PHYTOREMEDIATION OF METAL CONTAMINATED SOILS:
METAL TOLERANCE AND METAL ACCUMULATION IN PELARGONIUM SP.

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Presented to
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of
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by
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for the degree of
Doctor of Philosophy
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ABSTRACT

PHYTOREMEDIATION OF METAL CONTAMINATED SOILS:
METAL TOLERANCE AND METAL ACCUMULATION IN *PELARGONIUM* SP.

Tereza Violeta Dan                Advisor:  Dr. P. K. Saxena
University of Guelph, 2001

Phytoremediation is an innovative technology that uses plants to remove and/or degrade environmental contaminants such as heavy metals and organic compounds. Plants that possess the ability to accumulate high levels of metals in their biomass are referred to as metal accumulators. The overall objective of this research was to identify, characterize and evaluate the utility of metal accumulator plant species, specifically *Pelargonium* sp. Physiological characterization of metal tolerance using chlorophyll a fluorescence parameters (Fv/Fm, Fv/Fo) indicated that the scented geranium plants were capable of maintaining an efficient photosynthetic activity required for sustained metabolism and to overcome metal-ion mediated stress. The metal accumulation ability of scented geraniums was tested under greenhouse conditions. Scented geranium plants accumulated in excess of 0.08% Cd, 0.12% Ni, 0.30% Pb per kg of shoot dry weight (DW) and 2.70% Cd, 2.11% Ni, 6.10% Pb per kg of root DW. Additionally, scented geraniums exposed to a mixture of metals (Pb+Cd+Ni) have the ability to uptake in excess of 0.44% Cd + 0.52% Ni + 4.72% Pb per kg of root DW, as well as 0.07% Cd + 0.14% Ni + 0.17% Pb per kg of shoot DW. Scanning electron microscopy (SEM) and transmission electron microscopy
(TEM) studies coupled with X-ray microanalysis indicated that the ability of scented geraniums to tolerate metals was due, in part, to its ability to detoxify the metal ions by formation of complexes as well as sequestration of these metal ion complexes in vacuoles, intercellular spaces and as deposits on cell membranes. The efficacy of scented geraniums to tolerate complex contaminants (Pb, Cu and hydrocarbons) under field conditions further confirmed the utility of this plant species for phytoremediation. Identification and characterization of *Pelargonium* sp. provides the field of phytoremediation with an efficient system for remediation of metal polluted substrates.
ACKNOWLEDGEMENTS

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This work was only made possible by the important help that I have received from the members of the Tissue Culture Laboratory: Dr. Y. Mei, Dr. M. Perras, Dr. S. Murch, Mithila Jugulam, Mark Garnett, Kristen Choffe, Skye Campbell, Andrew McCartney, Jerrin Victor, and Tannis Slimmon. Your friendship and support have kept in track and made my adaptation to the Canadian climate much faster and enjoyable. Thanks for your technical expertise, which includes translating, endless knowledge about music, beer, Canadian politics and decaf coffee.

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I am most appreciative of my parents who have dedicated so much time of their life to me. Thank you for encouraging the growth that prepared me to undertake this degree. Finally, I am forever indebted to my husband, Liviu, for his understanding, endless patience and encouragement when it was most required. This would have meant nothing without you.
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<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\phi_{\text{PSII}}$</td>
<td>Qunatum yield of photochemistry</td>
</tr>
<tr>
<td>$\mu g$</td>
<td>micrograms</td>
</tr>
<tr>
<td>$\mu \text{mol}$</td>
<td>micromole</td>
</tr>
<tr>
<td>Car</td>
<td>Carotene</td>
</tr>
<tr>
<td>Chl a</td>
<td>Chlorophyll a</td>
</tr>
<tr>
<td>Chl b</td>
<td>Chlorophyll b</td>
</tr>
<tr>
<td>DW</td>
<td>Dry weight</td>
</tr>
<tr>
<td>Fm</td>
<td>Maximum fluorescence</td>
</tr>
<tr>
<td>Fo</td>
<td>Unquenchable portion of fluorescence</td>
</tr>
<tr>
<td>Fo'</td>
<td>Minimal fluorescence (light)</td>
</tr>
<tr>
<td>Fs</td>
<td>Steady fluorescence yield</td>
</tr>
<tr>
<td>Fs'</td>
<td>Steady fluorescence yield (light)</td>
</tr>
<tr>
<td>Fv</td>
<td>Variable fluorescence</td>
</tr>
<tr>
<td>Fv'</td>
<td>Variable fluorescence (light)</td>
</tr>
<tr>
<td>Fv/Fm</td>
<td>Efficiency of PS II activity</td>
</tr>
<tr>
<td>Fv/Fo</td>
<td>Size and number of active photosynthetic reaction centers</td>
</tr>
<tr>
<td>Fv'Fm'</td>
<td>Excitation transfer energy</td>
</tr>
<tr>
<td>IM</td>
<td>Indian mustard</td>
</tr>
<tr>
<td>LHC II</td>
<td>Light harvesting complexes</td>
</tr>
<tr>
<td>qP</td>
<td>Photochemical quenching</td>
</tr>
<tr>
<td>QPN</td>
<td>Non-photochemical quenching</td>
</tr>
<tr>
<td>SG</td>
<td>Scented geranium</td>
</tr>
<tr>
<td>SF</td>
<td>Sunflower</td>
</tr>
</tbody>
</table>
Environmental pollution with metals has accelerated dramatically since the beginning of the industrial revolution (Nriagu, 1991). The build-up of metals in the soil, aqueous waste streams and ground water renders polluted lands unfit for agriculture and human inhabitation. Heavy metals are primarily a concern because they cannot be destroyed by degradation. There are a number of conventional remediation technologies which are employed to remediate environmental contamination with heavy metals such as solidification/stabilization, soil flushing, soil washing, excavation, retrieval and off site disposal. But a majority of these technologies are costly to implement (Table 1) and cause further disturbance to the already damaged environment (Lasat, 2000).

Often, these polluted lands contain a mixture of numerous organic and inorganic contaminants (for example, biosludge disposal sites of petroleum industries) which makes it impractical for employing any simple remediation approach. In Canada, the federal government estimates there are more than: 5,000 contaminated sites (owned or managed by the federal government), approximately 10,000 abandoned mines, 90,000 active / abandoned drilling sites in the Prairie provinces, 6,000 abandoned tailing sites, approximately 875 millions of tons of radioactive tailings from uranium mines and 29,000 Provincial
Table 1. Cost of soil treatment (Glass, 1999a, Saxena, 1999).

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>COST ($/TON)</th>
<th>ADDITIONAL FACTORS/EXPENSES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitrification</td>
<td>75-425</td>
<td>Long-term monitoring</td>
</tr>
<tr>
<td>Landfilling</td>
<td>100-500</td>
<td>Transport/excavation/monitoring</td>
</tr>
<tr>
<td>Chemical treatment</td>
<td>100-500</td>
<td>Recycling of contaminants</td>
</tr>
<tr>
<td>Electrokinetics</td>
<td>20-200</td>
<td>Monitoring</td>
</tr>
<tr>
<td>Soli Washing</td>
<td>120-200</td>
<td>Transport/ monitoring</td>
</tr>
<tr>
<td>Low Temp. Thermal Desorption</td>
<td>4.5-200</td>
<td>Transport/ monitoring</td>
</tr>
<tr>
<td>Incineration</td>
<td>200-600</td>
<td></td>
</tr>
<tr>
<td>Vitrification</td>
<td>700</td>
<td></td>
</tr>
<tr>
<td>Pneumatic fracturing</td>
<td>8-$12</td>
<td></td>
</tr>
<tr>
<td>Excavation/Retrieval</td>
<td>270-460</td>
<td>Transport/ monitoring</td>
</tr>
<tr>
<td>Disposal</td>
<td>35-60</td>
<td></td>
</tr>
<tr>
<td>Disposal alone</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Brownfield sites (Environment Canada, 1999). Estimated clean up for remediating this sites range from $6$ billion to $20$ billion (Environment Canada, 1999). It was estimated that the potential cost for remediating contaminated soils in Canada is $6$-$20$ billion (Environment Canada, 1999), while in the United States the projected cost is $187$ billion (US EPA, 1999). Metals of concern include lead, cadmium, selenium, mercury, arsenic iron, manganese, nickel, zinc, and copper originating from industrial activities and mining (Table 2).

**Phytoremediation**

Phytoremediation can be defined as the process of utilizing plants to absorb, accumulate, detoxify and/or render harmless, contaminants in the growth substrate (soil, water and air) through physical, chemical or biological processes (Cunningham and Berti, 1993). The concept of using plants to clean up contaminated environments is not new. About 300 years ago, plants were first proposed for use in the treatment of wastewater (Hartman, 1975). At the end of the 19th century, *Thlaspi caerulescens* and *Viola calaminaria* were the first plant species documented to accumulate high levels of metals in leaves (Baumann, 1885). Byers (1935) found that plants of the genus *Astragalus* were capable of accumulating up to $0.6\%$ selenium in dry shoot biomass. Plants able to accumulate up to $1\%$ Ni in shoots were reported by Minguzzi and Vergnano (1948) and recently, Rascio, (1977) reported high Zn accumulation in shoots of *Thlaspi caerulescens*. The idea of using plants to extract metals from contaminated soil was reintroduced and developed by Utsunamyia (1980) and Chaney (1983), and the first field trial on Zn and Cd phytoextraction was
Table 2. Estimated releases of some metals in the Canadian environment (from McIntyre and Lewis, 1997).

<table>
<thead>
<tr>
<th>TOXIC SUBSTANCE</th>
<th>~TONNES RELEASED ANNUALLY</th>
<th>LOCATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium</td>
<td>159 T</td>
<td>Atmosphere</td>
</tr>
<tr>
<td></td>
<td>12 T</td>
<td>Aquatic</td>
</tr>
<tr>
<td></td>
<td>300 T</td>
<td>Slag, sludge, solid wastes disposed on land</td>
</tr>
<tr>
<td>Arsenic</td>
<td>15 T</td>
<td>Released by base metal smelters and refineries in liquid effluent into atmosphere solid waste</td>
</tr>
<tr>
<td></td>
<td>310 T</td>
<td></td>
</tr>
<tr>
<td></td>
<td>770 T</td>
<td></td>
</tr>
<tr>
<td>Nickel</td>
<td>100 T</td>
<td>Air emissions</td>
</tr>
<tr>
<td></td>
<td>64 T</td>
<td>Effluent</td>
</tr>
<tr>
<td></td>
<td>1800 T</td>
<td>Disposed of on land as sludges and solids</td>
</tr>
<tr>
<td></td>
<td>8700 T</td>
<td>Deposited as slags</td>
</tr>
<tr>
<td>Chromium</td>
<td>84 T</td>
<td>Atmosphere</td>
</tr>
<tr>
<td></td>
<td>27 T</td>
<td>Discharges to water</td>
</tr>
<tr>
<td></td>
<td>5000 T</td>
<td>Discharge</td>
</tr>
</tbody>
</table>
conducted in 1991 (Baker et al., 1991).

**Identification of metal-accumulator plants**

An ideal plant species for phytoremediation should have either one of the following characteristic combinations (US EPA, 2000): a) a low biomass plant with a very high metal accumulation capacity, or b) a high biomass plant with enhanced metal uptake potential. In addition to these characteristics, versatility of the candidate plant to tolerate and at the same time accumulate multiple metal contaminants and/or metal-organic mixtures would be an asset for any phytoremediation system (Chaney et al., 1989). Despite increasing reports, most species of metal-accumulator plants are metal-specific, have a small biomass, slow growth rate, and require careful management of agricultural techniques under field conditions (Gleba et al., 1999). It is also important to acknowledge the possible environmental impacts of introducing a new plant species that could represent an environmental risk (US EPA 2000). In addition, most metal accumulator plants are collected from natural habitats, and the use and performance of these plants in other regions could be limited by the lack of optimized agronomic practices in the new regions. In this context, the identification domesticated metal accumulator plant species, coupled with a better understanding of the physiological mechanisms underlying metal accumulation, would facilitate development of effective remediation systems.

Preliminary surveys of domesticated horticultural plants species as candidates for phytoremediation resulted in identification of plants of the genus *Pelargonium*. Additionally, Murch et al. (1997) found that *Pelargonium* sp. plants when treated
with a growth regulator (Thidiazuron) exhibited increased uptake of nutrients and heavy metals (such as iron). In view of these, plants belonging to the *Pelargonium sp.* were selected as candidate plants for assessing their efficiency in phytoremediation of metal contaminated soils.

**Hypothesis, objectives and research approach**

The overall hypothesis of this study was "scented geraniums have the ability to both tolerate and accumulate metals from the growth substrate". The overall objective of the research was to identify *Pelargonium* species that are capable of accumulating and sequestering high levels of toxic metals in their biomass (Figure 1). To accomplish this research the specific objectives were:

I. To evaluate the metal tolerance potential of *Pelargonium* sp. and to compare this plant species with *Brassica juncea* sp. (Indian mustard) and *Helianthus annuus* sp. (sunflower), known as metal tolerant and metal accumulator plants, with regards to the metal tolerance/accumulation under similar experimental conditions.

II. To assess the potential for metal uptake and accumulation when exposed to low, medium and high concentrations of metal ions under greenhouse and field conditions in *Pelargonium* sp.

III. To characterize the metal uptake mechanism in scented geranium plants.

IV. To identify the metal localization/sequestration within the cell.

Several *Pelargonium* species were chosen and screened for their capacity to tolerate metal stress. The capacity to tolerate cadmium, nickel and lead was evaluated under greenhouse conditions using chlorophyll *a* fluorescence kinetics.
(Chapter 3). On the basis of this study, *Pelargonium* sp. that were found to be
highly tolerant to various concentrations of cadmium, nickel and lead, were
tested for their potential for metal uptake and accumulation (Chapter 4). The
metal localization studies (Chapter 5) provided an insight into the possible sites
of metal sequestration in *Pelargonium* exposed to metals. Finally field studies
were designed to evaluate the utility phytoremediation of *Pelargonium* sp.
‘Frensham’ for phytoremediation of metal contaminated soils (Case Study).
Figure 1. Schematic representation of the overall approach to identify plant species that are capable of tolerating and accumulating high levels of metals.
CHAPTER 2

LITERATURE REVIEW

Every living organism has the ability to withstand a specific quantity of essential and non-essential metals present in the environment, and utilize them for their growth processes, but at higher level these metals can be toxic (Bradshaw, 1991). In contrast to essential metals, which serve as metabolic precursors for the plants, the elements classified as non-essential elements do not have any known physiological function in the plant. The most commonly occurring non-essential elements in the environment can be grouped into one major category, termed as heavy metals.

There are two routes of entrance of metals into the environment: natural processes or human activities. Natural contamination originates from either excessive withering of mineral and metal ions from rocks or from displacement of certain contaminants from the ground water or subsurface layers of the soil (Pendias-Kabata and Pendias, 1984). Humans have been introducing trace metals into the environment since they first gained knowledge of their many useful properties. Despite the benefits that society has had after this discovery, the harsh consequences of metal pollution were encountered at the turn of the 19th century, when the Industrial Revolution resulted in a greater demand of various metals (Nriagu, 1991).

The major sources of metal pollution arises mainly from combustion of fossil fuels (coal, oil, natural gas), metal manufacturing plants and foundries, mines
urban/agricultural runoff (Acton and Gregorich, 1995) and sewage effluent use of agricultural chemicals such as pesticides, herbicides and fertilizers (Seward and Richardson, 1990; Alexander, 1995).

The metal species commonly found in the soil as a result of aforementioned human activities include copper (Cu), lead (Pb), zinc (Zn), nickel (Ni), cobalt (Co), mercury (Hg), cadmium (Cd) and arsenic (As) (US EPA 2000, Environment Canada 2000). In order to alleviate the problems caused by these pollutants several criteria have to be taken into consideration such as: characteristics of the contaminant land, form and concentration of the contaminant as well as the end use of the remediated land (Saxena et al., 1999).

In general, the conventional technologies colloquially termed as ‘pump-and-treat’ and ‘dig-and-dump’ techniques are limited in their applicability to small area and have their own inherent limitations (Vangronsveld and Cunningham, 1998). At sites where the contaminants are slightly higher than the industrial criterion (governmental regulations), the use of conventional technologies is not economically viable due to the cost involved (Table 1). So far, irrespective of the technology being selected, the cost estimates for utilizing conventional remediation techniques have remained high (Lasat, 2000, Glass, 1999a). The overall remediation budget includes design, construction, operation and maintenance costs of the process associated with each technology in addition to mobilization, demobilization and pre- and post-treatment costs which are determined on a site-to-site basis (KrishnaRaj et al., 2000). Also, in the case of most ex situ treatment technologies, excavation and transport costs need to be
Table 1. Soil concentration ranges and regulatory guidelines for some toxic metals (from Lasat, 2000)

<table>
<thead>
<tr>
<th>METAL</th>
<th>SOIL CONCENTRATION RANGE (mg kg⁻¹)</th>
<th>REGULATORY LIMITS (mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pb</td>
<td>1.00-6,900</td>
<td>600</td>
</tr>
<tr>
<td>Cd</td>
<td>0.10-345</td>
<td>100</td>
</tr>
<tr>
<td>Cr</td>
<td>0.05-3,950</td>
<td>100</td>
</tr>
<tr>
<td>Hg</td>
<td>&lt;0.01-1,800</td>
<td>270</td>
</tr>
<tr>
<td>Zn</td>
<td>150.00-5,000</td>
<td>1,500</td>
</tr>
</tbody>
</table>
factored in, to arrive at the final cost for remediating a contaminated site. Market survey indicates that the world remediation market ranged between is expected to grow to approximately US$ 50 billion by the year 2002 (Glass, 1999b).

**Phytoremediation**

This technology, which uses the age-old concept of utilizing nature (plants) to cleanse nature (soil and water) has been termed 'phytoremediation'. Phytoremediation can be defined as the process of utilizing plants to absorb, accumulate, detoxify and/or render harmless, contaminants in the growth substrate (soil, water and air) through physical, chemical or biological processes (Cunningham and Berti, 1993). In the last decade, phytoremediation has gained a great deal of interest in both public and private sectors as a cost-efficient alternative to the conventional remediation technologies. The use of plants for remediating heavy metal contaminated soils has multifold advantages (Saxena et al., 1999; Glass, 1999a) such as:

- growing plants is relatively inexpensive
- large scale application, as plants can be sown or planted in large areas large
- plants provide an aesthetic value to the landscape of contaminated sites
- plants concentrate the contaminants within their tissues, thereby reducing the amount of hazardous waste
- concentrated hazardous waste would require smaller reclamation facilities for extracting the heavy metals
- increased aeration of the soil which in turn enables microbial degradation of organic contaminants and microbe-assisted uptake of metal contaminants
- reduced top soil erosion due to plant stand
- enhancement of rhizospheric micro-fauna and flora for maintaining a healthy ecosystem (Chaney et al., 1983)

Disadvantages of phytoremediation (US EPA, 2000)
- root system it is required that the contaminants be in contact with the root zone of the plants. Either the plants must be able to extend the roots to the contaminants, or the contaminated media must be moved to within range of the plants.
- growth rate and seasonality will prolong the phytoremediation time of site as compared with other more traditional cleanup technologies.
- contaminant concentration only sites with low medium level contamination within the root zone are the best candidates for phytoremediation processes.
- performance monitoring demonstrates whether phytoremediation has successfully cleaned up contaminated soil or groundwater to a predetermined standard. This requires continuous measurements of changes in the mass balance of contaminant concentrations in plants, soil, and/or ground water, or by measuring the plant stress response.
- risk monitoring is necessary to assure that accumulation of contaminants in plants does not cause unacceptable ecological risks
- regulatory acceptance is currently being developed (DOE, 1999; US EPA; 2000)

Phytoremediation approaches

Although the basic concept of utilizing plants to remediate contaminated sites
Table 2. Major factors limiting the success and applicability of phytoremediation
(from Lasat, 2000 and US EPA, 2000)

<table>
<thead>
<tr>
<th>PLANT-BASED BIOLOGICAL LIMITATION</th>
<th>REGULATORY LIMITATIONS</th>
<th>OTHER LIMITATIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Low plant tolerance</td>
<td>1) Lack of cost and performance data</td>
<td>1) Contaminant beneath root zone</td>
</tr>
<tr>
<td>2) Lack of contaminant translocation from root to shoot</td>
<td>2) Regulators unfamiliarity with the technology</td>
<td>2) Lengthy process</td>
</tr>
<tr>
<td>3) Small size of remediating plants</td>
<td>3) Disposal of contaminated plant waste</td>
<td>3) Contaminant in biologically unavailable form</td>
</tr>
<tr>
<td></td>
<td>4) Risk of food chain contamination</td>
<td>4) Lack of remediating plant species</td>
</tr>
</tbody>
</table>
Table 3. Root Depth for Selected Phytoremediation Plants (from US EPA, 2000)

<table>
<thead>
<tr>
<th>PLANT</th>
<th>MAXIMUM ROOT DEPTH</th>
<th>TARGET CONTAMINANTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indian mustard</td>
<td>To 12 inches</td>
<td>Metals</td>
</tr>
<tr>
<td>Grasses</td>
<td>To 48 inches</td>
<td>Organics</td>
</tr>
<tr>
<td>Poplar trees</td>
<td>To 15 feet</td>
<td>Metals, organics, chlorinated solvents</td>
</tr>
</tbody>
</table>
remains the same, phytoremediation technology can be subdivided into five distinct subtechnologies:

- **phytoextraction**, involves specific plant species which can absorb and accumulate metal contaminants and/or excess nutrients in harvestable root and shoot tissue, from the growth substrate (soil). This approach is suitable to remove most metals (such as Pb, Cd, Ni, Cu, Cr, V) and excess nutrient from contaminated soils. Examples of plants species used are plants belonging to Brassicaceae family such as *Thlaspi* sp., (Baker et al., 1994) *Brassica* sp., (Kumar et al., 1995) and *Alyssum* sp. (Kramer et al., 1997)

- **rhizofiltration**, utilizes plant roots to take up and sequester metal contaminants and or excess nutrients from aqueous growth substrates (waste water streams, nutrient-recycling systems). This approach is suitable for remediating most metals (such as Pb, Cd, Ni, Cu, Cr, V), excess nutrients, and radionuclides (such as U, Cs, Sr) contaminated water. Examples of plant species used are *Helianthus* sp., *Brassica* sp., (Dushenkov et al., 1995) *Populus* sp., *Lemna* sp., and *Thlaspi* sp. (Salt et al., 1995).

