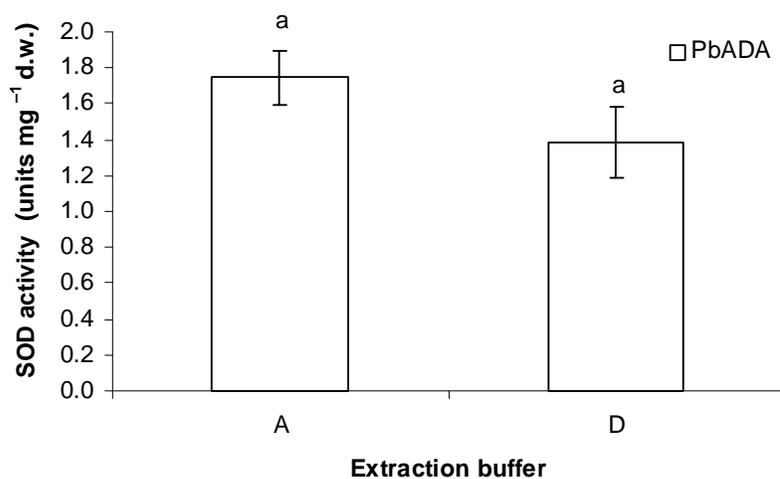


Effect of Triton X-100 concentrations in extraction Buffer A on SOD activity in blade tissue of *in vitro* grown *Symphytum officinale*. Values are means \pm S.E. of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$).

Composition of buffer:

Buffer A: 100 mM potassium phosphate + 1% (w/v) PVPP, pH 7.8.

APPENDIX I



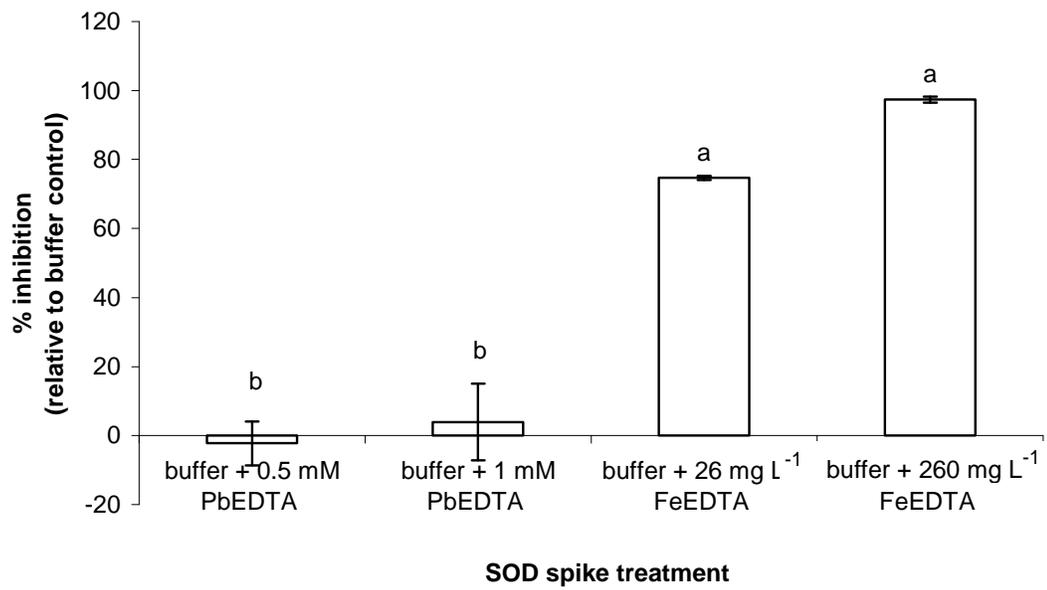
Comparison of different extraction buffers on SOD activity in roots of 3-4 month-old sand-grown *Symphytum officinale* plants exposed to modified HC nutrient solution (pH 7.0) with 500 μ M Pb(NO₃)₂ and 500 μ M ADA. Values are means \pm S.E. of three replicates. Values sharing the same letter are not significantly different ($P \geq 0.05$). HC = Huang and Cunningham.

Composition of buffers:

Buffer A: 100 mM potassium phosphate + 1% (w/v) PVPP, pH 7.8

Buffer D: Buffer A + 0.625 mM EDTA

APPENDIX J



Percentage inhibition caused by various spikes added to 0.1 M potassium phosphate buffer (pH 7.8) used in the NBT assay. Values are means \pm S.E of three replicates. Values not sharing the same letter are significantly different ($P \leq 0.05$).

APPENDIX K

ARABIDOPSIS THALIANA STUDIES: LEAD MUTANTS

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1 METHODS

A combination of guidelines from Lehle seeds (2002), Weigel and Glazebrook (2002), Chen *et al.*, (1997) and Scholl *et al.*, (1998) were followed for the methods in this section.

1.1 Germination

Approximately 500 seeds (0.01 g) of wild type *Arabidopsis thaliana* (ecotype Columbia) seeds were weighed into 1.5-mL-capacity Eppendorf tubes and soaked in non-sterile dH₂O for 30 minutes. After this, the water was replaced with a series of solutions: one mL of 95% (v/v) ethanol for exactly five minutes, one mL rinse of non-sterile dH₂O (twice), one mL of 10% (v/v) bleach with one drop of Tween 20 per 100 mL for exactly five minutes, and finally after transfer to the laminar flow, rinsed five times with one mL of with sterile dH₂O. Seeds suspended in 0.5 mL of sterile dH₂O, were plated to the agar periphery of three 250 mL containers with a Pasteur pipette at a rate of 80-120 seeds per container, or 250 seeds across an entire nine cm Petri plate.

Seeds were stratified at 4°C for four days (in the dark) prior to germination under light (16 hour photoperiod at $26.5 \text{ mol m}^{-2} \text{ s}^{-1}$, 22°C) for five days. The Pb concentration chosen for mutant screening was based on which media resulted in the majority of wild-type seeds not germinating or with abnormal morphology.

Agar media for germination and screening of *A. thaliana* seedlings consisted of 25 mL of modified HC nutrient solution containing 0, 50, 100, 250, 500, 750 $\text{M Pb(NO}_3)_2$ plus 2% (w/v) sucrose and 0.8% (w/v) bacto-agar. Media containing 1000 $\text{M Pb(NO}_3)_2$ was not used as the media did not set. All media were made with nanopure dH₂O and adjusted to pH 4.5.

1.2 Mutant selection and analysis

Using 50 $\text{M Pb(NO}_3)_2$ agar media in Petri plates both wild type and M2 mutants were screened for putative Pb mutants; those with root tissue greater than 6-7 mm on this Pb medium were identified as *putative mutants* (after five days) and were rescued to MS media (pH 5.8) containing 2% (w/v) sucrose and without $\text{Pb(NO}_3)_2$. In total, 20,000 seeds were screened. After two weeks the putative mutants were planted to seedling soil mix (Yates, Auckland NZ) and allowed to set seed. To confirm that the mutation is specific to Pb tolerance, rather than enhanced germination, putative mutants and wild type seeds were germinated horizontally on modified HC media (no Pb) for three days and then transferred to vertical plates containing ten mL of modified HC media supplemented with 50 $\text{M Pb(NO}_3)_2$ media. Mutants were identified as those with longer roots than the wild-type on this Pb media.

2 RESULTS AND DISCUSSION

2.1 Selecting a lead concentration for mutant screening

Increasing concentrations of $\text{Pb(NO}_3)_2$ (0, 50, 100, 250, 500, 750, and 1000 M) were incorporated into an agar based medium using the same nutrient composition as modified HC nutrient solution (pH 4.5) but also supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar. However, only 0-750 $\text{M Pb(NO}_3)_2$ was used as the 1000 $\text{M Pb(NO}_3)_2$ concentration failed to set.

Root growth inhibition in wild type *A. thaliana* seeds increased with Pb concentration in agar medium. After five days, root growth and germination was almost or completely impeded with concentrations above 100 μ M Pb(NO₃)₂. At 50 μ M Pb(NO₃)₂ root growth was reduced by 68%, but only by 5% for stem length ($P \leq 0.05$) (Table K). Pb(NO₃)₂ at 50 μ M was selected for mutant screening as it allowed visibly perturbed germination and root growth to be visualized for mutant selection based on root growth inhibition.

2.2 Mutant screening of EMS exposed *Arabidopsis thaliana* seeds

Mutants can be identified by selecting seedlings with root lengths greater than the wild type seedlings on the same media. How much longer should the roots be? This is defined by the tolerance index. According to Chen *et al.*, (1997), the tolerance index (TI) of *A. thaliana* to Pb(NO₃)₂ is defined by:

$$TI = \frac{\text{root length of wild type } A. thaliana \text{ grown on media with Pb}}{\text{root length of wild type } A. thaliana \text{ without Pb}}$$

In this case the TI index was an average of 0.322. To achieve a mutant TI index of 0.5, as suggested by Chen *et al.*, (1997), putative mutants with a root length of between 6-7 mm (if wild type root lengths were 12.10 mm on control medium) or relative to the root length of EMS seeds on control media (without Pb).

Over 20,000 seeds were screened for Pb mutants using root length as the screening tool. Three putative mutants were identified and rescued for seed production. Only one of the three putative mutants set seed. These putative mutant seeds only germinated faster than wild type seeds (Plates K1 and 2); upon transplantation to vertical root growth plates, root length of these putative mutants were the same as wild type seedlings on the 50 μ M Pb(NO₃)₂ media (Plate K3). Thus of the 20,000 seeds screened, no true mutants could be identified.