- **phytostabilization**, involves use of plants especially roots and/or plant exudates to stabilize, demobilize and bind the contaminants in the soil matrix thereby reducing their bioavailability. This approach is suitable for both organic and metal contaminated soils (Vangronsveld and Cunningham, 1998).

- **phytovolatilization**, uses the plants ability to absorb and subsequently volatilize the contaminant into the atmosphere (Vangronsveld and
Cunningham, 1998). This approach is suitable for remediating metals such as Hg and Se from contaminated soils. Recently, Rugh et al. (2000) has genetically engineered plants (*Arabidopsis thaliana* L., *Nicotiana tabacum* and *Liriodendron tulipifera*) with bacterial genes that convert organic and ionic mercury compounds to the volatile and less toxic forms.

- **Phytodegradation/ phytostimulation**, utilizes the rhizospheric associations between plants and soil microorganisms to degrade complex organic-metal contaminant mixtures. This approach is suitable for remediating TNT, PAH, petroleum hydrocarbons from contaminated soils (Schnoor et al., 1995). Examples of plant species used are: *Medicago* sp. and several grasses (Gordon et al., 1997).

**Phytoremediation of metal contaminated substrates**

The term of “hyperaccumulator” has been first used by Brooks et al. (1977) to describe plants that can survive (tolerate) or even thrive in soils contaminated with toxic metals. By definition metal hyperaccumulators are herbs, shrubs or even trees with the ability to concentrate heavy metals in their biomass to levels greater than 100 times the normal without exhibiting phytotoxicity symptoms (Lasat, 2000). Baker et al. (1994) suggested that for a plant to be termed as a must accumulate > 0.1% (by dry weight) of Co, Cu, Cr, Pb or Ni and > 1% (by dry weight) of Mn or Zn in its natural habitat. To date, approximately 400 plant species from at least 45 plant families have been reported to hyperaccumulate metals (Salt and Guerinot, 2001). Most hyperaccumulators accumulate Ni, about 30 absorb either Co, Cu, and/or Zn, even fewer species accumulate Mn and Cd, and there
Table 4. Applications of Phytoremediation (from Glass, 1999).

<table>
<thead>
<tr>
<th>APPLICATION</th>
<th>CONTAMINANTS</th>
<th>TYPES OF PLANTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytoextraction</td>
<td>metals, inorganics, radionuclides</td>
<td>variety of natural and selected hyper-accumulators, e.g., <em>Thlapsi, Alyssum, Brassica</em></td>
</tr>
<tr>
<td>Phytostabilization</td>
<td>metals, organics</td>
<td>various</td>
</tr>
<tr>
<td>Phytostimulation</td>
<td>organics; e.g., TNT, PAHs, petroleum hydrocarbons</td>
<td>grasses, alfalfa, many other species including common food crops, trees</td>
</tr>
<tr>
<td>Phyto-volatilization</td>
<td>volatile metals (e.g., Hg) and inorganics</td>
<td>wetland and terrestrial plants, transgenics bearing bacterial genes</td>
</tr>
<tr>
<td>Phyto-transformation</td>
<td>organics, including nitroaromatics</td>
<td>various, including poplars, parrot feather, milfoil</td>
</tr>
<tr>
<td>Phytomining</td>
<td>precious metals</td>
<td>hyperaccumulators</td>
</tr>
<tr>
<td>Rhizofiltration</td>
<td>metals, radionuclides</td>
<td>aquatic metal hyperaccumulators, e.g. duckweed, pennywort, also Brassica, sunflower</td>
</tr>
<tr>
<td>Hydraulic Barriers</td>
<td>organics, inorganics, metals</td>
<td>poplar, willow trees</td>
</tr>
<tr>
<td>Vegetative Caps</td>
<td>organics, inorganics, wastewater, landfill leachate</td>
<td>trees such as poplar, plants (e.g. alfalfa) and grasses</td>
</tr>
<tr>
<td>Constructed Wetlands</td>
<td>metals, acid mine drainage, industrial and municipal wastewater</td>
<td>free-floating or aquatic macrophytes; reeds, cattails, bamboo</td>
</tr>
</tbody>
</table>
Cd, and there are no known natural Pb-hyperaccumulators (Reeves and Baker, 2000). The accumulation of metals could represent a defense mechanism against insect and herbivores (Boyd and Martens, 1994).

**Plant species for phytoremediation, an ideal prototype**

The potential for any plant species to remediate successfully heavy metal contaminated sites depends on all of the following prerequisite factors: a) the amount of metals that can be accumulated by the candidate plant, b) the growth rate of the plant in question, and c) the planting density (Saxena et al., 1999). The growth rate of a plant in a chemically contaminated soil is important from the perspective of biomass. The rate of metal removal from the soils can be calculated if information on the above mentioned parameters is available. An ideal plant species for phytoremediation should have either one of the following characteristic combinations (US EPA, 2000): a) a low biomass plant with a very high metal accumulation capacity, or b) a high biomass plant with enhanced metal uptake potential. In addition to these characteristics, versatility of the candidate plant to tolerate and at the same time accumulate multiple metal contaminants and/or metal-organic mixtures would be an asset for any phytoremediation system.

**Metal hyperaccumulators for terrestrial ecosystems**

The first report of a hyperaccumulator plant goes as far back as 1885 when Baumann analysed for zinc specimens of *Viola calaminaria* and *Thlaspi calaminare* growing over the calamine deposits of Aachen, Germany. In 1948, Vergagno Gambi discovered the unusual hyperaccumulation of nickel by the
Tuscan serpentine plant *Alyssum bertolloni*. Hyperaccumulators are taxonomically well represented throughout the plant kingdom (Baker et al., 1994). For example, *Sebertia acuminata* (a small tree) exudes sap that contains up to 25% Ni by dry weight (Baker et al., 1994). Another example is *Thlaspi caerulescens* a member of the Brassicaceae family that can accumulate up to 4% zinc in its tissue without any visible signs of damage (Brown et al., 1994). Despite a large distribution throughout the plant kingdom, most of the commonly known hyperaccumulators belong to the Brassicaceae family (Kumar et al., 1995). They probably inherited this characteristic from wild members of the family, which are known to thrive in metal rich environments and accumulate metals in their roots and shoots (Baker 1978; Baker et al., 1994).

Environment Canada's Environmental Technology Advancement Directorate has recently released the PHYTOREM data base, an interactive electronic database of more than 700 plants, lichens, algae, fungi and bryophytes that have demonstrated an ability to tolerate, accumulate or hyperaccumulate a range of 19 different metals (Environment Canada, 1999). Species that show considerable potential to date include sunflowers, ragweed, cabbage, Indian mustard, geranium and jack pine. Accompanying this database are 35 different search fields containing additional geographical, regulatory and eco-physiological data on each species. This allows the owners and managers of contaminated sites to choose the species that suit their site conditions, and take the steps necessary to secure regulatory approval for their use (. An additional database (Phytopet® - available in CD format)- has been developed in order to describe plants species with a
demonstrated ability for tolerance to petroleum hydrocarbons and the capacity to reduce contaminant levels in terrestrial or wetland environments (Farrell et al., 2000). These plants grow in the Canadian prairies and boreal plains, but may have useful applications in other environments as well. The rigorous of the seasonal changes of Canada's climate have forced Canadian species to be highly adaptive. These plants may possess genetic material that supports the development of agricultural crops that can withstand greater temperature ranges. The database contains also botanical surveys of a number of "historical" (weathered) hydrocarbon contaminated (adjacent and non-contaminated) sites in Alberta and Saskatchewan.

**Factors influencing heavy metal availability and uptake by plants**

The composition of the soil at a contaminated site can be extremely diverse and the heavy metals present can exist as components of several different fractions (Salt et al., 1995): (1) in soil solution, as free metal ions and soluble metal complexes, (2) adsorbed to inorganic soil constituents at ion exchange sites, (3) bound to soil organic matter, (4) precipitated such as oxides, hydroxides, carbonates, and (5) embedded in structure of the silicate minerals. Soil sequential extractions are employed to isolate and quantify metals associated with different fractions (Tessier et al., 1979). For phytoextraction to occur, contaminants must be associated with the first two fractions listed above (Lasat, 2000). Some metals, such as Zn and Cd, occur primarily in exchangeable, readily bioavailable form while Pb, occur as soil precipitate, which a less bioavailable form (Lasat, 2000). Plants grown in metal-enriched substrate take up metal ions at varying degrees. This uptake is largely influenced by the
bioavailability of the metals which is, in turn, determined by both external (soil-associated) and internal (plant-associated) factors.

**Soil associated factors**

The success of any phytoremediation scheme relies on the availability of metals in the soil, which in turn is controlled by: a) chemical (pH, Eh, CEC, metal speciation), b) physical (size, texture, clay content, % organic matter) and c) biological (bacteria, fungi) processes and their interactions (Ernst et al. 1992).

**pH**

The chemical forms of heavy metals in soil are affected by modifications to the soil pH. An increase in pH (basic range) results in higher adsorption of Cd, Zn, Cu to soil particles and reduces the uptake of Cd, Zn, Pb by plants (Kuo et al., 1985). On other hand, acidification increases the metal absorption by plants through a reduction of metal adsorption to soil particles (Brown et al., 1994). For example, the metal cation concentration was estimated at 9080 μg/l cations in an acidic soil, compared to 17 μg/l cations at neutral pH (Pendias- Kabata and Pendias 1984). Soil pH affects not only metal bioavailability, but also the process of metal uptake by roots. This effect could be metal specific. For example, in *Thlaspi caerulescens*, Zn uptake in roots showed a small pH dependence, while in the case of Mn and Cd the uptake was more dependent on soil acidity (Brown et al.,1995).

**Redox potential (Eh)**

The redox potential of the soil is a measure of the tendency of the soil solution to accept or donate electrons. As the redox potential decreases, heavy
metal ions are converted from insoluble to soluble forms, thus increasing bioavailability (Pendias-Kabata and Pendias, 1984). It is likely that a lower pH and Eh of the soil would enhance the mobility of most metals.

**Cation exchange capacity (CEC)**

The cation exchange capacity of the soil is a measure of the ability of the soil to retain metal ions. The cation exchange capacity increases with increasing clay content in the soil while the availability of the metal ions decreases (Pendias-Kabata - Pendias and Pendias, 1984). Thus, the higher the cation exchange capacity (CEC) of the soil, the greater the sorption and immobilization of the metals. In acidic soils, metal desorption from soil binding sites into solution is stimulated due to H+competition for binding sites. Modulating the CEC would, therefore, result in increased or decreased availability of metals to plants.

**Soil type**

The bioavailability of heavy metals in the soil also depends on the texture of the soil. A gradient of metal ion availability exists in varying soil types with the availability being lowest in clay soils, followed by clay loam, and finally loam and sand. Similarly, heavy metal concentrations in soil are also dependent on the soil order; Gleysols and Luvisols have the highest concentration, followed by Brunisols and Podzols. However, this observation can also be related to soil texture because Gleysols and Luvisols have a higher clay content, compared to Brunisols and Podzols (Webber and Singh, 1995). Normally, a higher level of heavy metal can be retained in fine-textured soils such as clay and clay loam, compared to coarse textured soils such as sand. This is in part due to the low
bioavailability of these metal ions, or reduced leaching as metals are bound to the soil matrix in fine-textured soils (Webber and Singh, 1995). The complexation of heavy metals with organic matter, humic acid in particular, has been well documented (Friedland, 1990). A high organic matter content enhances the retention of the metals, drastically reducing the metal availability.

**Chelates**

An essential component of the bioavailability process is the exudation of metal chelating compounds by plant roots (ex. phytosiderophores). These chelators are synthesized by plants and can mobilize heavy metals such as copper, lead and cadmium by formation of stable complexes (Mench et al., 1995). Chelators are usually low molecular weight compounds such as sugars, organic acids, amino acids and phenolics that can change the metal speciation and thus, metal bioavailability.

Apart from the chelating agents produced by plants, addition of synthetic chelating agents to contaminated soils was shown to increase substantially the metal solubility in the soil (Salt et al., 1995, Cunningham and Berti, 1993). It is likely that in contaminated soils, chelator application enhances the formation of metal-chelate complexes reducing the sorption of metals to the soil particles (Huang et al., 1997). To date, numerous studies have focussed on evaluating the effect of adding synthetic chelates such as ethylene diaminetetracetic acid (EDTA), ethyleneglycoltetracetic acid (EGTA) and citrate on the uptake of metals by plants (Salt et al., 1998). For example the addition of EDTA to a Pb contaminated soil increased the shoot Pb concentration of corn (*Zea mays*) and
pea (*Pisum sativum*) from 500 mg/kg to more than 10,000 mg/kg (Huang et al. 1997) with a concomitant, more than 1000-fold, increase in available metal content of the soil solution (Cunningham and Berti, 1993). Increased absorption of heavy metal from soil into the roots of *Brassica juncea* has also been attributed to chelators (Blaylock et al., 1997). Chelator-assisted phytoextraction approach is applicable to several metals of interest (Zn, Cd, Ni, Se, As, Cr, U) (for an in depth review, see Salt et al., 1998).

However, the adverse effects of applying EDTA or other chelators such as the leaching of PbEDTA to subsurface water has been encountered in an *in situ* phytoextraction field test (Minnesota) and in and *ex situ* phytoextraction, were the leachate collected had 160 -180 mg Pb/L (Chaney, PhytoNet Network, 2001).

**Plant associated factors**

The genetic make-up of the plant greatly influences its metal uptake potential (Chen et al., 1997). Irrespective of which approach of phytoremediation is used, two major plant-associated factors need to be satisfied in order to devise a successful remediation strategy (Saxena et al., 1999). These characteristics, viz., metal tolerance and metal hyperaccumulation potential, clearly define phytoremediation candidate plants. The metal accumulation potential of different plant species varies markedly and is highly correlated to their genotype. Huang et al. (1997) have found that Pb accumulation varies significantly in different species grown in similar environments. It is therefore crucial that a selection scheme be employed to select the best hyperaccumulator genotypes even within
a specific species to ensure the success of phytoremediation procedure.

Rhizospheric processes

The conditions that exist at root-soil interface (rhizosphere) differ in many aspects from those of the bulk soil and are, in part, responsible for the manifestation of certain plants adaptations. Conditions in the rhizosphere have a significant influence on the mineral nutrient uptake and water relations (Marschner, 1995). The extent to which plant roots can modify these conditions is extremely important for the survival of plants under adverse conditions (Marrschner, 1995). The pH of the rhizosphere can, in some instances, differ from the bulk soil pH by up to two units. Any modifications to the rhizospheric pH values would, in turn, have an impact on the cation/anion uptake ratio, the release of carbonated and the excretion of organic acids. Acidification of the rhizosphere through exudation of protons from the roots has also been shown to enhance uptake and accumulation of metals (Marschner, 1995).

Rhizospheric microorganisms also play a significant role in metal availability. For instance, several strains of *Pseudomonas* and *Bacillus* were able to increase the amount of Cd accumulated by 2-week-old *Brassica juncea* seedlings grown in hydroponics (Salt et al., 1995). Finally, the association between plant root system and mycorrhizal colonies has been suggested to influence metal uptake in plants (Burke et al., 2000). The plant-mycorrhizal associations in phosphorus-deficient plants led to enhanced uptake of phosphates as well as copper and zinc (Lambert et al., 1976). A similar enhancement in cadmium and lead uptake was observed when plants were infected with vesicular arbuscular mychorrizae.
(Kilham and Firestone, 1983; Joner and Leyval, 1997). A large number of ectomycorrhizal (ECM) fungi have also been found to be effective in increasing heavy metal availability to their host plant (Marschner, 1995). For example, birch seedlings inoculated with the ECM fungi *Lactarius rufus* or *Sclerotinia flavidium* had increased Ni levels under high Ni exposure. The increased tolerance to heavy metals in this scenario was a result of sequestration of heavy metals in the fungal structure either at the extramatrical mycelium or in the sheath, which resulted in lower concentration of metal ions in the soil solution around the host roots.

**Agronomic practices**

Chaney et al. (1999) has discussed the importance of employing effective agronomic practices. These authors investigated the effect of soil acidification on Zn and Cd phytoextraction and proposed the use of \((\text{NH}_4)_2\text{SO}_4\) as a soil additive to provide nutrients (N and S) needed for high yield, and to acidify the soil for greater metal bioavailability. These authors found that after phytoremediation the soil can be limed to elevate the pH near a neutral value, so that normal farm uses or ecosystem development could resume. However, premature liming may increase soil capacity for metal binding and restrict the potential for phytoextraction. The addition of organic fertilizers could generate the same effects. Phosphorus is a major nutrient, and plants respond favorably to the application of P fertilizer by increasing biomass production. The addition of P fertilizer, however, can also inhibit the uptake of some major metal contaminants, such as Pb, due to metal precipitation as pyromorphite and chloro-pyromorphite (Chaney et al., 2000).
Physiology of metal tolerance mechanism

Tolerance mechanisms are of primary importance for accumulator plant species, as these plants accumulate large amounts of metal ions and sequester them appropriately without incurring cellular/tissue damage. Important tolerance mechanism identified to date, include, production of intracellular binding facilitate toxic metal ions sequestration, alteration of metal compartmentalization patterns to avoid damage and/or disturbances to basic cellular processes, modulation of cellular metabolism to alleviate damage due to accumulation of toxic metals, and modification of membrane structure (Thurnann and Collins 1989; Verkleij and Schat 1990; Ernst et al., 1992, Woolhouse 1993; Cobbert and Goldsbrough, 2000).

a. Production of intracellular binding compounds

Plants are stationary in a fluctuating environment, and therefore undergo a constant modulation of internal levels of metal ions. In general, plants synthesize and accumulate a variety of metal chelating compounds upon exposure too exceedingly high levels of available metals. Natural chelating compounds viz. amino acids and their derivatives, citric acid, malic acid and/or phytochelatins are produced by the plants to alleviate metal-induced toxicity symptoms (Prasad 1999; Rauser, 1999).

The family of proteins called metallothioneins (MT) also serves to bind metal ions by coordinating metal ions to closely spaced cysteine thiol groups. The role of metallothioneins and phytochelatins in imparting metal tolerance in plants is well established, has been reviewed in depth (see Rauser 1990; Steffens, 1990, Robinson, 1994, Cobbert and Goldsbrough, 2000). In brief, phytochelatins are a
unique family of thiol-containing metal binding polypeptides. These polypeptides are synthesized in the plant in response to treatment with metals, and their biosynthesis is connected with glutathione metabolism rather than to ribosomal protein synthesis (Rauser, 1990). Among the metals, Cd is the strongest inducer of phytochelatins while higher concentration of Zn also elicits a similar induction response. Phytochelatins appear to be the primary metal binding polypeptide in plants, and the enzyme catalyzing their biosynthesis, phytochelatin synthase is constitutively expressed in plants.

Phytochelatins may be also involved in trace-metal homeostasis, which can detoxify excess metals. Although the reasons for the apparent dichotomy in metal sequestration by animals (metallothioneins) and plants (phytochelatins) remains poorly understood, recent evidence of metallothionein-like proteins in plants suggests that phytochelatins may not be the exclusive heavy metal binding polypeptides in plants (Steffens, 1990).

The role of organic acids, like citric acid or malic acid, as metal buffering substances has been suggested by a number of researchers (Reeves 1992; Prasad; 1999; Rauser, 1999). The mechanisms by which this may occur remain unclear as the accumulation of these organic acids upon metal exposure may merely reflect disturbances in metabolism (Gotbold, 1984).

b. Alteration of metal compartmentalization patterns

Although metal tolerance may be prevalent at the cellular, tissue, organ level in plants, the metal compartmentalization processes taking place at the level of tissues and organs that contribute to survival of the intact plant. Significant
differences have been reported between tolerant and non-tolerant plants of the same species, in the distribution of metals ions in their tissues (Baker, 1978; Locklema et al., 1986; Verkleij and Prast, 1989). Organic complexes mediate transport of heavy metal ions from root to shoot via xylem, at least partially. It is therefore possible that low metal concentration in the shoots of any species could be a direct result of a reduced metal transport, instead of decreased translocation rate (Ernst et al., 1992).

At the subcellular level, the central vacuole often contains high concentration of heavy metals: Zn and Ni (Brooks et al., 1991), Cu and Pb (Mullins et al., 1985) or Cd (Rauser and Ackerley, 1987). Altered compartmentalization patterns at the subcellular level, due to changes in tonoplast transferase system, could likely play an important role in differential tolerance to heavy metals in plants (Ernst et al., 1992).

c. Alteration of cellular metabolism

Plants have the ability to modify their metabolic processes in order to survive and grow in adverse conditions including the presence of phytotoxic metals (Cobbert and Goldsbrugh, 2000). It has been hypothesized that changes in metabolism, other than the production of chelators or metal compartmentation could provide the plants with an adsorptive advantage (Prasad, 1999). Hyperaccumulator plant species on the other hand may survive in a metal rich environment by avoiding damage to metal sensitive metabolic processes by activation of alternative pathways or through over-production of cytosolutes, which detoxify the metal ions). Alternatively, enhanced metabolism and increased levels
of metabolic energy for metal sequestration could represent potential mechanism involved in the survival of hyperaccumulator plants. An increased production of metal sensitive enzyme could also help counteract the metal inactivation of these critical enzymes, thereby maintaining biosynthetic processes and normal growth and (Verkleij and Schat, 1990).

d. Alteration of membrane structure

The final potential mechanism for metal ion-tolerance is the alteration of membrane structure. Evidence for metal ion induced changes in membrane structure originates from the studies quantifying ion leakage from roots or cells exposed to a variety of metal ions. Several ions including, Ni, Co, and Zn enhance K efflux from Zea mays root segments, while Cd enhances solute leakage from leaf discs of Phaseolus vulgaris (Cumming and Taylor, 1990). Copper induced potassium loss has also been observed in Agrostis capillaris (Woolhouse, 1983), Silene vulgaris (De Vos and Schatt, 1989), and Mimulus guttatus (Strange and McNair, 1991). These reports provide circumstantial evidence for heavy metal induced alterations in plasma membrane structure.

**Experimental characterization of metal tolerance: chlorophyll fluorescence kinetics**

In recent years, the technique of chlorophyll fluorescence has become ubiquitous in plant echophysiology studies (Van Kooten and Snel 1990; Krause 1991; Horton et al., 1996; Govindjee, 1995). The principle underlying chlorophyll fluorescence analysis is relatively straightforward. Light energy absorbed by chlorophyll molecules in a leaf can undergo one of three fates: it can be used to
drive photosynthesis (photochemistry), excess energy can be dissipated as heat or it can be re-emitted as light - chlorophyll fluorescence. These three processes occur in competition, such as any increase in the efficiency of one it will result in a decrease in the yield of the other two. Hence, by measuring the yield of the chlorophyll fluorescence, information about the changes in the efficiency of photochemistry and heat dissipation can be gained.

The absorption of light causes the energy level of chlorophyll to be raised and thus electrons are displaced into higher orbitals. Most of the excitation energy of chlorophyll is transferred to the reaction centers of the photosystem and used to drive the reactions of photosynthesis (oxidation of water, oxygen evolution, NADP reduction, membrane proton transport and ATP synthesis).

Although the total amount of chlorophyll fluorescence is very small (about 1-2% of total light absorbed) measurement is relatively easy. The spectrum of fluorescence is different to that of absorbed light, with peak of fluorescence emission being of longer wavelength than that of absorption. Therefore, fluorescence yield can be quantified by exposing a leaf to light of defined wavelength and measuring the amount of the re-emitted light at longer wavelengths. The most prominent pigments that absorb this energy are chlorophyll a and chlorophyll b. At room temperature almost 90% of the chlorophyll fluorescence is emitted from the PS II antenna complexes, the rest is PS I. However, only PS II fluorescence varies with changes in the photochemistry (Govindjee et al., 1995) and the strongest chlorophyll a fluorescence maximum from LHCII is at 685 nm. The typical response of a dark
adapted leaf is represented in Figure 1 and the description of the fluorescence parameters is summarized in Table 3.

**Physiology of metal uptake by hyperaccumulators**

**Mechanism of absorption**

The unusually high level of metal found in hyperaccumulating plants can be attributed to the physiological, biochemical and genetic processes inherent to these species. For the purpose of this review, we shall limit the discussion to the metal accumulation process, which has been recognized as an extreme physiological response in metal tolerant plants (Thurmann and Collins, 1989; Ernst et al., 1992; Woolhouse, 1983). The uptake of metals by plant roots involves several processes like: a) root interception of metal ions, b) entry of the metal ion into the roots through mass flow and diffusion, and c) translocation of the metal ions from the root to the shoot (Marschner, 1995).

The root uptake process is achieved by mobilizing metal bound to soil particles in the soil solution. The mobilization is reached in several ways (Salt et al., 1995): (a) metal chelating molecules (phytosiderophores) secreted in to the rhizosphere, (b) specific plasma membrane bound metal reductases and (c) proton extrusion from roots.

The transport across of metal ions the plasma membrane has also gained attention recently (Fox and Guerinot, 1998). It is likely that the entry of metal ions inside the plants, either through the symplast (intercellular) or the apoplast (extracellular), depends on the type of metal and the plant species under investigation. For example, cadmium is thought to enter the tissues by both
Figure 1. Kinetics of chlorophyll a fluorescence yield.

ML indicates the illumination of the continuous modulated light, SP indicates a brief saturating pulse (<3s), +AL indicates the illumination of the continuous of the actinic light, - AL indicates the when the actinic light is switched off, and +FR is the illumination of the far red light (Schreiber et al., 1986).
Fluorescence Yield (relative units)

$F_M$ - $F_0$

$60 \text{ s}$

ML SP $\pm$ AL

SP $-\text{AL}$ $+\text{FR}$

$q_N \cdot F_V$

$q_P \cdot F_V'$

$F_M'$

$F_O'$

Time
Table 5. Chlorophyll fluorescence nomenclature (from DeEll et al., 1999).

<table>
<thead>
<tr>
<th>SYMBOL / NAME</th>
<th>TYPES OF PLANTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>F fluorescence intensity</td>
<td>Actual intensity at any time</td>
</tr>
<tr>
<td>Fo minimal fluorescence</td>
<td>Fluorescence intensity with all PS II reaction centers open while the photosynthetic membrane is in the non-energized state, $q_P = 1$ and $q_N = 0$ (dark or low light adapted)</td>
</tr>
<tr>
<td>(dark)</td>
<td></td>
</tr>
<tr>
<td>Fm maximal fluorescence</td>
<td>Fluorescence intensity with all PS II reaction centers closed ($q_P = 0$ and $q_N = 1$). This is the classical maximum fluorescence level in the dark or low light adapted</td>
</tr>
<tr>
<td>(dark)</td>
<td></td>
</tr>
<tr>
<td>Fv variable fluorescence</td>
<td>Maximum variable fluorescence in the state when all the non-photochemical processes are at minimum ($F_m - F_o$)</td>
</tr>
<tr>
<td>(dark)</td>
<td></td>
</tr>
<tr>
<td>Fv/ Fm (dark)</td>
<td>Excitation transfer energy, LHC II to PS II reaction centers</td>
</tr>
<tr>
<td>Fo' minimal fluorescence</td>
<td>Fluorescence intensity with all PS II reaction centers open in any light adapted state, $q_P = 1$ and $q_N &gt; 0$</td>
</tr>
<tr>
<td>(light)</td>
<td></td>
</tr>
<tr>
<td>Fm' maximal fluorescence</td>
<td>Fluorescence intensity with all PS II reaction centers closed in any light adapted state ($q_P = 0$ and $q_N &gt; 0$)</td>
</tr>
<tr>
<td>(light)</td>
<td></td>
</tr>
<tr>
<td>Fv' variable fluorescence</td>
<td>Maximum variable fluorescence in any light adapted state ($F_m' - F_o'$)</td>
</tr>
<tr>
<td>(light)</td>
<td></td>
</tr>
<tr>
<td>Fv'/Fm' (light)</td>
<td>Excitation transfer energy, LHC II to PS II reaction centers</td>
</tr>
<tr>
<td>$q_P$ photochemical quenching</td>
<td>$(F_m' - F) / (F_m' - F_o')$</td>
</tr>
<tr>
<td>$q_N$ non-photochemical quenching</td>
<td>$1 - (F_m' - F) / (F_m' - F_o')$</td>
</tr>
<tr>
<td>$\phi_{PS II}$ Quantum yield of photochemistry</td>
<td>$(F_m' - F) / F_m'$</td>
</tr>
</tbody>
</table>
metabolic and non-metabolic processes. Cutter and Raius (1974) found that a large fraction of Cd was taken up by barley tissues was through of exchange absorption and through diffusion coupled with sequestration, without any concomitant active metabolic uptake. Entry of toxic ions into plant tissues may also occur through specific ion transporters, competing with essential ions of similar radii. Zinc has an electronic structure similar to that of Cd and, in certain instances, was found to depress Cd uptake, while in other cases Zn stimulated Cd uptake (Hardiman et al., 1984). Recently, Vassil et al. (1998) observed that the uptake of metal-chelate complexes in Brassica juncea was enhanced by removing Zn and Ca ions from the plasma membrane. In that situation, the physiological barrier (root plasma membrane) to metal uptake was alleviated with a resultant increase in uptake of metals.

Metal accumulation in root tissue can be accomplished either through deposition of the metal ions along the cell wall and/or inside the cell in the vacuoles (Salt et al., 1995; Salt and Kramer, 2000). The sequestration of specific metal ions or metal-chelate complexes in the root cells is highly dependent on the metal ion in question. For example, Pb is generally found to be associated with cell walls outside the plasmalemma in the form of Pb precipitates and Pb crystals (Malone et al. 1974). Similar to Pb, large concentration of Cd (Hardiman et al. 1984) has also been associated with cell walls, while Zn was primarily sequestered in the vacuole (Brooks et al. 1991).

**Mechanism(s) of translocation**

The mechanisms of metal translocation seem to be very similar to those for
essential nutrients such as Fe or Ca, in as much as the transport from the root to the shoot takes place principally through the xylem. According to Hardiman et al. (1984), Cd enters the stele through leakage and its translocation rate to the shoot is dependent on the root concentration. It is, therefore, reasonable to expect that a large part of metals would be retained in the stem during translocation through the xylem fluid. Prezemeck and Haase (1991) suggested phytochelatin-mediated metal binding in the xylem sap as a possible mechanism for metal translocation. However, Salt et al. (1995) found that the translocation of Cd through the xylem sap was not dependent of the production of phytochelatins in roots.

Several types of compounds have been proposed to be involved in metal absorption and translocation in hyperaccumulator plant species (Salt and Kramer, 2000). Among these, low molecular weight chelators such as citrate (Lee et al., 1978) and free histidine in *Alyssum lesbiacum* (Kramer et al., 1996) have been reported to play an important role in metal tolerance by forming nickel-histidine complexes. This complexes can reduce the solution concentration of the free metal ion preventing metal toxicity at cellular and sucellular level. Malate and malonate were reported to be the predominant nickel ligands complexes isolated from *Alyssum bertolonii* (Brooks et al., 1991). Similarly, high malate concentrations were also reported in the leaves of zinc accumulaor *Thlaspi caerulescens* (Lee et al., 1978). The presence of Pb-EDTA complexes in the xylem exudates of *Brassica juncea* suggests a potential involvement of chelators in metal transport within the plants (Vassil et al., 1998). It is likely that,
phytochelatins are involved in trace-metal homeostasis, while organic acids, like citric acid or malic acid, merely act as metal buffering substances. The mechanisms by which this may occur still remain unclear, as the accumulation of chelators such as organic acids upon metal exposure, might in part reflect disturbances in metabolic status of the plant (Gotbold et al., 1984). Apart from these factors, heavy metal ion translocation within the plant is also influenced by other factors such as transpiration rate, root uptake, radial transport, and xylem loading (Salt et al., 1995).

**Phytoremediation— potential candidates**

According to the literature reviewed, in 1997 there were only several plant species that had their potential for phytoremediation evaluated and confirmed by basic research, laboratory trials and field scale testing that have also gained regulatory approval. Two of these plant species were *Brassica juncea* (Indian Mustard) and *Helianthus annuus* (sunflower).

**Brassica species**

*Brassica* species close relatives of the well-known metal-accumulating wild mustards have received much attention. Studies carried with different varieties of *Brassica juncea* (Indian mustard) have shown that these plants were able to take up and concentrate toxic heavy metal (Pb, Cu, Ni) to a level up to several percent of their dried shoot biomass. Kumar et al. (1995) showed that while most crop *Brassica* tested were able to accumulate Pb, some cultivars of *Brassica juncea* (L.) Czern. showed a strong ability to accumulate Pb in the roots and to transport the metal to the shoots. These plants could accumulate up to 34 mg/kg
DW of lead in their shoots and up to 190 mg/kg DW in their roots, which represent 3.5 and 19.0% respectively of the dried tissues. Their study also revealed that *B. juncea* was able to concentrate other metals such as Cr, Cd, Ni, Zn and Cu in the shoots from the feeding solution. For this reason, *Brassica juncea* is often used as choice candidate in field trials conducted on contaminated soils.

**Sunflower**

By screening roots of hydroponically cultivated plants for their ability to remove and concentrate heavy metals from the solution, Dushenkov et al. (1995) identified certain varieties of sunflower (*Helianthus annuus*) as being the most efficient plants for rhizofiltration. In their study, Kumar et al. (1995) showed that sunflower plants could accumulate 5.6 mg of lead per g of dry weight tissues from a hydroponic solution which was about 10 times more lead than other species tested namely sorghum, amaranth, tobacco and corn. Sunflower plants were also used in a greenhouse-based hydroponic reactor filled with U-contaminated water at concentrations as high as 350 ppb. In that case, the plants were capable of reducing the water contamination by 95% within 24 hours, accumulating U in their root tissues at concentration 5,000 to 10,000 times greater than the concentration in the water (Phytotech Inc. 1995).

**Conclusions**

The emerging field of phytoremediation offers us a low-cost alternative to conventional remediation technologies for controlling the persistent global problem of environmental pollution. However the success of the
Phytoremediation technology primarily depends on the selection of the ideal plant species.

It is suggested that further identification and characterization of metal accumulators (plants in natural habitats or domesticated plant species) and enhancing the metal accumulation potential of identified species will facilitate the utility of this technology on a commercial scale.
CHAPTER 3A.

METAL TOLERANCE OF SCENTED GERANIUM (Pelargonium sp. ‘Frensham’): EFFECTS OF CADMIUM, NICKEL AND LEAD ON CHLOROPHYLL FLUORESCENCE KINETICS

Abstract

The ability of scented geraniums (Pelargonium sp. ‘Frensham’) to tolerate metal stress was assessed using chlorophyll a fluorescence kinetics. The effects of various concentrations of cadmium, nickel and lead in the culture solution on photosynthetic efficiency in scented geranium was evaluated in comparison to two well established metal accumulators, the Indian mustard (Brassica juncea) and the sunflower (Helianthus annuus), under greenhouse conditions. The efficiency of the photosynthetic apparatus was affected to varying degrees at all metal concentrations for the plants tested. High concentrations of cadmium (1000 mg L\(^{-1}\)) did not significantly affect the efficiency of photosystem II activity, expressed as the ratio of variable fluorescence to maximal fluorescence (F\(_{\text{v}}\)/F\(_{\text{m}}\)), which remained high (0.738) in scented geraniums, but decreased significantly (P<0.05) in Indian mustard (0.089) and sunflower (0.026) plants following 4 days of metal exposure. Similar trends were observed for nickel treatments. The effect of lead was minimal. Also, the number and size of active photosynthetic reaction centers, as measured by the F\(_{\text{v}}\)/F\(_{\text{o}}\) ratio, was not significantly affected by metal exposure in scented geranium plants, while the ratio significantly decreased in Indian mustard and sunflower seedlings. The results suggest that scented geranium plants were able to overcome metal stress through a) maintaining an efficient photosystem II activity, which is required for plant metabolism and physiological functions, as well as to overcome metal ion
mediated stress, and b) restricting damage to the photosynthetic apparatus (reaction centers) by metal ions.

**Introduction**

Plants represent the principal entry point of metals into a food chain ultimately consumed by man and other animals. Although most plants are affected by elevated levels of metal ions, few higher plants have developed the ability to survive and thrive in metal rich soils (Baker et al., 1994). Some of these plants, apart from surviving on metal-contaminated soils, can also uptake and sequester these metals in their biomass without incurring physiological damage. The use of such metal accumulating plants for decontaminating metal-polluted soils has resulted in the development of phytoextraction technology (Kumar et al., 1997). Phytoextraction involves the use of such metal-accumulator plants that are capable of tolerating and accumulating elevated levels [>0.1 % dry weight (DW)] of metals in the above-ground parts, i.e., stems and leaves, without manifesting signs of phytotoxicity. Thus, the identification of new metal tolerant and accumulator species has become an integral part of phytoremediation research and technology development.

The presence of excessive amounts of metals in soils commonly elicit many stress symptoms in plants, such as alteration of growth, especially root growth (Moya et al., 1993), disturbances in photosynthesis (Greger and Ögren, 1991; Ferreti et al., 1993), carbohydrate metabolism (Malik et al., 1992), water relations (Poschenrieder and Barcelo, 1999; Poschenrieder et al., 1989), mineral nutrition
(Rubio et al., 1994), and ultimately, senescence resulting in death (Ernst et al., 1992). However, quantification of the physiological effects of metals on plants is technically complex, time consuming, and usually involves destructive techniques (Losch and Knochl, 1999). To characterize and study tolerance, the identification of markers, preferably non-destructive ones, is mandatory.

Chlorophyll a fluorescence has been previously shown to be valuable in screening for several abiotic stresses, such as heat, chilling, drought, and salinity (Weigel, 1985; Becerril et al., 1988; Lanaras et al., 1993; KrishnaRaj et al., 1993). The current study was designed to evaluate the metal tolerance of scented geranium plants. Non-destructive chlorophyll fluorescence kinetics has been utilized as a means of quantifying the physiological effects of metal stress in the current investigation. The specific objectives were: a) to compare the effects of metal stress on scented geranium with two other metal hyperaccumulator species, viz. Indian mustard (Brassica juncea) and sunflower (Helianthus annuus), b) to quantify the physiological effects of metal stress on the photosynthetic apparatus of scented geranium plants, and c) to develop a non-destructive standard for assessing metal tolerance in plants.

Materials and methods

Preparation of plant material and metal exposure

Three different plant species, viz. scented geranium (Pelargonium sp. 'Frensham'), Indian mustard (Brassica juncea), and sunflower (Helianthus annuus), were used for all experiments in this study. The effects of three
different metals, i.e., cadmium, nickel and lead on the physiological status of the three plant species were assessed independently. Cuttings of scented geraniums made from greenhouse-grown plants were rooted in 3.5 cm plastic pots containing approximately 150 g of well-washed (with deionized water until the washing solution became clear) coarse Perlite (Premier Horticulture Inc., Mississauga, Canada). Seeds of Indian mustard and sunflower were soaked in water for 2 h and then sown in well-washed coarse Perlite placed in the 3.5 cm plastic pots. Plants (one plant of the test species in each pot) were placed in 5.5 cm plastic saucers (three scented geranium pots or two Indian mustard or two sunflower pots in each saucer). All three species were grown in a greenhouse equipped with supplementary lighting (16/8 h photoperiod; 350 μmol m⁻²s⁻¹) and controlled temperature (18-22°C) for 30 d with irrigation (mist beds).