It was interesting to note that roots of Pb-germinated seedlings followed a different morphological growth pattern compared with that of the control (no Pb) (after seven days, outside the screening period); Pb-germinated seedlings produced a stunted tap root with lateral roots; the control plants (no Pb) only produced a tap root (Plate K4 and 5, Figure K1 and 2). This may have some bearing on the effectiveness of the mutant selection regime (see more below).

Table K. Effect of Pb concentration on root length of *Arabidopsis thaliana* seedlings after five days in modified HC nutrient agar medium (pH 4.5) supplemented with 2% (w/v) sucrose, 0.8% (w/v) agar and various concentrations of $\text{Pb}(\text{NO}_3)_2$. Values are means \pm S.E. of ten replicates. Values not sharing the same letter are statistically different from each other ($P \leq 0.05$). Statistics can not be tabulated for 100-750 M $\text{Pb}(\text{NO}_3)_2$ as differences in values were close to zero. HC = Huang and Cunningham.

$\text{Pb}(\text{NO}_3)_2$ level (M)	Root length (mm)	Stem length (mm)
0	12.10 ± 1.17^a	1.95 ± 0.44^a
50	3.90 ± 0.33^b	1.85 ± 0.53^a
100	≤ 1	≤ 1
250	≤ 1	≤ 0.5
500	-	-
750	-	-

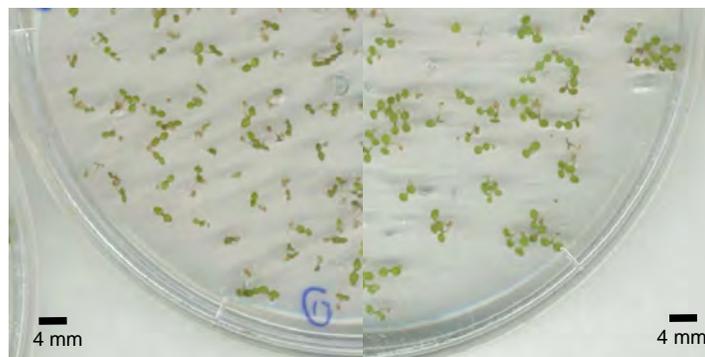
Plate K. Comparison of putative mutant with wild-type seed germination and root growth.

1. Wild-type seedlings (control) after three days on modified HC nutrient agar (pH 4.5) supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar.

2. Putative mutant seedlings after three days on modified HC nutrient agar (pH 4.5) supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar.

3. Five days after transfer to vertically-placed plates containing modified HC nutrient agar (pH 4.5) supplemented with 50 μ M $\text{Pb}(\text{NO}_3)_2$, 2% (w/v) sucrose and 0.8% (w/v) agar. Wild-type seedlings (control) were placed on same plate; sections that are not indicated are seedlings from the putative mutant.

HC = Huang and Cunningham.



1.

2.



3.



4.



5.

Plate K (continued).

Root growth of *Arabidopsis thaliana* seedlings after seven days in modified HC nutrient agar (pH 4.5) supplemented with 2% (w/v) sucrose, 0.8% (w/v) agar and (4) no Pb (control) and (5) 50 μ M $\text{Pb}(\text{NO}_3)_2$. HC = Huang and Cunningham.

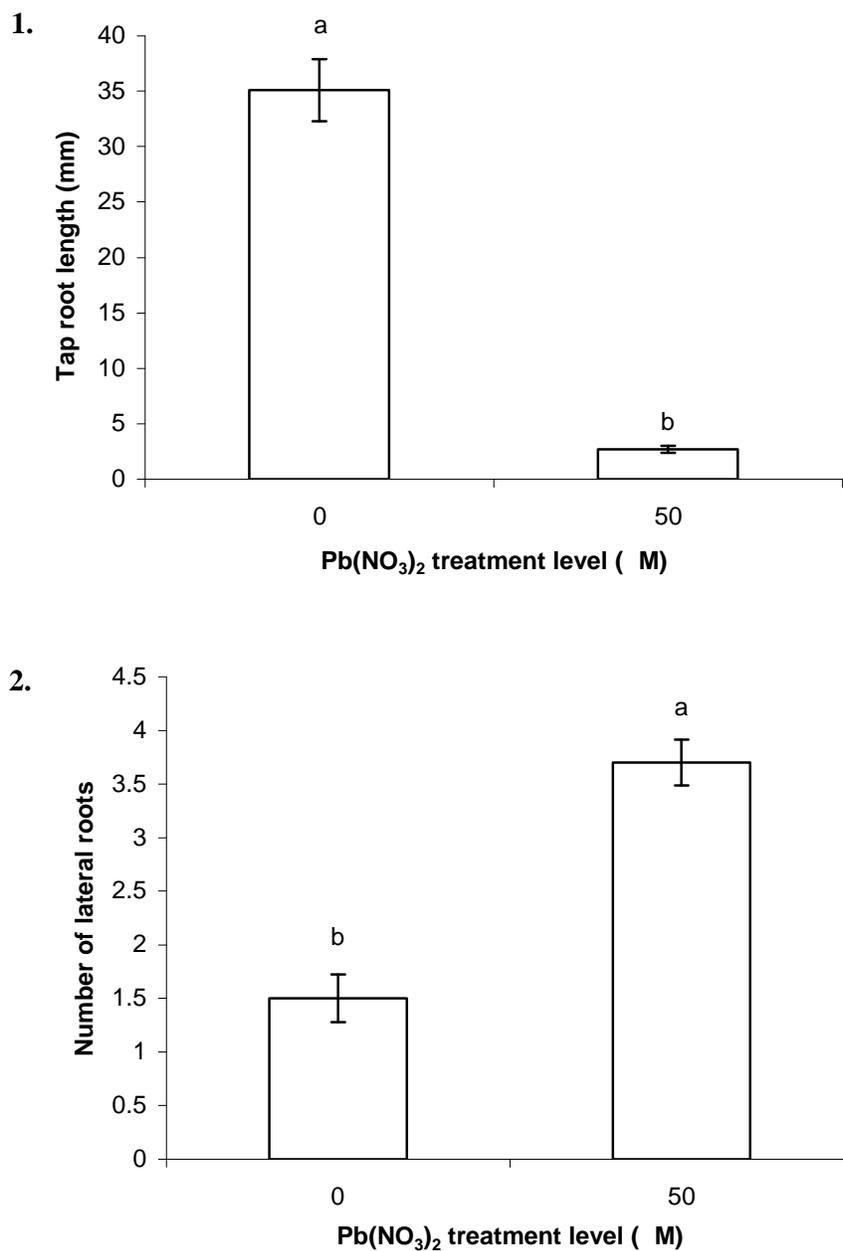


Figure K. Effect of Pb(NO₃)₂ treatment level on (1) tap root length and (2) number of lateral roots after seven days in modified HC nutrient agar medium (pH 4.5) supplemented with 2% (w/v) sucrose, 0.8% (w/v) agar and 0 or 50 M Pb(NO₃)₂. Values are means ± S.E. of ten replicates. Values not sharing the same letter are statistically different from each other (P≤0.05). HC = Huang and Cunningham.

3 CONCLUSIONS AND FUTURE STUDIES

It was anticipated that more than three putative mutants should have been isolated. According to Chen *et al.*, (1997), 25 putative Pb mutants were selected from 500,000 seeds; using this ratio, it was expected that up to ten putative mutants could be found amongst the 20,000 seedlings screened.

The decision to follow the procedure from Weigel and Glazebrook (2002) and Chen *et al.*, 1997 for heavy metal or metalloid mutant selection, may have played a part in the lack of Pb-tolerant mutants found. Weigel and Glazebrook (2002) recommended a mutant screening period of five days, probably before the root density of the 250 seedlings plated prohibited mutant identification. But after five days, Pb treatment did not simply cause root growth inhibition; it appeared to induce a different growth response (compared to the control). Only the primary root could be seen at this time. The lateral roots, which upon reflection of the results may have been necessary for mutant selection, was not seen at this time. These developed after seven days, outside the selection time. In *A. thaliana* seedlings, it appears that the inhibition of primary root growth and observed increase in lateral root formation may be an adaptive response to damaged root tips due to Pb stress. This more dense and compact root morphology has been apparently observed in *Fagus* and *Trifolium* roots under after exposure to Pb, Al, Cd and Cu (Breckle, 1991).

The key question arising from this technique was “how can one accurately identify putative mutants based on degree of root inhibition (section 2.1.2 above) if it is not merely inhibition, but an induction of a different root growth pattern (for both wild type and EMS seedlings) on Pb media?” One possibility is that the overall lateral root growth should be instead have been compared. However, this would require a radical screening and practicality adjustment: (i) a longer germination time required for lateral root growth, (ii) all plates would be vertically placed, and most importantly, and (iii) significantly more plates as at least ten times less seeds per plate would be required to clearly visualize root growth. These steps require far greater resources (in terms of number of plates and time) than was allowed in the study.

In future studies, X-ray fluorescence spectrometry (XRFS) could be used for screening *A. thaliana* mutants. This technique enables the detection of potential mutants at shoot level and from large quantities of seedlings (100,000). This is because the technique involves growing seedlings in soil, so there is no need for sterile practices (aseptic germination) and no limitation associated with root density (Delhaize *et al.*, 1993).