Two weeks prior to initiation of the experiment, the plants were watered with half-strength Hoagland’s solution followed by flushing with water. During the treatment period (14 days), both the seedlings and the rooted cuttings were treated with 50 ml of half-strength Hoagland’s solution, which was devoid of phosphate and sulphate, but supplemented with the various metal salts. The metal treatments were provided as nitrates: cadmium as Cd(NO₃)₂·4H₂O at 0, 250, 500, 750, 1000 mg L⁻¹, nickel as Ni(NO₃)₂·6H₂O at 0, 250, 500, 750, 1000 mg L⁻¹ and lead as Pb(NO₃)₂·6H₂O at 1000, 1500, 2000, 2500 mg L⁻¹. The pH of the metal solutions was adjusted to 6.0-6.5. The treatments were repeated at 24 h intervals, over the 14-d treatment period.
**Chlorophyll a fluorescence**

Chlorophyll fluorescence was measured using parameters described by Öquist and Wass (1988). Briefly, the leaves were dark-adapted for 30 min with a Hansatech clip (3 cm diam. with a central window of 0.4 cm diam. Hansatech Instruments Ltd., King Lynn, England). The fluorescence parameters were measured using a portable fluorometer (Plant Efficiency Analyzer, Hansatech Instruments Ltd., King Lynn, England) at 3,000 μmol m⁻²s⁻¹ light (650 nm) for 5s. The measurements (Fv, Fm, Fo) were recorded on newly formed, fully expanded leaves, and were repeated on the same leaves on alternate days, over the 14-d treatment period. The Fv/Fm and Fv/Fo ratios were calculated from measurements of Fv, Fm and Fo. The ratio of variable fluorescence to maximal fluorescence (Fv/Fm) is an indicator of the efficiency of the photosynthetic apparatus, while the ratio of variable fluorescence to unquenchable portion of fluorescence (Fv/Fo) is an indicator of the size and the number of active photosynthetic reaction centers. An Fv/Fm ratio of > 0.80 is characteristic of a healthy plant, while a ratio of < 0.3 represents a physiologically restricted (dead / senescent) plant (Smillie and Hetherington, 1983).

**Experimental design and statistical analysis**

All the experiments were designed as a completely randomized block design (CRBD) and repeated at least twice. Fluorescence measurements were made on 3 leaves per plant on 4 individual plants of scented geraniums (12 replicates) and 2 leaves per plant on 4 individual plants of Indian mustard and sunflower (8
replicates). Analysis of Variance (ANOVA) was carried out using General Linear Models procedure of the Statistical Analysis System Version 6.12 (SAS Inc. 1995) to determine if there were significant differences in fluorescence parameters as a result of the metal treatments. Significant differences between the means were assessed by Tukey's honestly significant difference test (Tukey's _HSD_ ) at _P_ < 0.05. Linear regression analysis was performed to obtain the prediction equation to determine the days to effect complete senescence for each metal treatment level for Cd, Ni and Pb.

**Results**

Exposure of scented geraniums to solutions of cadmium, nickel and lead for a two week period resulted in minimal expression of stress related morphological symptoms. In contrast, Indian mustard and sunflower seedlings exposed to similar concentrations of metals exhibited severe wilting and necrotic spots on leaves by day 3. Also, Indian mustard and sunflower plants exhibited symptoms of early chlorosis and senescence after only 3 days of treatment. Premature flowering was observed in the case of Indian mustard plants in response to metal treatments.

**Cadmium**

Throughout the 14-day treatment period, the Fv/Fm ratio decreased gradually in scented geranium plants exposed to cadmium. After two days of treatment with low (250 mg L⁻¹), and medium (500 mg L⁻¹) concentrations of cadmium, scented geranium plants exhibited no symptoms of phytotoxicity (Fv/Fm ratio of 0.800), while the ratio decreased negligibly on exposure to higher concentrations (750 mg L⁻¹, 1000 mg L⁻¹ cadmium) compared to control plants (Figure 1A). The
decline in the Fv/Fm ratio in scented geranium was primarily due to a decrease in Fv (an approximate 58 % decrease compared to control plants) while Fm value exhibited only minor changes. In contrast to scented geraniums, the Fv/Fm ratio decreased drastically after only 2 days of metal exposure (Figure 1B, 1C) for both Indian mustard and sunflower plants exposed to all levels of cadmium tested. In Indian mustard and sunflower plants exposed to low levels of cadmium, the ratio decreased significantly to 0.675, and 0.518, respectively, after two days of exposure as compared to their respective controls plants (0.795 and 0.813). Furthermore, the sunflower plants succumbed to the elevated levels of metal stress after four days of exposure (Fv/Fm ratio of 0.026), and Indian mustard plants were physiologically restricted (senescence / death) after six days (Fv/Fm ratio of 0.032). The decline in Fv/Fm ratio in Indian mustard and sunflower was primarily due to a significant decrease in Fv (approximately 99 %) as a result of cadmium exposure.

To predict the number of days to attain physiological death (Fv/Fm ratio of 0.3) in scented geraniums, prediction equations were generated (Figure 2). The prediction equations indicated that scented geranium plants will be physiologically restricted after 23 days of exposure to cadmium at concentrations as high as 1000 mg L⁻¹. The Fv/Fo ratio in scented geraniums remained unaltered compared to controls after two days of exposure to low or medium concentrations of cadmium (4.4) but, the ratio decreased to 2.5 (Figure 3A) after 8 days of exposure to 750 mg L⁻¹ of cadmium. The decrease in the Fv/Fo ratio was primarily due to an increase in the level of unquenchable portion of fluorescence (Fo). In comparison to scented
Figure 1. Effects of various concentrations of cadmium on Fv/Fm ratio for (A) scented geranium, (B) Indian mustard and (C) sunflower plants. Treatment means (±SE) were compared using Tukey’s test at P<0.05 (n=12).
geraniums, there was a drastic reduction in Fv/Fo ratio (2.83 and 2.10, respectively) in Indian mustard and sunflower, after 2 days of exposure to low levels of cadmium (Figure 3B, 3C) as compared to their respective controls plant (4.02 and 4.32, respectively). The variable fluorescence levels for Indian mustard decreased by 99 % as compared to the control plants after 6 days, while the Fo value decreased by only 22 %. Similarly, in sunflower Fv decreased by 99.5 % compared with control plants in 4 days, while Fo value decreased by 12 %.

Nickel

The Fv/Fm ratio of scented geraniums decreased gradually following exposure to nickel over a 14 days treatment (Figure 4A). After 4 days of exposure with the low and moderate metal concentrations the ratio remained constant (Figure 4A). However, after day 8 there was an appreciable decrease in the Fv/Fm ratio in plants exposed to higher nickel concentrations (0.663). By day 14, the Fv/Fm ratio of these plants declined significantly to 0.429. This decrease in Fv/Fm ratio was due to a significant decline in the level of variable fluorescence (63 % decrease).

In contrast to the response of scented geraniums at high nickel exposures, Indian mustard and sunflower plants exhibited a significant decline in Fv/Fm ratio at all levels of nickel exposure, and the decrease was significant even at low concentrations of metal exposure (Figure 4B, 4C). At the highest level of nickel exposure, the Fv/Fm ratio declined significantly after day 4 in both sunflower (0.06; Figure 4C) and Indian mustard (0.026; Figure 4B) indicating physiological death. In sunflower the decline in Fv/Fm ratio was primarily due to 95 % decrease in the Fv.
Figure 2. Prediction equations (regression analysis for number of days to death) for scented geranium (SCG), Indian mustard (IM) and sunflower (SF) plants exposed to various concentrations of cadmium.
Figure 3. Effects of various concentrations of cadmium on Fv/Fo ratio for (A) scented geranium, (B) Indian mustard and (C) sunflower plants. Treatment means ($\pm$SE) were compared using Tukey's test at $P<0.05$ (n=12).
The prediction equations indicated that scented geraniums would be physiologically restricted after 20 days of exposure to the highest level of nickel treatment (1000 mg L⁻¹) tested (Figure 5).

The Fv/Fo ratio for scented geranium plants (Figure 6A) decreased gradually at low (4.29) and moderate (4.38) levels of metal exposure compared with control plants (4.4). However, at the same levels of metal treatment, a significant decline in the Fv/Fo ratio was observed after day 6, when the ratio decrease to 3.7. After 14 days, the ratio declined markedly as a result of a substantial increase in levels of Fo (82 % increase in Fo). On the other hand, the Fv/Fo ratio for Indian mustard and sunflower exposed to low concentrations of nickel for two days were significantly less than controls (2.83 in Indian mustard, and 2.10 in sunflower). At higher concentrations of nickel exposure (1000 mg L⁻¹), the decrease in the Fv/Fo ratio was more severe (1.06 in Indian mustard; Figure 6B and 0.43 in sunflower; Figure 6C).

**Lead**

The scented geranium plants were highly tolerant to lead compared to both Indian mustard and sunflower. The Fv/Fm ratio decreased from 0.82 to 0.50 (after 14 d) in scented geranium (Figure 7A), compared to a decline in the ratio from 0.80 to 0.03 (within 6 d) for Indian mustard (Figure 7B), and 0.81 to 0.03 (within 4 d) for sunflower (Figure 7C), following daily treatment with the highest level of lead.

The ratio of variable fluorescence to unquenchable portion of fluorescence (Fv/Fo) remained unchanged (4.36) in the scented geraniums plants exposed to
low levels of lead, compared to controls (4.6) after 4 days of exposure. In the in Fv/Fo ratio in both Indian mustard and sunflower even at low levels of lead exposure (2.54 and 2.06, respectively) after only 2 days of exposure (Figure 8 B and C) compared to their respective control plants (3.90 and 4.72, respectively). The Fv value for Indian mustard decreased by 99 % as compared with the control plants after 6 days, while the Fo value decreased by 32.77 %. Similarly, in sunflower Fv decreased by 80.38 % compared with control plants in 4 days, while Fo value decreased by 26.7 %.

The prediction equations indicated that treatments with 2500 mg L⁻¹ over 14 d would be essential to inhibit metabolic activity in scented geraniums (Figure 10).

**Discussion**

The use of metal-accumulating plants for remediation generated a new technology termed phytoremediation (Chaney, 1983). In order to make this technology more viable, an ideal hyperaccumulator plant species has to satisfy two prerequisites: tolerance and accumulation. In addition, a better understanding of the mechanism(s) of the metal tolerance is essential for the development of efficient phytoremediation strategies. This study represents the first attempt to characterize the physiological effects of heavy metal exposure in geraniums, which have been found to accumulate metals in previous studies (Murch et al., 1997).

The chlorophyll a fluorescence parameters have been widely used to assess the impact of environmental stress on plants, because they offer a rapid method for identification of injury in the absence of visible symptoms (Bolhar- Nordenkampf et
Figure 4. Effects of various concentrations of nickel on Fv/Fm ratio for (a) scented geranium, (b) Indian mustard and (c) sunflower plants. Treatment means (±SE) were compared using Tukey’s test at P<0.05 (n=12).
Figure 5. Prediction equations (regression analysis for number of days to death) for scented geranium (SCG), Indian mustard (IM) and sunflower (SF) plants exposed to various concentrations of nickel.
Figure 6. Effects of various concentrations of nickel on Fv/o ratio for (A) scented geranium, (B) Indian mustard and (C) sunflower plants. Treatment means (±SE) were compared using Tukey's test at P<0.05 (n=12).
Figure 7. Effects of various concentrations of lead on Fv/Fm ratio for (A) scented geranium, (B) Indian mustard and (C) sunflower plants. Treatment means (±SE) were compared using Tukey’s test at P<0.05 (n=12).
Days of treatment
Figure 8. Prediction equations (regression analysis for number of days to death) for scented geranium (SCG), Indian mustard (IM) and sunflower (SF) plants exposed to various concentrations of nickel.
Figure 9. Effects of various concentrations of lead on Fv/Fo ratio for (A) scented geranium, (B) Indian mustard and (C) sunflower plants. Treatment means (±SE) were compared using Tukey’s test at P<0.05 (n=12).
toxicity symptoms before any morphological signs could be observed, provides the field of phytoremediation a very efficient technique to screen for metal tolerant plants.

DeEll et al. (1999) has emphasized the advantages of using chlorophyll fluorescence parameters for detection of several stress induced changes in plants, and in particular the ratios Fv/Fm and Fv/Fo. These ratios have been frequently reported as suitable parameters for determining stress-induced effects on plant metabolic status (Greger and Ögren, 1991; Lanaras et al., 1993).

The most significant finding was the relatively limited physiological damage, as expressed by chlorophyll fluorescence parameters, observed in scented geranium plants exposed to metals at levels that were toxic to the other species. The decline in the PS II efficiency expressed by the Fv/Fm ratio, observed in Indian mustard and sunflower, is likely a result of major irreversible damage to the photosynthetic apparatus in response to metal stress.

A decline in the Fv/Fm ratio was a result of a reduced Fv value, which in turn indicates interference in the reduction of the electron acceptor QA by the PS II. A decrease in Fv would therefore reflect changes in the photochemistry of PS II (Papageorgiou, 1975).

Earlier, Baszynski et al. (1980) observed an inhibition in PS II activity in chloroplasts of cadmium treated plants. Similar, inhibition of PS II by cadmium, nickel and lead has been reported previously, particularly in experiments conducted with isolated systems (in vitro).

Cadmium has been found to inhibit electron transport (Baszynsky et al., 1980;
Becerril et al., 1988) and affect different sites in the Calvin cycle (Weigel, 1985; Greger and Ögren, 1991), while nickel has been shown to interfere with the biosynthesis of chlorophyll (Stobart et al., 1985). Similarly, lead was reported to induce pigment biosynthesis and chloroplast structure (Prasad and Strzalka, 1999).

An interesting observation is that nickel phytotoxicity symptoms were observed earlier (day 1 in the case of Indian mustard and sunflower, and day 10 in the case of scented geranium) as compared to cadmium (day 2 in the case of Indian mustard and sunflower, and day 12 in the case of scented geranium). This could be explained by a higher mobility for Ni than for Cd in all three plants. Therefore, excessive accumulation would affect the rate of uptake and distribution of the major nutrients in the plant, and consequently would be responsible for mineral deficiencies and depression of the plant growth (Rubio et al., 1994).

In the case of scented geranium plants, the minor reduction in the Fv/Fo ratio indicates no apparent change in the rate of electron transport from PS II to the primary electron acceptors. In contrast, Indian mustard and sunflower plants exhibited a severe reduction in the Fv/Fo ratio at all levels tested.

Similarly, the Fv/Fo ratio has been reported to decrease drastically in sensitive plants exposed to environmental stresses like high temperature and water stress (Briantis et al., 1986).
The damage caused by heavy metals to the PS II reaction centers obstruct the captured light energy from being utilized in the electron transport system and can result in formation of a highly reactive oxygen species (Dietz et al., 1999).

Alterations in Fv/Fo caused by modifications in the unquenchable fluorescence (Fo) have also been related to decreases in the energy transfer from the chlorophyll a antenna to the reactions centers, and a disturbance in the reaction center activity (Sheoran et al., 1990). Consequently, an increase in Fo will determine a reduction in the variable fluorescence, which can be interpreted as a decline in the ability to reduce plastoquinone.

In both, Indian mustard and sunflower, there was a severe reduction in Fv/Fo value, which is indicative of either a decline in the rate of photochemistry as the primary electron acceptor pool (QA) became increasingly oxidized, or a reduction of the pool size of the primary electron acceptors associated with PS II activity (Krause, 1991).

Clijsters and Van Asshe (1985) have suggested heavy metal-mediated inhibition of photosynthesis especially through inhibition of the electron transport on the water splitting enzyme. Leaks of these electrons could be involved in the reduction of oxygen to form free radicals, that would selectively degrade the unsaturated fatty acids from the plant membranes (Marschner, 1995). Slivinskaya (1991) reported that sunflower plants exposed to nickel induced peroxidation of lipid membranes. The membrane damage was further associated with extensive degradation of the intercellular membranes and organelles, particularly of the chloroplast.
In the case of scented geranium plants exposed to metals, there was no apparent reduction in the rate of electron transfer from PS II to the primary electron acceptors as expressed by the minor changes in the Fv/Fo ratio. A possible explanation for the survival of the scented geranium plants may be a higher photosynthetic efficiency, which would allow the plant to tolerate heavy metal exposure.

It is likely that the increased metal tolerance in scented geraniums is due to several mechanisms that operate in tandem. First, maintaining a constant photosynthetic activity during the first week of exposure is probably achieved through inactivation of the toxic metals by formation of metal complexes and compartmentation. This would prevent cadmium, nickel and lead from interfering with more sensitive sites of cellular metabolism. Secondly, the ability of scented geraniums to maintain an efficient photosynthetic activity required for sustaining the growth and development as well as for overcoming the ion metal mediated stress.

Earlier, Murch et al. (1997) have shown that Pelargonium sp. plants had the ability to overcome a growth regulator-mediated (thidiazuron) stress, by increasing overall metabolic activity. This increased rate of metabolism, in turn, resulted in increased metabolic energy (ATP) and reducing power (NADPH), which are required for the biosynthetic processes related to growth and maintenance for overcoming chemical stress.

There is a possibility that a similar scenario exists in scented geraniums exposed to metal stress wherein the plants ability to overcome metal stress is
through increased metabolic activity, which limits damage to photosynthetic reaction centers. It is likely that more than one mentioned mechanism(s), singly or in combination, might be responsible for the superior performance of scented geranium plants exposed to high metal concentrations.

**Conclusion**

Scented geranium plants have the potential to tolerate a wide range of cadmium, nickel and lead concentrations under greenhouse conditions. Such tolerance is probably due to multiple mechanisms, such as cellular uptake, active detoxification and sequestration, and an increased metabolic rate, thus maintaining optimal photosynthetic activity even under metal stress.

The superior performance of scented geranium plants in substrates that contained various types of metal contamination (cadmium, nickel and lead) under controlled environmental conditions is of major relevance for the improvement of phytoremediation technology, offering a new accumulator species that can tolerate a wide range of metals, situation that is often encountered at the contaminated sites. In addition, scented geranium plants represent a unique model system for the study of multiple mechanism(s) of metal tolerance in plants.

In addition the use of chlorophyll fluorescence kinetics has been proven to be very effective in to characterizing metal tolerance in a non-destructive manner. Chlorophyll fluorescence may also aid in developing an effective procedure to identify and characterize new metal tolerant species. The use of chlorophyll fluorescence kinetics could be extended in monitoring the field performance of
plants used in phytoremediation, offering a possibility to assess the physiological status of the plants over time.
Metal Tolerance of *Pelargonium* sp. 'Citrosa': Effects of Cadmium, Nickel and Lead on Chlorophyll Fluorescence Kinetics

Abstract

The ability of scented geraniums (*Pelargonium* sp. 'Citrosa') to tolerate metal stress was assessed under greenhouse conditions using chlorophyll a fluorescence kinetics. Plants were grown in an artificial soil system and exposed to elevated concentrations of nickel (1000 mg L\(^{-1}\)), cadmium (1000 mg L\(^{-1}\)) or lead (2500 mg L\(^{-1}\)) over a 14 days treatment period. The efficiency of photosystem II activity, expressed as the ratio of variable fluorescence to maximal fluorescence (F\(_{v}/F_{m}\)) was not affected by any of the metals, and remained high (0.80) throughout the treatment period. The number and size of active photosynthetic reaction centers, as measured by the F\(_{v}/F_{o}\) ratio, was not significantly affected by cadmium and lead, but decreased in the case of nickel. Analysis of the fluorescence quenching under light adapted conditions showed that all metals affected the efficiency of the excitation energy captured by the open PS II reaction centers (F\(_{v'}/F_{m'}\)). At the same time, a decrease in the photochemical quenching (q\(_{P}\)) was observed for nickel and lead, but not in the case of cadmium. The decreased q\(_{P}\) and F\(_{v'}/F_{m'}\) is consistent with the decline in the quantum efficiency of PSII (\(\phi_{PSII}\)), which led to a down-regulation of the photosynthetic electron transport. The non-photochemical quenching (q\(_{NP}\)) increased in the case of lead treated plants, suggesting that the non-radiative dissipation of the excitation energy as heat could be a possible metal tolerance...
strategy. It is suggested that the overall efficiency and integrity of the photosynthetic apparatus of Citrosa plants was maintained under the high metal stress by modification of the electron transport through PS II which would be in concert with the modifications of the demand for ATP and NADPH in the Calvin cycle.

Introduction

Living organisms, including plants, are exposed to metal pollutants and manifest several physiological changes. In plants, heavy metal toxicity has been described to affect various physiological processes such as growth (Hagemeyer, 1999), water relations (Poschenreider and Barcelo, 1999) and, in particular, photosynthesis (Clijters and Van Assche, 1985; Prasad and Strzalka, 1999). In addition, heavy metals can irreversibly replace other metal ions in essential photosynthetic co-factors, such as the water splitting complex (Mn) and the plastocyanin (Cu) (Poschenreider and Barcelo, 1999). Furthermore, the substitution of the central ion (Mg) of the chlorophyll by heavy metals has been reported to prevent the photosynthetic light-harvesting processes in the affected molecules, resulting in a breakdown of photosynthesis (Kupper et al., 1996).

Also, heavy metals exert their toxicity through membrane damage by altering the structure and function of the thylakoid membrane lipids (Devi and Prasad, 1999). These changes are mainly attributed to changes in lipid content and peroxidation of the chloroplast membrane (Slivinskaya, 1991). Any changes in the lipid content would affect the fluidity of the membranes, leading to alteration of membrane physiological functions, particularly the ionic permeability.
(Baszynski et al., 1988). Furthermore, the changes in the fluidity of the membranes have been reported to disturb the organization of PS II complexes, leading to a drastically decrease of the PS II activity (Fodor et al., 1995) and, consequently to the inactivation of the photosynthetic carbon reduction cycle enzymes and the inhibition of CO₂ fixation (Krupa and Baszynski, 1989).

Several plant species posses unique survival mechanisms that allow them to tolerate exposure to high levels of toxic metals (Baker and al., 1994). The basic strategy of metal tolerance is the reduction of the cytosolic concentration of free metal, which is achieved by various mechanisms such as: (1) metabolic changes (enhanced metabolism and increased level of metabolic energy), (2) intracellular compartmentation (mostly sequestration in the vacuole together with organic acids, aminoacids) and (3) complexation (with polypeptides and synthesis of phytochelatins and metallothioneins) (Rauser, 1995).

In this context, the understanding of the complex physiological changes that occur in the major processes in a plant, in particular photosynthesis, is highly warranted. In the past 15 years in vivo chlorophyll fluorescence measurements have been commonly used to study the functioning of the photosynthetic apparatus when exposed to various environmental stresses (DeEll et al., 1999). Fluorescence kinetics studies have been found to be effective for measuring tolerance (Homer et al., 1980), in screening for metal tolerant plants and for characterizing metal tolerance mechanisms (Moustakas et al., 1993).

The present investigation was designed to assess the tolerance of Pelargonium sp. ‘Citrosa’ to high levels of nickel, cadmium and lead utilizing the
a pulse modulated fluorometer to measure the dark and light adapted fluorescence. The specific objectives of this study were: a) to assess the effects of metal stress on the efficiency of the photosynthetic apparatus b) to dissect out and identify potential metal tolerance mechanism that impart tolerance to ‘Citrosa’ scented geraniums.

**Materials and methods**

**Preparation of plant material and metal exposure**

*Pelargonium* sp. Citrosa plants (*Pelargonium* sp. ‘Citrosa’) were used for all experiments in this study. The effects of three different metals, i.e., nickel, cadmium and lead, on the physiological status of *Pelargonium* sp. Citrosa were assessed independently. Preparation of the plant material was similar to the conditions described in Chapter 3A. In brief, cuttings of scented geraniums made from greenhouse-grown plants were rooted in 3.5 cm plastic pots containing approximately 150 g of well-washed (with deionized water until the washing solution became clear) coarse Perlite (Premier Horticulture Inc., Mississauga, Canada) and placed in 5.5 cm plastic saucers. The plants were grown in a greenhouse equipped with supplementary lighting (16/8 h photoperiod; 350 μmol m⁻²s⁻¹) and controlled temperature (18-22°C) for 30 d with irrigation (mist beds) and fertilization.

During the treatment period (14 days), the rooted cuttings were treated with 50 ml of half-strength Hoagland’s solution, which was devoid of phosphate and sulphate, but supplemented with the various metal salts. The metal treatments
were provided as nitrates: cadmium as Cd(NO₃)₂·4H₂O at 0 and 1000 mg L⁻¹, nickel as Ni(NO₃)₂·6H₂O at 0 and 1000 mg L⁻¹ and lead as Pb(NO₃)₂·6H₂O at 0 and 2500 mg L⁻¹. The pH of the metal solutions was adjusted to 6.0-6.5 (with 0.1N HCl or 0.1N NaOH). The treatments were repeated at 24 h intervals, over the 14-d treatment period.

**Pigment estimation**

Newly formed, fully opened leaves from metal treated and control plants were collected for pigment estimation. Leaf segments were weighed (approximately 1 gram of tissue) and immersed in test tubes containing 10 ml of 80 % acetone (Arnon, 1945). Absorption was measured, using a spectro-photometer (UV-265FW, Shimadzu), at 645 and 663 nm and chlorophyll content was calculated using the equation: chlorophyll_a + b = 8.02(A₆₆₃) + 20.21(A₆₄₅). Three replicates were made of each measurement. The tissue was then homogenized with a pestle and mortar (on ice), and the homogenate was filtered through a Whatman No. 4 filter paper in the dark. Pigments (chlorophyll a, chlorophyll b and carotene) content were determined spectrophotometrically and expressed as μg·mg⁻¹ LFW (leaf fresh weight). Results of pigment estimates are mean of 12 replicates ± SE.

**Chlorophyll a fluorescence**

Chlorophyll fluorescence was measured using parameters described in Chapter 3A. The fluorescence parameters were measured using a portable fluorescence monitoring system (FMS 2, Hansatech Instruments Ltd., King's Lynn, England). The measurements were recorded on newly formed, fully
expanded leaves, and were repeated on the same leaves on alternate days, over the 14-d treatment period. The minimum fluorescence (Fo) with all PS II reaction centers open was measured using the modulated light that was sufficiently low (<0.1 μmol m⁻² s⁻¹) not to induce any significant variable fluorescence. The maximum fluorescence level (Fm) with all PS II reaction centers closed was determined by a pulse of saturated light at 2.5 s at 15 000 μmol m⁻² s⁻¹ in dark adapted leaves. Then the leaves were continuously illuminated with a white actinic light at an irradiance of 200 μmol m⁻² s⁻¹. The steady state value of fluorescence was thereafter recorded and a second saturating light pulse at 15 000 μmol m⁻² s⁻¹ was imposed to determine the maximal fluorescence in the light adapted state (Fm'). The actinic light was then removed and the minimal fluorescence in the light-adapted state (Fo') was determined with a 2.5 s far red light.

Using both, light and dark fluorescence measurements the following parameters were calculated: (1) the maximum quantum efficiency of PS II photochemistry in the dark adapted state (Fv/Fm), (2) the ratio of variable fluorescence to unquenchable portion of fluorescence (Fv/Fo) which is an indicator of the size and the number of active photosynthetic reaction centers, (3) the efficiency of excitation energy captured by open PS II reaction centers (Fv'/Fm'), (4) the photochemical quenching coefficient qP = (Fm'-Fs)/(Fm'-Fo'), (5) non-photochemical quenching coefficient qN =1-(Fm'- Fo')/(Fm-Fo) and (6) the quantum yield of PS II electron transport in the light adapted state ϕPS II = (Fm'-Fs)/Fm'. All fluorescence nomenclature was based on Van Kooten and
Snel (1990). All fluorescence measurements were made consistently during the same time of each treatment day.

**Experimental design and statistical analysis**

All the experiments were designed as a completely randomized block design (CRBD) and repeated at least twice. Fluorescence and pigment measurements were made on 3 leaves per plant on 4 individual plants per treatment (12 replicates). In the case of fluorescence experiments, according to the one way ANOVA, the results from individual experiments could not be pooled. The experiments were done at different time periods and therefore differences among absolutes values were probably due to seasonal variation. The trends were, however, similar in all experiments and the data from one representative experiment is presented. Analysis of Variance was carried out using General Linear Models procedure of the Statistical Analysis System Version 6.12 (SAS Inc. 1995) to determine if metal treatment had a significant effect on the parameters measured. Significant differences between the means were assessed by Tukey’s honestly significant difference test (Tukey’s HSD) at $P<0.05$.

**Results**

Exposure of scented geraniums to elevated levels of various metals in solution for a two-week period resulted in minimal expression of stress related morphological symptoms (chlorosis, wilting). Cadmium and nickel treatment elicited earlier morphological stress symptoms were observed earlier by day 10, as compared to the lead treated plants (day 12).
Pigment

The plants exposed to nickel exhibited no significant changes in the chl a/b ratio, chl a and b, respectively (Table 1). On the contrary, in the cadmium treated plants the total chlorophyll decreased significantly by 52.20 %, and the ratio of chlorophyll a/b also decreased by 28.42 % (Table 1). Chlorophyll a and b contents were also reduced as compared to their control, indicating that changes in the total chlorophyll content were due to alterations in both pigments levels. Lead exposure induced a minor decrease (13.14 %) in the chlorophyll a/b ratio, and this decrease was mainly due to the reduction in the chlorophyll a content. Carotene levels were unaffected by the presence of any of the metals used in this study.

Dark adapted chlorophyll a fluorescence kinetics

In the case of nickel, throughout the 14-day treatment period, the Fv/Fm ratio remained (Figure 1A) in the range of 0.800. The toxic effect of nickel on the physiological status of plant was noticed only after 12 days of metal exposure, when the Fv/Fm ratio decreased by 14.3 % as compared to their control. However, by day 14 there was no significant difference between the nickel treated and untreated plants. The maximum efficiency of PSII photochemistry (Fv/Fm) showed no considerable changes (6.11 % decrease as compared to control) after 14 days of exposure to an elevated level of cadmium (Figure 1B). The Fv/Fm ratio remained unaltered throughout the treatment period in the case of lead also (Figure 1C). However, it is important to note the slight decrease trend in FvFm ratio over the first half of treatment, followed by a recovery on the
second.

PSII photochemistry (Fv/Fm) showed no considerable changes (6.11 % decrease as compared to their control) after 14 days of exposure to an elevated level of cadmium (Figure 1B). The Fv/Fm ratio remained unaltered throughout the treatment period in the case of lead also (Figure 1C). However, it is important to note the slight decrease trend in FvFm ratio over the first half of treatment, followed by a recovery on the second. The Fv/Fo ratio in nickel treated plants declined following the 14 days of metal treatment. However the decrease in the Fv/Fo ratio was minor, only 6.11 % decrease as compared to their control (Figure 2A). Similarly, no significant changes were observed in the Fv/Fo ratio after the 14 days of cadmium (Figure 2B) and lead (Figure 2C) exposure.

**Light adapted phase of chlorophyll a fluorescence kinetics**

The nickel induced stress was evident in the case of the efficiency of excitation energy capture by the open PS II reaction centers (Fv'/Fm' ratio) which decreased started to decrease after 4 days of exposure (Figure 3A). Plants treated with cadmium showed a gradual decrease in the Fv'/Fm' ratio for the first 10 days of exposure, followed by a 30.28 % decrease after day 12 and a 63.05 % decrease by day 14 (Figure 3B). In the case of lead, the Fv'/Fm' ratio declined after 6 days of exposure (by 18% as compared to their control) and the ratio continued to decline in the following days, and by day 14 the ratio decrease by 58 % (Figure 3C). The photochemical quenching coefficient (qP) decreased
Table 1. The effect of cadmium, nickel and lead on pigment content (μg/g FW) in *Pelargonium* sp. Citrosa, values are means of 12 replicates ± SE. Treatments with the same letter are not statistically significant according to Tukey's test at P<0.05.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>CHL A</th>
<th>CHL B</th>
<th>CAROTENE</th>
<th>CHL A/B RATIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>446.4±44.7a</td>
<td>243.7±99.5a</td>
<td>128.1±19.7a</td>
<td>1.8</td>
</tr>
<tr>
<td>Ni (1000 mg/L)</td>
<td>375.9±55.1a</td>
<td>213.9±70.3a</td>
<td>117.1±8.7a</td>
<td>1.8</td>
</tr>
<tr>
<td>Control</td>
<td>574.9±48.7a</td>
<td>291.4±36.8a</td>
<td>179.9±23.8a</td>
<td>2.0</td>
</tr>
<tr>
<td>Cd (1000 mg/L)</td>
<td>243.2±24.3b</td>
<td>172.2±14.4b</td>
<td>112.4±11.9b</td>
<td>1.4</td>
</tr>
<tr>
<td>Control</td>
<td>496.4±44.1a</td>
<td>282.8±34.2a</td>
<td>129.0±19.7a</td>
<td>1.7</td>
</tr>
<tr>
<td>Pb (2500 mg/L)</td>
<td>323.5±55.1b</td>
<td>212.7±99.5a</td>
<td>109.7±13.3a</td>
<td>1.5</td>
</tr>
</tbody>
</table>
Figure 1. Effects of nickel, cadmium, and lead on Fv/Fm ratio for *Pelargonium* sp. 'Citrosa' (metal and control). Treatment means (±SE) were compared using Tukey's test at P< 0.05 (n=12).
significantly in the case of nickel exposure, over the duration of the experiment (Figure 4A) and, by day 14, \( q_P \) decreased by 21.04%. Cadmium treatment did not significantly influence the \( q_P \) levels over the 14 days of exposure (Figure 4B). However, it is important to notice that a major decrease (38.97%) occurred in day 4, beyond which the plants seemed to recover from the metal induced stress. Lead exposure had no significant effect on \( q_P \) during the first 10 days of experimentation, but \( q_P \) values significantly decreased (26.91%) occurred after day 12 (Figure 4C).

The non-photochemical quenching coefficient (\( q_{NP} \)) decreased significantly with increasing nickel exposure except for days 8 and 10 (Figure 5A). In the case of cadmium exposed plants \( q_{NP} \) remained consistently higher as compared to their respective control (Figure 5B). Interestingly, in the case of lead the \( q_{NP} \) remained higher as compared to their control up to day 10 (Figure 5C).

Consistent with the \( q_P \) decrease there was a decrease in the quantum yield of PSII (\( \varphi_{PSII} \)) in the case of nickel exposure (Figure 6A), while in the case of cadmium \( \varphi_{PSII} \) increased consistently, but still lower than controls throughout except day 14 (Figure 6B) and increased significantly when plants were exposed to lead (Figure 6C).

**Discussion**

Interest in the subject of metal tolerance has been growing recently due to the increasing levels of metal contaminants in the environment and the potential toxicity to both, animals and plants. The capacity of some plants to survive to high concentrations of certain heavy metals has been the focus of several studies, in an
Figure 2. Effects of nickel, cadmium, and lead on Fv/Fo ratio for *Pelargonium* sp. ‘Citrosa’ (metal and control). Treatment means (±SE) were compared using Tukey’s test at P< 0.05 (n=12).
Days of Treatment

- Ni
- Cd
- Pb
Figure 3. Effects of nickel, cadmium, and lead on Fv'/Fm' ratio for *Pelargonium* sp. ‘Citrosa’ (metal and control). Treatment means (±SE) were compared using Tukey’s test at P< 0.05 (n=12).
Figure 4. Effects of nickel, cadmium, and lead on $q_P$ ratio for *Pelargonium* sp. 'Citrosa' (metal and control). Treatment means (±SE) were compared using Tukey's test at $P<0.05$ (n=12).
Figure 5. Effects of nickel, cadmium, and lead on $q_{NP}$ ratio for *Pelargonium* sp. ‘Citrosa’ (metal and control). Treatment means (±SE) were compared using Tukey’s test at $P < 0.05$ (n=12).
Figure 6. Effects of nickel, cadmium, and lead on \( \varphi \) PS II ratio for *Pelargonium* sp. 'Citrosa' (metal and control). Treatment means (±SE) were compared using Tukey's test at \( P<0.05 \) (n=12).
attempt to elucidate the underlying mechanism that metal tolerance (Woolhouse, 1983, Thurmann and Collins, 1989). The PS II activity as reflected by the Fv/Fm ratio appeared to be unaffected by the presence of the metals over the 14 day treatment period. Moreover, in the case of nickel the levels of pigments were not affected, which indicates that nickel is not interfering with the pigment biosynthesis or that only a reduced degradation of the pigment is being generated. This may be due to production of intracellular binding compounds in order to facilitate toxic metal ion sequestration and to avoid damage and/or disturbances to basic cellular processes, such as photosynthesis (Verkleij and Prast, 1989). This finding indicates that in the case of cadmium and lead treated plants the photosynthetic apparatus maintained a high efficiency of PS II photochemistry, in spite of the chlorophyll loss (Lu and Zhang, 1998).

The Fv/Fo ratio indicated no apparent change in the rate of electron transport from PS II to the primary electron acceptors in the case of cadmium and lead exposed plants. Nickel however, was responsible for a minor decrease that is indicative of either a decline in the rate of photochemistry as the primary electron acceptor pool (Qa) became increasingly oxidized or a reduction of the pool size of the primary electron acceptors associated with PS II activity (Krause, 1991). In the present investigation, changes in the Fv/Fo ratio were due to the modifications in the unquenchable fluorescence (Fo) that affected the energy transfer from the chlorophyll a antenna to the reactions centers (DeEll et. al., 1999). Another possible explanation for the changes in the Fv/Fo ratio of the nickel treated plants is the substitution of the central atom of chlorophyll,
magnesium by the nickel. by the presence of nickel. This substitution prevents photosynthetic light-harvesting in the affected chlorophyll molecules, resulting in a disruption of photosynthetic reactions (Kupper et al., 1996).

During photosynthesis, photochemical and non-photochemical processes can dissipate the light energy absorbed by the chloroplast. The decline in the efficiency of excitation energy capture by open PS II reaction centers (Fv'/Fm') caused by all three metals in this study, reflects a negative change in the effectiveness and the antenna size of PS II (Krause, 1991). The results from the fluorescence quenching analysis under light adapted conditions showed that, cadmium and lead induced a significant decline in qP, which may indicate an increase in the proportion of the closed PS II reaction centers or in the proportion of the redox state of QA (Dietz et al., 1999). Increase in the proportion of the redox state QA will generate a decrease in the excitation energy available used for photochemistry (Halves et al., 1991). However, the total oxidation of the electron acceptor of PS II leads to an acceleration of the electron transport through PS II (DeEll et al., 1999). Interestingly, in the plants exposed to cadmium no major changes were observed in the qP, indicating that Citrosa plants have the capability of preventing the excessive reduction of PS II reaction centers, maybe by the production of a large pool of free radical scavengers (Fracheboud et al., 2000).

The decreased qP and Fv'/Fm' generated a decrease in the quantum yield of PS II electron transport in order to regulate the electron transport. Such regulation might serve to equilibrate the production of ATP and NADPH with the
decrease demand of the Calvin cycle. Limitation of the ATP and NADPH consumption may cause a high pH gradient, and therefore a limited electron transport (Larson et al., 2000). In the case of plants that were exposed to cadmium is possible that the lack of changes in the $q_P$ might reflect an increase in the overall metabolic activity. This increased rate of metabolism, in turn, resulted in increased metabolic energy (ATP) and reducing power (NADPH), which are required for the biosynthetic processes related to growth and maintenance for overcoming the stress imposed by the high level of toxic metals accumulated in the plants (Ouzounidou, 1996). Oxidation of NADPH continuously restores the terminal electron acceptor and maintains photochemical de-excitation of reaction centers in a steady state (Sheoran, 1990).

Additionally, the proton motive force through the coupling of electron transport to the ATP synthesis regulates the amplitude of the photosynthetic electron transport by feedback inhibition (Siedlecka and Baszynski, 1993). Such regulation serves as a dissipation mechanism for the excess excitation energy, when the rate of ATP and NADPH synthesis exceeds the carbon metabolism (Krause and Cornick, 1987).

The non-photochemical processes ($q_N$) are believed to represent the non-radiative dissipation of excitation energy (Horton, 1996). The increased level of $q_N$ observed in the plants exposed to lead would be in agreement with the increase in the proportion of the redox state $Q_A$, therefore favoring non-photochemical quenching processes at the cost of photochemical utilization of excitation energy ($Fv'/Fm'$) (Larson et al., 2000). Consequently this will lead to a
down regulation of PS II in order to avoid the over-reduction of QA (Lu and Zhuang, 1998). According to Kruppa et al. (1993) the increased qN could be due to the metal poisoning of the Calvin cycle, which results in limitation of ATP and NADPH consumption, causing a high pH gradient and a limited electron transport. On the contrary, the plants treated with cadmium maintained an unaffected qN that could be related to the increased PS II efficiency as depicted by the quantum efficiency of PS II.

**Conclusion**

The responses of *Pelargonium* sp. 'Citrosa' to the exposure to nickel, cadmium and lead suggest that this plant species is metal tolerant. In the case of nickel, tolerance was achieved probably by diminishing the mobility of the toxic ion and consequently, by minimizing the possibility of interfering with pigment biosynthesis. Cadmium and lead tolerance was due a higher PS II efficiency and, probably an overall increase of the metabolic activity, which maintains optimal photosynthetic activity despite the high levels of metal exposure. It is likely that tolerance to metals is accomplished via a cascade of events that include production of intracellular binding compounds, effective metal compartimentation and changes in the cellular metabolism that are crucial for limiting the damage to the photosynthetic apparatus.
CHAPTER 4.

CADMIUM, NICKEL AND LEAD UPTAKE AND ACCUMULATION IN SCENTED GERANIUM
(PELARGONIUM SP. ‘FRENSHAM’)

Abstract

The ability of scented geraniums (Pelargonium sp. ‘Frensham’) to uptake cadmium, nickel and lead was assessed under greenhouse conditions. Plants were grown in an artificial soil system and exposed to increasing concentrations (0-1000 mg/L Cd(NO₃)₂·4H₂O, 0-1000 mg/L Ni(NO₃)₂·6H₂O and 0-2500 mg/L Pb(NO₃)₂·6H₂O) over a 14 day treatment period. Scented geranium plants accumulated 27,000 mg of cadmium; 21,100 mg of nickel and above 60,000 mg of lead per kg DW of root tissue and 750 mg of cadmium; 1,190 mg of nickel and in excess of 3,000 mg of lead per kg DW of shoot tissue. Additionally, scented geraniums exposed to a mixture of metals (lead+cadmium+nickel) had the ability to uptake in excess of 4.72% Pb + 0.44% Cd + 0.52% Ni per kg of root DW, as well as 0.17% Pb + 0.07% Cd + 0.14% Ni per kg of shoot DW. The high levels of cadmium, nickel and lead accumulation in scented geranium biomass is likely due to varied functional detoxification mechanism that limit damage to the metabolically sensitive sites.
Introduction

The anthropogenic entry of metals into the environment is a consequence of decades of uncontrolled industrial processes (Nriagu, 1991). Heavy metals are elements with a specific weight of higher than 5 g per cm$^3$. In nature metals are found throughout the earth, in rocks, soils and sediments, primarily trapped in some stable form. Yet, through natural processes such as weathering and erosion, small amounts of metals are removed from bedrock and circulate in ecosystems. On the other hand, humans have been introducing metals into the environment through combustion of fossil fuels (coal, oil, natural gas), metal manufacturing plants, mines and smelting of ores, urban/agricultural run-off and sewage effluents (Friedland, 1990).

While metal ions such as copper, zinc and recently, nickel (Brown et al., 1987) are considered essential micronutrients for plants, the same metal ions could become phytotoxic at higher levels. On the contrary, metal ions with no known physiological function in plants, such as cadmium and lead, could trigger phytotoxicity symptoms while present even at very low levels in the soil (Barcello et al., 1988). Although most plant species are affected by the presence of metal ions in the environment, a few higher plants have evolved populations with ability to tolerate and thrive in metal-rich soils. These plants, known as metal-accumulators, can sequester excessive amounts of metal ions (> 0.1% DW) in their biomass without incurring damage to basic metabolic functions (Cunningham et al., 1997). Because of the potential to survive, uptake and sequester very high levels of metal
ions in their tissue without exhibiting phytotoxicity symptoms, metal-accumulator plants have become the basis of the development of ‘green’ technologies (referred to as phytoremediation) for decontamination of metal polluted soils.

Studies of the impact of metals on model plants that manifest metal accumulation have been focused on several plants species such as *Thlaspi* sp. (Baker et al., 1994), *Brassica* sp. (Blaylock et al., 1997; Huang et al., 1997) and *Alyssum* (Krammer et al., 1996). Although the list of metal accumulator plants is increasing steadily (more than 400 plant species) (Salt and Guerinot, 2001; Baker et al., 2000), most species are very metal-specific, have a small biomass, slow growth habit and require careful management and manipulation of agricultural techniques under field conditions (Gleba et al., 1999). Thus identification of new plant species with a high biomass and a robust growth habit, coupled with ability to tolerate and accumulate multiple metals has become an important aspect of phytoremediation.

The overall objective of the current investigation was to evaluate the potential of the *Pelargonium* sp. to uptake and accumulate various metal pollutants, cadmium, nickel and lead individually or in combination from the growth substrate. The specific objectives were: 1) to assess the morphology of plants under metal stress, 2) to quantify metal accumulation when plants were exposed to individual metals and 3) to quantify the metal accumulation when plants were exposed to a mixture of metals.
Material and methods

Preparation of plant material and metal exposure

Three different plant species, viz. scented geranium (Pelargonium sp. 'Frensham'), Indian mustard (Brassica juncea), and sunflower (Helianthus annuus), were used for all experiments in this study. Cuttings of scented geraniums and seedlings of Indian mustard and sunflower were prepared as described in Chapter 3A. In brief, cuttings of scented geraniums made from greenhouse-grown plants were rooted in 3.5 cm plastic pots containing approximately 150 g of well-washed (with deionized water until the washing solution became clear) coarse Perlite (Premier Horticulture Inc., Mississauga, Canada) and placed in 5.5 cm plastic saucers. The plants were grown in a greenhouse equipped with supplementary lighting (16/8 h photoperiod; 350 μmol m\(^{-2}\text{s}^{-1}\)) and controlled temperature (18-22°C) for 30 d with irrigation (mist beds) and fertilization.

The metal treatments were provided as nitrates: cadmium as Cd(NO\(_3\))\(_2\)·4H\(_2\)O at 0, 250, 500, 750, 1000 mg L\(^{-1}\), nickel as Ni(NO\(_3\))\(_2\)·6H\(_2\)O at 0, 250, 500, 750, 1000 mg L\(^{-1}\) and lead as Pb(NO\(_3\))\(_2\)·6H\(_2\)O at 0, 1000, 1500, 2000, 2500 mg L\(^{-1}\). The mixed metal treatment solution consisted of 50 ml of half-strength Hoagland’s solution devoid of phosphates and sulphates and supplemented with 1000 mg L\(^{-1}\) Cd(NO\(_3\))\(_2\), 1000 mg L\(^{-1}\) Ni(NO\(_3\))\(_2\), and 2500 mg L\(^{-1}\) Pb(NO\(_3\))\(_2\). The pH of the metal solutions was adjusted to 6.0-6.5 (with 0.1N HCL or 0.1N NaOH). The treatments were repeated at 24 h intervals, over the 14 d treatment period.
**Metal analysis**

Plants from all the treatments were harvested, washed thoroughly with distilled deionized water, and divided into root and shoot biomass. Metal from the biomass was extracted using the closed teflon vessel method as described by Topper and Kotuby-Amacher (1990). Briefly, the tissue was cut into small pieces and oven dried at 80°C for 2 d. Oven-dried material was weighed, and 1 g of the plant material was placed in teflon vessels. The plant material was digested by adding 10 ml of trace metal grade nitric acid (70%) and the teflon vessels were placed in an oven overnight (110°C). The volume of the extract was made up to 50 ml with distilled water. The metal content in the extract was estimated using a flame atomic absorption spectrophotometer (AAS), Model 55 Varian (Varian, Australia). Standard reference materials (pine leaves, citrus leaves) provided by the National Institute of Standards and Technology (Gaithersburg, MD) and blanks were used to assess the accuracy of the measurements.

**Experimental design and statistical analysis**

All the experiments were designed as a completely randomized block design (CRBD) and repeated at least twice. Metal analysis was carried out on six individual plants per level of metal exposure (n=6), and the experiments were repeated at least twice. Analysis of Variance was carried out using General Linear Models procedure of the Statistical Analysis System Version 6.12 (SAS Inc. 1995) to determine if there were significant differences in metal accumulation
treatments. Significant differences between the means were assessed by Tukey's honestly significant difference test (Tukey's HSD) at P<0.05.

Results

Exposure of scented geranium to various levels of cadmium and nickel induced morpho-phytotoxicity symptoms noticed on the plants exposed to the higher levels (Figure 1A and B). The symptoms were mostly observed on the mature leaves, which indicated signs of chlorosis and early senescence. Also, the plants exposed to the low levels of nickel showed chlorosis symptoms. Nickel phytotoxicity symptoms were observed earlier (day 10) as compared to cadmium (day 12). Increased stem lignification was observed in all the treated plants after the 14 days of metal exposure.

Cadmium

The uptake and accumulation of cadmium in the biomass of scented geranium plants increased with the exposure to increasing levels of cadmium treatment viz. low (250 mg L⁻¹, 500 mg L⁻¹) and medium levels (750 mg L⁻¹) followed by a decline at the highest level (1000 mg L⁻¹), for both roots and shoots. Roots of scented geranium plants exposed to low concentrations of cadmium accumulated 1,716 mg per kg DW, while exposure to 500 mg L⁻¹ of cadmium resulted in three-fold increase accumulation. The highest accumulation in roots (27,043 mg per kg DW) was observed in the 750 mg L⁻¹ cadmium treatment (Figure 2).

In the shoots of scented geranium plants, the uptake and accumulation followed a trend similar to the roots. At the low levels of cadmium exposure, the amount of
Figure 1. Morpho-phytotoxicity symptoms in scented geranium plants (Pelargonium sp. ‘Frensham’), Indian mustard (Brassica juncea) and sunflower (Helianthus annuus) following exposure to lead.
Figure 2. Accumulation of cadmium in shoots (A) and roots (B) of scented geranium plants (*Pelargonium* sp. 'Frensham'), exposed to various levels of cadmium for 14 days. Means were compared by the Tukey's test at $P<0.05$ ($n=6$).
Cadmium concentration in solution (mg/L)

Cadmium content (mg/kg DW)

(A) Graph showing cadmium content at different concentrations of cadmium in solution.

(B) Graph showing cadmium content at different concentrations of cadmium in solution.
metal accumulated reached 42 and 128 mg per kg DW. The highest level of metal accumulation (778 mg per kg DW) was detected at 750 mg L⁻¹ of cadmium in the culture solution (Figure 2). In contrast, accumulation of cadmium decreased in the shoot tissues of plants exposed to the highest level of cadmium (456 mg per kg DW).

**Nickel**

Accumulation of nickel in roots of scented geranium plants did not increase linearly with the corresponding applied nickel levels. The level of nickel found in the roots of plants exposed to 250 mg L⁻¹, was significantly higher (14,998 mg per kg DW) than the levels of nickel measured in the roots exposed to 500 mg L⁻¹ and 750 mg L⁻¹ (8,086 mg per kg DW and 12,120 mg per kg DW, respectively). At the highest level of nickel exposure (1000 mg L⁻¹) the amount of metal accumulation observed in the roots was 21,144 mg per kg DW (Figure 3).

In contrast to the pattern of nickel accumulation in the roots, the levels of nickel in the shoots increased linearly with the level of metal exposure (Figure 3). Even at the lowest level of nickel exposure, the amount of metal accumulated in the shoots of scented geraniums was considerably high (288 mg per kg DW). The shoots of the plants exposed to the highest level had the highest accumulation per kg of DW of observed in shoots and roots of scented geranium plants, as compared to the nickel levels, at all the levels tested.

**Lead**

The scented geranium plants accumulated > 3005 mg lead per kg dry weight of shoot (Figure 5 A), and > 60986 mg lead per kg dry weight of root tissue (Figure 5 B). Over the wide range of lead treatments tested, tissue metal contents of both
Figure 3. Accumulation of nickel in shoots (A) and roots (B) of scented geranium plants (*Pelargonium* sp. ‘Frensham’), exposed to various levels of nickel for 14 days. Means were compared by the Tukey's test at P<0.05 (n=6).
Nickel concentration in solution (mg/L)
Figure 4. Accumulation of lead, in shoots (A) and roots (B) of scented geranium plants (*Pelargonium* sp. ‘Frensham’), exposed to various levels of lead for 14 days. Means were compared by the Tukey’s test at $P<0.05$ (n=6).
Lead concentration in solution (mg/L)

Lead content (mg/kg DW)

A

B

0 1000 1500 2000 2500

0 10000 20000 30000 40000 50000 60000 70000 80000 90000
Figure 5. Accumulation of cadmium, nickel and lead in shoots (A) and roots (B) of scented geranium plants (*Pelargonium* sp. ‘Frensham’), exposed to various levels for 14 days. Means were compared by the Tukey’s test at $P<0.05$ (n=6).
shoot and root tissues increased with increasing metal concentrations in the treatment solution (Figure 5).

**Mixed metal accumulation**

Scented geranium plants accumulated metal ionsamounting to about 4.72% Pb + 0.44% Cd + 0.52% Ni per kg of root dry matter, as well as 0.17% Pb + 0.07% Cd + 0.14% Ni per kg of the shoot dry matter within 14 d (Figure 6), demonstrating their multi-metal-accumulating potential.

**Discussion**

An efficient phytoremediation system requires plant species that satisfy two distinct prerequisites, viz. metal tolerance and metal accumulation (uptake, detoxification and sequestration). In addition, the ideal plant for phytoremediation should possess the ability to survive and accumulate more than one metal from the growth medium comprising of various metal contaminants, which are generally found under field conditions. However, only a few plant species have been reported to accumulate high levels of cadmium and/or nickel (Brooks, 1998).

The major objective of this investigation was to evaluate the potential of scented geranium plants to uptake and accumulate cadmium, nickel and lead. The significant finding of the present investigation was the demonstrated ability of scented geranium plants to accumulate and sequester elevated levels of various metals within the 14-day treatment period. Additionally, the metal distribution pattern in the plant biomass indicated that the metal accumulation process was a metabolically controlled mechanism, which varied with the specific metal studied.

The cadmium content of the roots and shoots increased in accordance with the
rising supply of cadmium, with the exception of the highest concentration, wherein the level of toxic metal accumulated decreased. The transport of cadmium to the shoots has been shown to be directly proportional to the external cadmium concentration in most plant species (Greger and Lindberg, 1986). Such pattern of uptake is an indication that the toxicity threshold has not been exceeded even at the elevated levels of cadmium supply, and the metabolic control of cadmium uptake is not lost. These findings suggest involvement of both active and passive transport mechanism for cadmium uptake (Arduini et al., 1996). The high level of cadmium found in the shoots of scented geranium plants has far exceeded the level of 0.01% DW, which is considered to be the level defining cadmium hyperaccumulator plants (Brown et al., 1994).

In the case of nickel, the accumulation in the roots did not increase with the applied metal concentration, indicative of a predominantly active uptake of this metal by scented geranium. Accumulation of nickel in the shoots was linear for all the nickel concentrations tested, despite the fact that higher accumulation of nickel was observed in the roots of plants exposed to low concentrations of nickel nitrate (Tiffin 1972, 1977).

Over the wide range of lead treatments tested, tissue metal contents of both shoot and root tissues increased with increasing metal concentrations in the treatment solution suggesting a predominant passive uptake mechanism.

Further, an excessive uptake of a specific metal ion from the growth medium did not have a negative influence on the ability of scented geraniums to uptake and accumulate yet another metal ion. This distinguishes scented geraniums from most
metal-accumulator plant species, except certain *Thlaspi* sp. (Baker et al., 1994; Brown et al., 1994) that accumulate specific combinations of Co, Ni, Zn, and Indian mustard cultivars (Salt et al., 1995) which accumulate Pb, Zn, Cd, Cu, or Ni combinations. However in contrast to scented geraniums, both *Brassica* and *Thlaspi* do not have the ability to tolerate high levels of mixed metal contamination in the growth substrate, as most other species are very metal-specific. Our data demonstrates the potential for existence of more than one functional tolerance and metal-detoxification mechanism(s) in scented geranium.

The higher level of cadmium, nickel and lead found in the roots of metal accumulators is highly dependent on at least two components, namely: sequestration and/or translocation. Cadmium sequestration in roots has been associated with the formation of cell wall in growths (Vasquez et al., 1992a), precipitation of metal oxalates in root intercellular spaces (VanBalen et al. 1980) and apoplastic storage (Vasquez et al., 1992b). Alternatively, cadmium binding to low molecular weight peptides and proteins (Weigel and Jager, 1980) has also been suggested to be involved in root metal sequestration. In the case of lead lignification of cell walls and formation of electron opaque, metal-lignin complexes, might be one primary mechanism of lead tolerance and uptake in the roots of scented geranium plants exposed to lead (Vasquez et al., 1992a). Consequently, it is possible that an analogous situation exists in the case of cadmium sequestration in the roots of scented geranium plants.

Hardiman et al. (1984) suggested that cadmium ions enter the stele through leakage and their translocation rate to shoot is dependent on the root
concentration. We suggest that in the case of scented geranium plants a similar scenario exists, wherein the level of metal accumulation in the shoots was proportional to the one transported to the shoots. The different translocation patterns could be due also, to various forms in which cadmium and nickel are translocated in the scented geranium shoots. Nickel could be transported in association with citrate (Lee et al., 1977), as a nickel-peptide complex or as a nickel-histidine complex (Krammer et al. 1996), while cadmium as a cadmium-citrate association (Senden et al., 1992). In scented geranium plants, cadmium was taken up to a relatively lower degree than nickel, which could be explained by the different mobility of these metals (Rubio et al., 1994).

The increased level of metals accumulated in shoots of several accumulator plants is attributed to active detoxification mechanism(s) (Verkleij and Schat, 1980) that would involve the production of intercellular compounds, metal compartmentalization patterns or an increased cellular metabolism. It is therefore likely that a similar process operating independently or in tandem in scented geranium plants might be responsible for the active detoxification of metals in scented geranium plants.

**Conclusion**

The present investigation clearly demonstrates the metal (cadmium, nickel and lead) accumulation potential of scented geranium. This indicates the potential of this plant species for decontamination of multiple metal polluted sites. Apart from the hypertolerance and accumulation potential, these plants also have the following characteristics that render them highly suitable candidate plant for
phytoremediation: 1) a dense foliage for sequestering high levels of metals ions, 2) a prolific root system for absorption of metals from soils, 3) adaptability to a wide variety of soils with relatively low requirements for water and nutrients, and 4) economic return in the form of extractable essential oils from the harvested biomass. Under these circumstances, the accumulation of cadmium, nickel and lead in scented geranium plants is of major importance, offering both an efficient plant species for phytoremediation and a unique model system for the study of physiological stress response processes in plants.
CHAPTER 4B

METAL ACCUMULATION IN *PELARGONIUM* SP. 'CITROSA', *P. PEPPERMINT', *P. PEAK' AND IN *P. X HORTORUM CV. TANGO*

**Abstract**

The ability of scented geraniums *Pelargonium* sp. Citrosa, *P. Peppermint*, *P. Oak* and of the *P. Hortorum* cv. Tango to uptake cadmium, nickel and lead was assessed under greenhouse conditions. Plants were grown in an artificial soil system and exposed to high concentrations (0-1000 mg L$^{-1}$ Cd(NO$_3$)$_2$·4H$_2$O, 0-1000 mg L$^{-1}$ Ni(NO$_3$)$_2$·6H$_2$O and 0-2500 mg L$^{-1}$ Pb(NO$_3$)$_2$·6H$_2$O) over a 14 day treatment period. With the exception of *P. Peppermint*, which showed signs of phytotoxicity, all the other species tested tolerated and accumulated metals. The cadmium accumulation in shoots was in excess of 664 mg/kg DW (Citrosa), 514 mg/kg DW (Tango) and 368 mg/kg DW (Oak), while the root accumulated greater than 28,405 mg/kg DW (Citrosa), 13,270 mg/kg DW (Tango) and 12,744 mg/kg DW (Oak). Nickel accumulation in the shoots was in excess of 515 mg/kg DW (Citrosa), 191 mg/kg DW (Tango), 1,435 mg/kg DW (Oak). In the roots nickel accumulation was in excess of 5,044 mg/kg DW (Citrosa), 1,626 mg/kg DW (Tango), 48,046 mg/kg DW (Oak). In the case of lead treatment, the lead shoot accumulation was greater than 5,451 mg/kg DW (Citrosa), 10,955 mg/kg DW (Tango), 4,629 mg/kg DW (Oak) and the roots accumulated in excess of 13,257 mg/kg DW (Citrosa), 21,095 mg/kg DW (Tango), 28,549 mg/kg DW (Oak). This data indicates that the plant species tested differ in metal uptake and translocation patterns.
Introduction

Remediation of metal contaminated soils through the conventional technologies represents a significant expense for many industries and governmental agencies. In a search for a cost-effective alternative, Chaney (1983) proposed the use of accumulator plants to extract the metal from the contaminated soil-phytoextraction. The success of using plants to extract metal from contaminated soils requires identification of metal-accumulating species that have the capability to tolerate and accumulate high levels of heavy metals.

Plants grown in metal-enriched substrate uptake metal ions at varying degrees in response to both external and internal factors. A primary factor, which influences uptake, is the genetic make-up of the plant and it has been clearly demonstrated that metal accumulation potentials varies among plant species. Huang et al. (1997) has found that Pb accumulation varies significantly in different species in similar environments. In the last few years, screening for metal accumulating plants from high biomass species has become an important aspect of phytoremediation (Huang et al., 1997; Kumar, 1995). The metal content of six different Brassica sp. and six other field crops (Table 1) were found to vary significantly even under controlled greenhouse experimentation (Kumar et al., 1995). Results from these studies demonstrated that plant species differ significantly in the metal uptake and translocation pattern and metal concentration among plant species vary by more than 10 to 20 fold. Genotype mediated differences in accumulation of cadmium by inbred lines of corn (Zea mays) has also been reported (Council for Agricultural Science and Technology, 1980).
Following identification of *Pelargonium* sp. ‘Frensham’ as a metal tolerant and metal accumulator plant species it was hypothesized that other plants belonging to the *Pelargonium* sp. could tolerate and accumulate elevated levels of toxic metals in their biomass. In order to test this hypothesis, the potential for cadmium, nickel and lead uptake of four *Pelargonium* sp. viz.: *Pelargonium* sp. Citrosa, *Pelargonium* sp. Peppermint, *Pelargonium* sp. Oak and *Pelargonium* sp. x *hortorum* cv. Tango was evaluated.

**Material and methods**

**Preparation of plant material and metal exposure**

*Pelargonium* sp. Citrosa, *Pelargonium* sp. Peppermint, *Pelargonium* sp. Oak and *Pelargonium* sp. x *hortorum* cv. Tango cuttings were used for all experiments in this study. The uptake of three different metals, i.e., cadmium, nickel and lead, was assessed independently. Preparation of the plant material was similar to the conditions described in Chapter 3A. Cuttings of geraniums made from greenhouse-grown plants were rooted in 3.5 cm plastic pots containing approximately 150 g of well-washed (with deionized water until the washing solution became clear) coarse Perlite (Premier Horticulture Inc., Mississauga, Canada). Plants (one plant in each pot) were placed in 5.5 cm plastic saucers (three pots in each saucer). All species were grown in a greenhouse equipped with supplementary lighting (16/8 h photoperiod; 350 μmol m⁻²s⁻¹) and controlled temperature (18-22°C) for 30 d with irrigation (mist beds). During the treatment period (14 days), the rooted cuttings were treated with 50
ml of half-strength Hoagland’s solution, which was devoid of phosphate and sulphate, but supplemented with the various metal salts. The metal treatments were provided as nitrates: cadmium as Cd(NO₃)₂·4H₂O at 0 and 1000 mg L⁻¹, nickel as Ni(NO₃)₂·6H₂O at 0 and 1000 mg L⁻¹, and lead as Pb(NO₃)₂·6H₂O at 0 and 2500 mg L⁻¹. Citroса plants were exposed to a mixed metal solution that consisted of: Cd(NO₃)₂·4H₂O at 1000 mg L⁻¹+ Ni(NO₃)₂·6H₂O at 1000 mg L⁻¹+ lead as Pb(NO₃)₂·6H₂O at 2500 mg L⁻¹. The pH of the metal solutions was adjusted to 6.0-6.5 (with 0.1N HCL or 0.1N NaOH). The treatments were repeated at 24h intervals, over the 14-d treatment period.

**Metal analysis**

Plants from all the treatments were harvested, washed thoroughly with distilled deionized water, and divided into root and shoot biomass. Metal from the biomass was extracted using the closed teflon vessel method as described by Topper and Kotuby-Amacher (1990). The tissue was cut into small pieces and oven dried at 80°C for 2 d. Oven-dried material was weighed, and 1 g of the plant material was placed in teflon vessels. The plant material was digested by adding 10 ml of trace metal grade nitric acid (70%) and the teflon vessels were placed in an oven overnight (110°C). The volume of the extract was made up to 50 ml with distilled water and the metal content in the extract was estimated using a flame atomic absorption spectrophotometer (AAS), Model 55 Varian (Varian, Australia). Standard reference materials (pine leaves, citrus leaves) provided by the National Institute of Standards and Technology Gaithersburg, MD) and blanks were used to assess the accuracy of the measurements.
Table 1. Lead content (µg/g DW) of roots and shoots of crop Brassica and other plants (Modified from Kumar et al., 1995)

<table>
<thead>
<tr>
<th>PLANT SPECIES</th>
<th>LEAD ACCUMULATION (µg/g DW)</th>
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<tbody>
<tr>
<td></td>
<td>shoots</td>
</tr>
<tr>
<td><em>Brassica juncea</em> (l.) Czern</td>
<td>10.3</td>
</tr>
<tr>
<td>B. nigra (l.) Coch</td>
<td>9.4</td>
</tr>
<tr>
<td>B. campestris (l.)</td>
<td>7.2</td>
</tr>
<tr>
<td><em>B. carinata</em> (A. Br.)</td>
<td>4.6</td>
</tr>
<tr>
<td>B. napus (l.)</td>
<td>3.4</td>
</tr>
<tr>
<td>B. oleracea (l.)</td>
<td>0.6</td>
</tr>
<tr>
<td><em>Helianthus annuus</em> (l.)</td>
<td>5.6</td>
</tr>
<tr>
<td><em>Nicotiana tabacum</em> (l.)</td>
<td>0.8</td>
</tr>
<tr>
<td>Sorghum bicolor (l.)</td>
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</tr>
<tr>
<td><em>Amaranthus hybridus</em> (l.)</td>
<td>0.3</td>
</tr>
<tr>
<td>A. paniculata (l.)</td>
<td>0.4</td>
</tr>
<tr>
<td><em>Zea mays</em> (l.)</td>
<td>0.2</td>
</tr>
</tbody>
</table>
Experimental design and statistical analysis

All the experiments were designed as a completely randomized block design (CRBD) and repeated at least twice. Metal analysis was carried out on six individual plants (n=6) per level of metal exposure, and the experiments were repeated at least twice. Analysis of Variance was carried out using General Linear Models procedure of the Statistical Analysis System Version 6.12 (SAS Inc. 1995) to determine if there were significant differences in metal accumulation as a result of metal treatments. Significant differences between the means were assessed by Tukey's honestly significant difference test (Tukey's HSD) at P<0.05.

Results

After 6 days of exposure to various metals Pelargonium sp. Peppermint showed signs of wilting, chlorosis and senescence. In contrast, the other species tested showed no morphological signs of phytotoxicity until day 12.

Cadmium

Cadmium accumulated in shoots (Figure 1A) in excess of 664 mg/kg DW (Citrosa), 514 mg/kg DW (Tango) and 368 mg/kg DW (Oak), while in the roots (Figure 1B) the accumulation was in excess of 28,405 mg/kg DW (Citrosa), 13,270 mg/kg DW (Tango) and 12,744 mg/kg DW (Oak).

Nickel

Nickel accumulation in the shoots (Figure 2A) reached levels of 515 mg/kg DW (Citrosa), 191 mg/kg DW (Tango), and 1,435 mg/kg DW (Oak). In the roots (Figure 2B) nickel was accumulated in excess of 5,044 mg/kg DW (Citrosa), 1,626 mg/kg
DW (Tango), 48,046 mg/kg DW (Oak).

**Lead**

The level of lead accumulated in the shoots (Figure 3A) was 5,451 mg/kg DW (Citrosa), 10,955 mg/kg DW (Tango), 4,629 mg/kg DW (Oak) and in roots (Figure 3B) in excess of 13,257 mg/kg DW (Citrosa), 21,095 mg/kg DW (Tango), 28,549 mg/kg DW (Oak).

**Mixed metal accumulation**

Citrosa plants were further investigated for their potential of mixed metal accumulation. In shoots, the accumulation of metals decreased markedly as compared to the accumulation of individual metals. Shoots of Citrosa plants accumulated in excess of 200 mg/kg DW Cd, 1,000 mg/kg DW Ni and 300 mg/kg Pb (Figure 4A). The accumulation of mixed metal in the roots followed a similar trend as in the plants exposed to individual metals, with the exception of nickel that was accumulated at higher levels. The levels of metals in the roots were: 25,000 mg/kg DW Cd, 45,000 mg/kg DW Ni and 38,000 mg/kg DW Pb (Figure 4B).

**Discussion**

Metal accumulator plants that can extract and take up large amounts of metal from the soil have been known for centuries. However, many of these natural metal accumulators have evolved in harsh contaminated environment and tend to produce small plants with low biomass making them somewhat less desirable for large-scale remediation. Nonetheless, these plants are providing models for the research and screening of the plant kingdom for better phytoremediation candidates. In the last few years, a number of plants species have been selected.
Figure 1. Accumulation of cadmium in shoots (A) and roots (B) of *Pelargonium* sp. ‘Citrosa’, *Pelargonium* sp. ‘Peppermint’, *Pelargonium* sp. x *hortorum* cv. ‘Tango’, *Pelargonium* sp. ‘Oak’ when exposed to 1000 mg L $^{-1}$ cadmium. The values are means of 6 replicate plants ±SE (n=6).
Figure 2. Accumulation of nickel in shoots (A) and roots (B) of *Pelargonium* sp. 'Citrosa', *Pelargonium* sp. 'Peppermint', *Pelargonium* sp. *x hortorum* cv. 'Tango', *Pelargonium* sp. 'Oak' when exposed to 1000 mg L\(^{-1}\) nickel. The values are means of 6 replicate plants ±SE (n=6).
Figure 3. Accumulation of lead in shoots (A) and roots (B) of *Pelargonium* sp. 'Citrosa', *Pelargonium* sp. 'Peppermint', *Pelargonium* sp. *x hortorum* cv. 'Tango', *Pelargonium* sp. 'Oak' when exposed to 2500 mg L $^{-1}$ lead. The values are means of 6 replicate plants ±SE (n=6).
Figure 4. Accumulation of cadmium, nickel and lead in shoots (A) and roots (B) of *Pelargonium* sp. 'Citrosa' when exposed to a mixture of 1000 mg L$^{-1}$ cadmium, 1000 mg L$^{-1}$ nickel and 2500 mg L$^{-1}$ lead. The values are means of 6 replicate plants ±SE (n=6).
and tested for their metal uptake potential. Emphasis is placed on the selection of high biomass plants that can not only take up large amounts of metals but that can also grow easily even under harsh conditions.

The most important finding of this study was the identification of *Pelargonium* sp. 'Citrosa', *Pelargonium* sp. 'Oak' and *Pelargonium* sp. *x hortorum* cv. 'Tango' as potential metal accumulators. These results demonstrate that genotypic variation exists in *Pelargonium* sp. with regards to their metal uptake and metal translocation potential. In addition, certain species of *Pelargonium* accumulated preferentially one metal. For example, in the case of cadmium Citrosa plants accumulated high levels in the root as compared to cadmium accumulated in the roots of Tango and Oak plants. Interestingly, only a small fraction of this was transported into the shoots, suggesting the presence of both active and passive mechanism of uptake (Arduini et al., 1996). The accumulation of nickel was highest in the Oak plants (in both shoots and roots), followed by Citrosa plants. An interesting aspect was observed in the case of Tango plants exposed to nickel where the metal was excluded from the root uptake, suggesting an avoidance mechanism. Support for this hypothesis can be found in reports indicating large quantities of Cd localization in the cell wall of *Azolla* sp. and (Robinson et al., 1994). *Bechum bombei*, a highly Cu tolerant plant, collected from Cu rich soils, was found to have lower copper concentrations in the roots and shoots, indicative of restrictive metal uptake, that may be a result of altered membrane permeability (Reilly, 1969). On the contrary, in the case of Tango plants exposed to lead, the accumulation was high in both shoots and roots suggesting a different mechanism of uptake for lead than for
nickel and cadmium. One relevant was the finding that in the case of lead exposed plants all three species accumulated very high levels of lead in the shoots, suggesting that *Pelargonium* sp. have high potential for lead uptake. Using a sand culture system, Kumar et al. (1995) carried out an extensive screening for lead accumulator plants from the Brassicaceae family. These authors found that only some cultivars of *Brassica juncea* showed a strong ability to accumulate lead in the shoots. Similarly, Huang et al. (1997) screened several cultivars of corn and *Brassica* and reported that metal concentration in shoots of corn was similar to that in the shoots of the two best lead accumulating cultivars of *Brassica juncea*.

Exposure of Citrosa plants to mixed metal accumulation revealed a different response as compared to the plants treated with individual metals. In the roots of Citrosa plants exposed to the mixed metal solution the accumulation was higher for all the metals tested, suggesting the root uptake was not affected by the presence of cadmium, nickel and lead. On the contrary, the accumulation of cadmium and lead in shoots decreased, and a major limiting step is probably a lower metal translocation from roots to shoots. This could be interpreted as a competition of similar ions for metal uptake (Hardimann et al., 1985) or as a possible stress response mechanism, by which the plant is limiting the amount of toxic ions that could reach and damage the sensitive metabolic sites located into the shoots (Clijsters and Van Asche, 1985).

**Conclusion**

In conclusion, three of the species tested *Pelargonium* sp. ‘Citrosa’, *Pelargonium* sp. ‘Oak’ and *Pelargonium* sp. *x hortorum* cv. ‘Tango’ have a potential
for metal uptake, while *Pelargonium* sp. 'Peppermint' has a limited tolerance to high levels of the metal exposure. Our results demonstrate that, genotipic variation exists in *Pelargoniums* sp. plant species with regards to their metal uptake and translocation. Overall, *Pelargonium* sp. showed a higher potential for lead accumulation in the shoots as compared with cadmium and nickel accumulation. In particular, Citrosa plants showed a higher potential in tolerating and accumulating various metals. Further screening of *Pelargonium* sp. will allow us to identify and better characterize their potential use in phytoextraction.
Abstract

The cellular localization of lead was assessed using transmission electron microscopy coupled with an X-ray microanalyzer. Lead accumulation was observed in the apoplasm and in the cytoplasm, vacuoles, and as distinct globules (potentially as lead-lignin or lead-phosphate complexes) on the cell membrane and cell wall. The ability of scented geraniums to tolerate high lead accumulation in its biomass is due, in part, to limiting damage to photosynthetic apparatus and metal detoxification by formation of metal complexes.

Introduction

Lead uptake and accumulation through roots of higher plants has been demonstrated in a variety of plant species (Malone et al., 1974; Kumar et al., 1995; Huang et al., 1997). However, using plants to extract lead from contaminated soils requires a better understanding of the mechanisms of lead uptake, translocation and accumulation in plants (Huang et al., 1997).

The ability to sequester metal ions at subcellular compartments with little or no sensitive metabolic activity is considered to be a key factor in the mechanism of tolerance to many metals (Ernst, 1992). In particular, Malone (1974) and Lane and Martin (1982) have investigated the localization of lead and, one major finding of these studies was the fact that lead mainly accumulates in the cell walls. Time-
dependent investigations showed that lead rapidly accumulates on the root surface and in the radial and tangential walls of the root cap onions. After only 3 hours of exposure, lead was localized in the vacuoles of the ground meristematic tissue, while the central zone of the root tip remained almost free of lead (Wierzbicka, et al., 1987). When evaluating the transport mechanism of lead in roots of onions, Wierzbicka et al. (1987) suggested that there are two major barriers for apoplastic lead transport in roots: (1) the layers of protoderm and (2) hypodermic meristematic cells in the root meristem zone and the layer of endodermis in the mature root zone. The central zone seems to be a barrier for both, apoplastic and symplastic lead transport. In the cells with mature cell walls lead would only enter after severe damage of the plasmalemma (Tung and Temple, 1996). Several localization studies have demonstrated the presence of lead in vesicles inside plants cells, and it has been suggested that lead which enters the protoplast may be exported to cell walls by exocytosis (Malone, 1974; Wozny, 1982).

This investigation was designed to evaluate the suitability of scented geranions (Pelargonium sp. ‘Frensham’) to extract lead from the growth medium through assessing lead accumulation potential under greenhouse conditions. Also, evaluation of the lead localization and detoxification mechanism(s), as they play a major role in determining the threshold up to which or below which lead uptake will occur was carried out. In order to achieve this, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) coupled with X-ray microanalysis (EDX) for qualitatively and quantitatively determining the spectrum of elements in frozen hydrated (SEM-based, Sagner et al., 1998) or TEM sections.
The specific objective of this study was to identify the potential sites for lead localization and detoxification.

**Material and methods**

**Preparation of plant material and metal exposure**

Scented geranium cuttings (*Pelargonium* sp. 'Frensham') were prepared as described in Chapter 3A. In brief, Cuttings of scented geraniums made from greenhouse-grown plants were rooted in 3.5 cm plastic pots containing approximately 150 g of well-washed (with deionized water until the washing solution became clear) coarse Perlite (Premier Horticulture Inc., Mississauga, Canada). The plants were grown in a greenhouse equipped with supplementary lighting (16/8 h photoperiod; 350 μmol m⁻²s⁻¹) and controlled temperature (18-22°C) for 30 d with irrigation (mist beds) and fertilization.

Two weeks prior to initiation of the experiment, the plants were watered with half-strength Hoagland's solution followed by flushing with water. During the treatment period (14 days), both the seedlings and the rooted cuttings were treated with 50 ml of half-strength Hoagland's solution, which was devoid of phosphate and sulphate, but supplemented with the various metal salts. The metal treatment was provided as lead nitrate Pb(NO₃)₂·6H₂O at 2500 mg L⁻¹. The pH of the metal solutions was adjusted to 6.0-6.5 (with 0.1N HCL or 0.1N NaOH). The treatments were repeated at 24 h intervals, over the 14-d treatment period.
**Scanning electron microscopy**

Root segments (2 mm long) from control and lead treated plants were collected, mounted with Polyfreeze tissue freezing medium (Structure Prober Inc., West Chester, PA) onto a cryo-holder for the Emscope 2000A cryo-preparation unit. Samples were frozen by plunging into liquid nitrogen slush (-207°C) and fractured to expose a fresh surface. Water was sublimated for 30 min at -80°C to expose the underlying structure. Partially freeze dried samples were viewed in the Hitachi S-570 scanning electron microscope (-135 °C). Selected areas at varying depths from the root epidermis (0-180 μm) were analyzed by X-ray microanalysis with a NORAN Voyager X-ray microanalysis system equipped with a Germanium detector operating at 25 kV.

**Transmission electron microscopy and X-ray microanalysis**

Scented geranium plants were treated with 0 and 2500 mg/L Pb(NO₃)₂ as described in Chapter 3. Root segments (1-2 mm long) were harvested from the treated plants after 14 d, fixed in 2.5% glutaraldehyde in 0.1 M HEPES buffer, pH 6.8 and then post-fixed in 2% osmium tetroxide. After dehydration in alcohol, the material was embedded in LR white resin. Ultra-thin sections (100 nm thick) were prepared from the cross sectional surface of the root. The sections and electron opaque Lead deposits were examined without staining in an Hitachi H-7100 electron microscope, operating at 75 kV in STEM mode for imaging and to control beam location for X-ray microanalysis with a NORAN Voyager X-ray microanalysis system equipped with a Germanium detector. The control roots
were stained with lead citrate to obtain contrast in electron micrographs.

**Results**

In order to elucidate the functional metal detoxification mechanism(s) in scented geraniums, roots of scented geranium plants treated with 2500 mg/L Pb(NO₃)₂ were examined in a scanning electron microscope coupled with an X-ray microanalyzer. After stimulation of X-ray emission at various kV the characteristic spectra were used for identification of element composition. X-ray microanalysis of selected points of the freeze-fractured roots, at varying distances from the root epidermis, indicated the presence of a decreasing gradient of lead content towards the central axis of the root. Nearly, 32% of the total ionic content of the root tissue at 20 µm from the epidermis was accounted for as lead, compared to 4% at 180 µm from the epidermis (central cylinder of the root).

The control roots in contrast showed no evidence of lead deposits (Figure 1A & B). Cellular and subcellular location of lead by energy dispersive X-ray microanalysis using transmission electron microscopy provided convincing evidence for the presence of lead in the apoplasm (Figure 1C & D), as distinct globules embedded in the cell membranes, cell walls (Figure 1E & F), cytoplasm (Figure 1G & H) and vacuoles.

**Discussion**

Metal tolerant plants have varied mechanisms to alleviate the build-up of toxic metal ions in their cells such as, a) avoidance, b) reduced uptake of metal ions (Qureshi et al., 1986), c) efflux of metal ions from the cells, or d) deposition of metal
ions in the cell walls (Allen and Jarell, 1989; Sagner et al., 1998). It is likely that one or all of these mechanisms can operate within a plant to limit cellular damage by the toxic metal ions.

The present study provides evidence to substantiate the presence of both passive and active mechanisms of metal uptake and translocation through the root apoplasm to the symplasm, resulting in a gradient of lead ions leading to the shoot. Both apoplastic migration and symplastic transportation of lead has been shown to occur in onion root tips exposed to lead (Wierzbicka et al., 1987). The extent to which lead accumulated and how they are sequestered within different cell compartments plays an important role in the survival of the plant in these extreme conditions. Although, lead was found to be associated either with the cell walls (Poulter et al., 1985) or with vacuolar bodies in Anthoxanthum odoratum (Mullins et al., 1985), lead was predominantly distributed in the apoplasm of Norway spruce roots (Jentschke et al., 1998). Investigation of the localization of lead in Anthoxanthum odoratum clones showed that the differences observed in lead tolerance strongly support a role for cell walls in lead tolerance (Qureshi et al., 1981).

Histological evidence of distinct lead deposits in the cell walls together with following exposure to lead indicates that lignification of cell walls and the formation of electron opaque, metal-lignin complexes is one probable mechanism of lead tolerance and detoxification in scented geranium. The possibility also exists for cellular acid phosphatases to bind lead as lead polyphosphate complexes (Malone et al., 1974) and sequester them into newly forming cell walls.
Figure 1. Transmission electron micrographs (TEM) and X-ray microanalysis spectra (XMS) of scented geranium (Pelargonium sp. ‘Frensham’) roots treated daily with 0 mg/L and 2500 mg L\(^{-1}\) Pb(NO\(_3\))\(_2\) solutions.

**A**, TEM of control root sections stained with lead citrate (15,000x). **C**, TEM of intercellular space (30,000x), **E**, cell wall (40,000x) and **H**, cytoplasm (30,000x) of root sections from treated plants without staining. **B**, XMS of point x in **A**. **D**, **F**, XMS of points x in **C** and **E** respectively. Arrowhead denotes electron opaque lead deposit.
Abstract

The ability of scented geraniums (Pelargonium sp. ‘Frensham’) to uptake copper and lead was assessed under field conditions (1998, 1999). In 1998 plants were grown for approximately 8 weeks in a soil contaminated with low levels of metals: 346 mg/kg soil copper, 443 mg/kg soil lead and 40,000 mg/kg soil hydrocarbons. Scented geranium plants tolerated and accumulated in roots in excess of 59 mg/kg DW copper and 89 mg/kg DW lead, while in shoots the metal accumulation was in excess of 50 mg/kg DW copper and 25 mg/kg DW lead. In 1999, geranium plants were grown in a soil with 7,000 mg/kg copper and 2000 mg/kg lead. The level of copper and lead exceeded 100 mg/kg DW for both shoots and roots. This study confirms that accumulator plant scented geranium (Pelargonium sp. ‘Frensham’) has a high potential to accumulate and detoxify a mixed-metal contaminated soil under field condition.

Introduction

To date only a few reports have been published showing the results of field trials even though a number of field experiments have been initiated in the last few years and, most of them, still ongoing. A recent survey by Glass (1999) provides a detailed list of these phytoremediation field trials in the US. Results
obtained so far include:

The use of *Brassica juncea* by Phytotech, Inc to remove Pb from the soil on a site formally occupied by a battery manufacturer in Trenton, NJ. At project initiation, 40% of the plot exceeded the regulatory limit of 400 mg/kg and 7% was higher than 1000 mg/kg of soil. After three harvests, only 28% of the plot exceeded 400 mg/kg and no portion exceeded 1000mg/kg. The *Brassica* plants harvested at the site had taken up and accumulated 0.3% lead per weight in their tissues removing approximately 20-30 parts per million of lead from the top 18 inches of soil.

Phytotech has also used *Brassica juncea* plants for remediating a Pb contaminated site in Bayonne, NJ and a residential site in Dorchester, MA. At the Dorchester site, the soil initially contained hot spots of over 1000 mg/kg. Three 6-week growth periods of *B. juncea* was enough to substantially reduce the lead concentration at these hotspots.

The project conducted at Pig’s Eye Landfill site in St-Paul (MN) under the supervision of USDA showed that Alpine pennycress (*Thlaspi caerulescens*) was the best plant species for taking in cadmium, zinc and lead from the contaminated soil. Pennycress was accumulating up to 30,000 ppm of zinc in its leaves without any sign of damage to the plant or yield reduction. Most plants show signs of zinc toxicity when they accumulate more than 500 ppm zinc in their tissues. Pennycress was shown to take in zinc at the rate of 125 kg per hectare (kg/ha) per year (108 pounds/acre) and cadmium at the rate of 2 kg/ha per year (1.7 lbs./acre) when the plants were provided optimum growth conditions.
Initial field studies on the ability of plants to remove Cs-137 from contaminated soils are presently under way at Brookhaven National Lab, NJ and in Ashtabula, OH. Preliminary studies in pots have revealed that certain species of the mustard (Brassicaceae) and amaranth (Amaranthaceae) families were able to accumulate 3 times more Cs-137 in leaf and stem biomass than the concentration in the soil. In field trials, redroot pigweed (A. retroflexus L.) plants were able to accumulate over 900 pCi/gm, which was well over the goal of 300 pCi/gm. In these trials, pigweed showed better performance than B. juncea and Phaseolus acutifolius. Field trials on Cs-137 and Sr-90 contaminated soils are currently under way at the Idaho National Engineering Laboratory.

In a field trial on surface water from the Chernobyl, Ukraine nuclear disaster, contaminated with Cs-137 and Sr-90, Phytotech used sunflower plants grown on rafts in a pond contaminated with the radionuclides. The plants reduced dramatically the levels of radionuclides in the water during the 4-8 week experimental period. After the first 12 days, sunflower roots had accumulated Cs and Sr at concentrations 8,000 and 2,000 times the concentration of Cs and Sr present in the groundwater.

This investigation was designed to evaluate the potential for metal accumulation of Pelargonium sp. under field conditions. In addition, the accumulation potential of Pelargonium sp. was compared to other metal accumulator species, viz. Indian mustard (Brassica juncea) and sunflower (Helianthus annuus).
Material and methods

Preparation of plant material

Scented geraniums (*Pelargonium* sp. 'Frensham') were used for the first season of experimentation, while for the second season *Pelargonium* sp. ('Frensham' and Citrosa'), Indian mustard (*Brassica juncea*) and sunflower (*Helianthus annuus*) were used. Cuttings of scented geraniums and seedlings of Indian mustard and sunflower were prepared as described in Chapter 3A. In brief, cuttings of scented geraniums made from greenhouse-grown plants were rooted in greenhouse soil mixture (Promix). Seeds of Indian mustard and sunflower were soaked in water for 2 h and then sown in greenhouse soil mixture (Promix). The plants were grown in a greenhouse equipped with supplementary lighting (16/8 h photoperiod; 350 µmol m\(^{-2}\)s\(^{-1}\)) and controlled temperature (18-22°C) for 30 d with irrigation (mist beds) and then transplanted into the contaminated soil (field).

Metal analysis

The harvested plants were washed thoroughly with distilled deionized water, and divided into root and shoot biomass. Metal from the biomass was extracted using the closed teflon vessel method as described by Topper and Kotuby-Amacher (1990). Briefly, the tissue was cut into small pieces and oven dried at 80°C for 2 d. Oven-dried material was weighed, and 1 g of the plant material was placed in teflon vessels. The plant material was digested by adding 10 ml of
trace metal grade nitric acid (70%) and the teflon vessels were placed in an oven overnight (110°C). The volume of the extract was made up to 50 ml with distilled water. The metal content in the extract was estimated using a flame atomic absorption spectrophotometer (AAS), Model 55 Varian (Varian, Australia). Standard reference materials (pine leaves, citrus leaves) provided by the National Institute of Standards and Technology (Gaithersburg, MD) and blanks were used to assess the accuracy of the measurements.

Composite soil samples (approximately 5 subsamples of 10 g each) were collected at the same time with the plant harvesting. The samples were analyzed for total metal content using the Aqua regia method at the OMAFRA Soil and Nutrient Laboratory.

**Experimental design and statistical analysis**

The experiments were designed as a completely randomized block design (CRBD) and repeated at least twice (1998, 1999). Metal analysis was carried out on 12 individual plants and on 12 individual composite soil samples per week of exposure. Analysis of Variance was carried out using General Linear Models procedure of the Statistical Analysis System Version 6.12 (SAS Inc. 1995) to determine if there were significant differences in metal accumulation. Significant differences between the means were assessed by Tukey's honestly significant difference test (Tukey's HSD) at P<0.05.
Results

Scented geraniums showed no phytotoxicity symptoms over the 9 weeks trial (Figure 1) despite the mixture of contaminants present in the soil. Additionally, geranium plants accumulated metal ions (Table 1) as the time of exposure increased (metal content in root and shoot increasing with increasing duration of growth in the contaminated soil) even after 9 weeks of growth. The content of heavy metals in the soil after phytoremediation with geranium plants was reduced with 20 % for copper and 3 % for lead.

In the second season of field trial the soil metal content was higher (approximately 7,000 mg/kg in the case of copper and 2,000 mg/kg for lead). The decrease in the soil metal content followed a similar pattern as in the first growing season (Figure 2). In the first 4 weeks of exposure there was a decline (almost 30% in the case of copper and 33% in the case of lead as compared to the first week of exposure) in the soil metal content, followed by an increase in the next weeks of exposure.

The accumulation of metals in the plants increased as the plants were exposed for a longer period of time, reaching similar values (in both shoots and roots) of 50 mg/kg DW of Cu and 25 mg/kg DW of Pb. In the second season of the field trial, geranium plants accumulated higher levels both metals, as the concentration of copper and lead in the soil was higher. Copper was accumulated in the range of 100 mg/kg DW in both shoots (Figure 3A) and roots (Figure 3B), while lead levels varied between 50 and 100 mg/kg DW in shoots (Figure 4A) and roots (Figure 4B). Indian Mustard plants and sunflower plants
TABLE 1. Metal uptake (mg/kg DW) by scented geranium plants (*Pelargonium* sp. 'Frensham') grown on contaminated soil containing low-concentrations of lead and copper, hydrocarbons (the results are average of 12 replicate plants ±SE).

<table>
<thead>
<tr>
<th>TIME (WEEKS)</th>
<th>COPPER</th>
<th>LEAD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shoots</td>
<td>Roots</td>
</tr>
<tr>
<td>Week 2</td>
<td>19.80±2.70</td>
<td>12.9±0.50</td>
</tr>
<tr>
<td>Week 3</td>
<td>20.99±1.10</td>
<td>28.36±1.10</td>
</tr>
<tr>
<td>Week 4</td>
<td>38.66±0.60</td>
<td>35.94±0.70</td>
</tr>
<tr>
<td>Week 5</td>
<td>36.04±1.50</td>
<td>34.36±0.20</td>
</tr>
<tr>
<td>Week 6</td>
<td>37.00±0.02</td>
<td>39.28±0.09</td>
</tr>
<tr>
<td>Week 7</td>
<td>40.20±0.08</td>
<td>28.30±1.40</td>
</tr>
<tr>
<td>Week 8</td>
<td>30.18±2.78</td>
<td>48.42±7.41</td>
</tr>
<tr>
<td>Week 9</td>
<td>50.17±2.58</td>
<td>59.3±4.50</td>
</tr>
</tbody>
</table>

* n=8 due to the presence of outliers
TABLE 2. Soil metal contents (mg/kg) after phytoremediation with lemon scented geranium (*Pelargonium* sp. 'Frensham') exposed to a mixture of multi-metal contaminants and 40 000 ppm hydrocarbons for 9 weeks, in a field trial.

<table>
<thead>
<tr>
<th>Week</th>
<th>Copper</th>
<th>Lead</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 0</td>
<td>346.75</td>
<td>443.40</td>
</tr>
<tr>
<td>week 2</td>
<td>317.00</td>
<td>485.25</td>
</tr>
<tr>
<td>week 3</td>
<td>289.00</td>
<td>463.67</td>
</tr>
<tr>
<td>week 4</td>
<td>275.50</td>
<td>430.33</td>
</tr>
<tr>
<td>week 5</td>
<td>304.65</td>
<td>373.00</td>
</tr>
<tr>
<td>week 6</td>
<td>378.00</td>
<td>365.00</td>
</tr>
<tr>
<td>week 7</td>
<td>260.00</td>
<td>386.50</td>
</tr>
<tr>
<td>week 8</td>
<td>209.00</td>
<td>552.00</td>
</tr>
<tr>
<td>week 9</td>
<td>309.50</td>
<td>402.00</td>
</tr>
</tbody>
</table>
Figure 1A. Phytoremediation of recycled material soil using *Pelargonium* sp. 'Frensham' and *Pelargonium* sp. ‘Citrosa’ (Field trial, June 1998).

Figure 1B. Remediation of lead and copper from recycled material using *Pelargonium* sp. 'Frensham' and *Pelargonium* sp. ‘Citrosa’ (Field trial, June 1999).
Figure 2. Soil copper and lead content after phytoremediation with *Pelargonium* sp. 'Frensham' and *Pelargonium* sp. 'Citrosa' (Hamilton 1999).
Figure 3. Accumulation of copper in shoots (A) and roots (B) of *Pelargonium* sp. 'Frensham'. The plants were grown on contaminated soil containing lead and copper and hydrocarbons (the results are average of 12 replicate plants ±SE) (Hamilton 1999).
Figure 4. Accumulation of lead in shoots (A) and roots (B) of *Pelargonium* sp. 'Frensham'. The plants were grown on contaminated soil containing lead and copper and hydrocarbons (the results are average of 12 replicate plants ±SE) (Hamilton 1999).
started to display symptoms of phytotoxicity as early as week 2 of exposure and by week 4 and 6 respectively, Indian mustard and sunflower plants died.

**Discussion**

Phytoextraction is a term used to indicate the process by which plants can remove significant quantities of substances. Basically, phytoextraction depends on high concentrations of the target elements in plant biomass, especially in above ground portions and production of relatively large biomass. These two features determine the efficiency of the process (US EPA, 2000). To date, there have been few demonstrations of phytoextraction with such plants under greenhouse and most importantly, field conditions (DOE, 2000).

The most significant finding of this study was the accumulation of copper and lead in the shoots from the soil contaminated with low levels of metals, while tolerating the very high levels of organic contamination (approximately 40,000 ppm). An important finding was the fact that *Pelargonium* sp. accumulated similar levels of metals in the shoots as in the roots, despite the low levels of metals in the soil. An interesting observation in this present study was the elevation of metal contents in soils after several weeks of metal exposure. It is suggest that scented geranium plants release root exudates into the growth medium, which mobilizes the metal ions from unavailable forms to more available forms, that can be readily taken up by the plants facilitating easy uptake by the plant (Lasat, 2000). One of the potential processes that might be involved in rhizosphere alteration of metal ion toxicity is exudation of metal chelating ligands into the rhizosphere (Cumming and Taylor, 1990). The increase in soil metal
levels could be attributed in part to either the non-homogeneity of the soil sample or the increased metal mobilization in the soil matrix fostered by scented geranium root exudates. Plants that exude large amounts of metal chelating substances, such as: organic acids, sugars, amino acids, peptides, and phenolic compounds, (Rauser, 1999; Salt and Kramer, 2000), may increase the mobility of metals within the soils leading to an increased or decreased availability of metal ions for uptake by the plants (Blaylock et al., 1997).

**Conclusion**

These observations confirm the metal tolerance and metal accumulation potential of scented geraniums and its utility as a candidate plant for remediation of mixed contaminant soils (organic and multi-metal contaminated soils). The unique ability of *Pelargonium* sp. in remediating complex contaminated substrates under field conditions further indicates the efficacy of this group of plants for phytoextraction. In addition, the dense foliage, prolific root system and low requirements for water and nutrient uptake increase the efficacy of *Pelargonium* sp. phytoremediation.
CHAPTER 6

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

Mounting pressures due to increasing human population, growing economies and excessive resource-use have all resulted in a cumulative increase in environmental pollution. Concomitant with the increase in contaminated land and water systems, there has been an increase in remediation costs. The US Environmental Protection Agency (Cleaning Up the Nation's Waste Sites: Markets and Technology Trends, EPA 1999) estimates that there are more than 217,000 contaminated sites from past governmental remediation activities, and has estimated a staggering $187 billion cost for cleaning up these sites. According to OECD (Organization for Economic Co-operation and Development) estimates, the total global market for environmental products and services will grow at an annual rate of 20% to $500 billion in 2000. In general, the cost estimates for utilizing most of these technologies, colloquially termed as ‘pump-and-treat’ and ‘dig-and-dump’ techniques, have remained high. Additionally, these technologies have several disadvantages including ineffective or variable treatment efficiencies and underproduction.

As one of the emerging, low cost and ecologically friendly technologies phytoremediation addresses these limitations associated with conventional approaches. Moreover, plant-based remediation is primarily an in situ remediation technology and therefore the costs associated with its application are relatively low ($25-$100 per ton in the case of soils and 0.60-$6.00 per 1,000 gal treated, Glass,
An ideal plant species for phytoremediation, as described earlier, should have one of the following characteristic combinations (Friedland, 1990, US EPA, 2000): a) a low biomass plant with a very high metal accumulation capacity, or b) a high biomass plant with enhanced metal uptake potential. Based on these background requirements, the present study was designed to identify and characterize plant species that have the potential to accumulate and sequester high levels of toxic metals in their biomass. First, the capacity to tolerate cadmium, nickel and lead was evaluated under greenhouse conditions using chlorophyll a fluorescence kinetics as an experimental characterization of heavy metal tolerance. The *Pelargonium* sp. ‘Frensham’ and *Pelargonium* sp. Citrosa were found to be highly tolerant to concentrations of cadmium, nickel and lead. Additionally, several of the metal localization sites were identified and some of the physiological mechanism that imparts the tolerance and accumulation to metals in *Pelargonium* sp. ‘Frensham’ were characterized.

From the chlorophyll fluorescence studies it was evident that scented geranium plants have the potential to tolerate a wide range of cadmium, nickel and lead concentrations under greenhouse conditions. Such tolerance is probably due to multiple mechanisms, such as cellular uptake, active detoxification and sequestration, and an increased metabolic rate, thus maintaining optimal photosynthetic activity even under metal stress.

The metal accumulation studies demonstrated the potential of scented geranium for cadmium, nickel and lead accumulation, indicating the efficacy of this plant species for decontamination of multiple metal polluted substrates.
Apart from the hypertolerance and accumulation potential demonstrated for individual metals, these plants (*Pelargonium* sp. ‘Frensham’ and Citrosa sp.) demonstrated versatility in tolerating and at the same time accumulating multiple metal contaminants. Under these circumstances, the accumulation of cadmium, nickel and lead in scented geranium plants is of major importance, offering both an efficient plant species for phytoremediation and a unique model system for the study of physiological stress response processes in plants.

The metal localization studies provided evidence to substantiate the presence of both passive and active mechanisms of metal uptake and translocation through the root apoplasm to the symplasm, resulting in a gradient of lead ions leading to the shoot. The extent to which metal ions accumulated and how they are sequestered within different cell compartments plays an important role in the survival of the plant in these extreme conditions.

To summarize, the major accomplishments were:

1. *Pelargonium* sp. ‘Frensham’ and *Pelargonium* sp. Citrosa have been identified as metal tolerant species when exposed to a wide range of toxic metals under greenhouse conditions.

2. *Pelargonium* sp. ‘Frensham’ maintained a superior metal tolerance capacity when compared to Indian mustard and sunflower (known as metal tolerant and metal accumulator plants) under similar experimental conditions.

3. *Pelargonium* sp. demonstrated a high potential for metal uptake when exposed to individual metals or to a mixed metal solution.
4. *Pelargonium* sp. ‘Frensham’ accumulated metals by a combination of both passive and active mechanisms of metal uptake.

5. Several sites of metal localization at the cellular level have been identified such as cell wall deposition and vacuolar sequestration.

This study has only touched on our current understanding and use of plants for clean up of metal contaminated substrates. The various findings to date suggest that multiple challenges and opportunities will be encountered until phytoremediation will become an established technology. Without a better understanding of many aspects of the basic science, it will be difficult to develop and apply efficient phytoremediation systems for decontamination of polluted sites. Future research should be focussed on:

- large scale phytoremediation trials / demonstration trials *in situ* and for a longer period of time (4-5 years) to allow the monitoring and evaluation of the performance of *Pelargonium* sp.

- developing and refining phytoremediation protocols for *Pelargonium* sp. that will address: issues such as crop practices and impact on the other components of the ecosystem (microorganisms, plants, animals and humans)

- enhancing metal tolerance, uptake, translocation and sequestration through genetic engineering technologies

- characterization of the metal accumulation mechanism in *Pelargonium* sp. at the cellular level

As with any growing technology, the major requirement for success of the phytoremediation technology, is the need to establish appropriate governmental
regulations to monitor and evaluate the performance of phytoremediation systems. In the last years, even as scientific progress is made the social view of environmental contamination and remediation is changing. Continuously, as clean up of environmental contamination becomes an important priority, there is an increasing understanding of the economic realities associated with solving this aspect. Changes in legal standards, methods and measurements used to assess and prioritize remediation reflect this understanding, and these changes could allow the development of phytoremediation as a viable remediation technology of the future.
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APPENDIX 1: THESIS PUBLICATIONS

Chapter 2


Chapter 3

Chapter 4
Dan TV, KrishnaRaj S and Saxena PK (2000b) Cadmium and nickel uptake and accumulation in scented geranium (Pelargonium sp. ‘Frensham’). Water Air Soil Pollut. (accepted)

Chapter 5