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(54) **MODE D'EMPLOI D'ESPECES DE PELARGONIUM EN TANT  
QU'HYPERACCUMULATEUR POUR REMEDIER A UN SOL  
CONTAMINE**

(54) **METHOD OF USING PELARGONIUM SP. AS  
HYPERACCUMULATORS FOR REMEDIATING  
CONTAMINATED SOIL**

(57) La présente invention porte sur un procédé permettant de remédier efficacement à la contamination de sols par certains ions métalliques, qu'il s'agisse d'ions individuels ou d'un mélange. Le procédé en question consiste à utiliser des plantes du genre *Pelargonium* afin d'accumuler dans leurs racines et leurs pousses de très grandes quantités d'ions métalliques. Ces plantes, lorsqu'elles poussent sur des sols contenant un ou plusieurs types d'ions métalliques, qu'il s'agisse de types individuels ou d'un mélange complexe, ont la propriété d'absorber les ions métalliques par leurs racines et de les déplacer dans leurs pousses. Ce processus entraîne l'extraction des ions métalliques hors du sol. On peut utiliser les pousses récoltées et la biomasse tirée de ces pousses pour extraire des huiles aromatiques essentielles. Le procédé proposé consiste également à utiliser les plantes susmentionnées pour réhabiliter les sites à vocation agricole d'industries pétrolières, généralement contaminés par un mélange de ions métalliques et de contaminants organiques. Ladite plante surpasse toutes les propriétés exigées d'un hyperaccumulateur idéal, à savoir: croissance généralement vigoureuse, importante biomasse (essentiellement les feuilles), système racinaire efficace, aptitude à survivre et à absorber un vaste gamme de ions métalliques. aptitude à retenir les parties de plantes sénescents, sans parler des avantages économiques potentiels liés aux huiles aromatiques essentielles obtenues à partir de la biomasse.

(57) A process for effectively remediating soils contaminated with individual or mixture of metal ions is described. The process involves utilizing plants of the genus *Pelargonium*, particularly *Pelargonium* sp., to hyperaccumulate metal ions in their roots and shoots. These plants when grown on soils, which contain one or more of the metal ions, individually or in a complex mixture, will uptake the metal ions through their roots and translocate them to the shoots. This process thereby removes the metal ions from the soil. The harvested shoot and root biomass can be used for extraction of essential aromatic oils, and the residual oil-extracted biomass will be available for extraction and recycling of the metals. The process also describes the use of the above said plant(s) for remediating land-farming sites of petroleum industries, which are generally contaminated with a mixture of metal ions and organic contaminants. The plant surpasses all the requirements of an ideal hyperaccumulator such as, robust growth habit, large shoot biomass (primarily leaves), effective root system, ability to survive and uptake of a wide array of metal ions, ability to retain senescing plant parts, in addition to potential economic returns in the form of essential aromatic oils from harvestable biomass.





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<b>(21) International Application Number:</b> PCT/CA98/01027 <b>(22) International Filing Date:</b> 4 November 1998 (04.11.98) <b>(30) Priority Data:</b> 60/064,238 4 November 1997 (04.11.97) US <b>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application</b> US 60/064,238 (CIP) Filed on 4 November 1997 (04.11.97) <b>(71) Applicant (for all designated States except US):</b> UNIVERSITY OF GUELPH [CA/CA]; Room 214, Reynolds Building, Guelph, Ontario N1G 2W1 (CA). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> KRISHNARAJ, Sankaran [IN/CA]; 230-78 College Avenue West, Guelph, Ontario N1G 4S7 (CA). SAXENA, Praveen, K. [CA/CA]; 32 Fieldstone Road, Guelph, Ontario N1L 1B4 (CA). PERRAS, Michel, R. [CA/CA]; 8 Arthur Place, Kitchener, Ontario N2G 1L5 (CA).	<b>(74) Agent:</b> DEETH WILLIAMS WALL; National Bank Building, Suite 400, 150 York Street, Toronto, Ontario M5H 3SH (CA). <b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). <b>Published</b> <i>With international search report.</i>	
<b>(54) Title:</b> METHOD OF USING <i>PELARGONIUM</i> SP. AS HYPERACCUMULATORS FOR REMEDIATING CONTAMINATED SOIL		
<b>(57) Abstract</b> <p>A process for effectively remediating soils contaminated with individual or mixture of metal ions is described. The process involves utilizing plants of the genus <i>Pelargonium</i>, particularly <i>Pelargonium</i> sp., to hyperaccumulate metal ions in their roots and shoots. These plants when grown on soils, which contain one or more of the metal ions, individually or in a complex mixture, will uptake the metal ions through their roots and translocate them to the shoots. This process thereby removes the metal ions from the soil. The harvested shoot and root biomass can be used for extraction of essential aromatic oils, and the residual oil-extracted biomass will be available for extraction and recycling of the metals. The process also describes the use of the above said plant(s) for remediating land-farming sites of petroleum industries, which are generally contaminated with a mixture of metal ions and organic contaminants. The plant surpasses all the requirements of an ideal hyperaccumulator such as, robust growth habit, large shoot biomass (primarily leaves), effective root system, ability to survive and uptake of a wide array of metal ions, ability to retain senescing plant parts, in addition to potential economic returns in the form of essential aromatic oils from harvestable biomass.</p>		

**TITLE: METHOD OF USING *PELARGONIUM* SP. AS HYPERACCUMULATORS FOR REMEDIATING CONTAMINATED SOIL**

**BACKGROUND OF THE INVENTION**

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Domestication of plants for human consumption has resulted in altering most crops and crop-related plant's ability to uptake metals from the soil medium. The ultimate aim of many decades of plant breeding practices has been to reduce the accumulation of unfavourable substances in plant parts that are destined for human or animal consumption. However, a few wild and native plant species have, to date, retained their ability to uptake undesirable toxic substances from the growth medium on which they establish. Some of these plant species often accumulate very high concentrations of metal ions in their foliage. These plants are commonly classified as "hyperaccumulators". Hyperaccumulators are plants which can accumulate toxic metal ions, such as nickel, copper, cobalt and lead at very high concentrations in shoot or root tissues (>0.1% of the dry matter). These plants are normally found growing in soils containing unusually high concentrations of these metals in special geological formations, for example, the presence of zinc accumulating *Thlaspi* plants growing in zinc-rich soils near the Germany and Belgium border. To date, only a handful of hyperaccumulating plant species have been identified for their potential to uptake different metal species (see Table A.).

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The term phytoremediation refers to the effective utilization of such metal-hyperaccumulating plant species which have the ability to uptake, bind, and detoxify environmental contaminants, such as metal ions and organics, through plant-mediated biological, biochemical and physical means. The current focus of researchers is to identify and select better plant species for phytoremediation, species that can be classified as hyperaccumulators and that also possess a large biomass into which the plants can accumulate and sequester large quantities of toxic metal ions. The identified plants must be hardy and suitable for the temperate North American environment. As stated by Brown et al. (1995), the hyperaccumulation mechanism involves the translocation of the metals from soil to shoot tissues in excess of 100 mg/kg for Cadmium, 1,000 mg/kg for Nickel and 10,000

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mg/kg for Zinc, Copper and Cobalt hyperaccumulators are defined as plants capable of accumulating more than 0.1% (1,000 mg/kg) of these metals in their dried tissue (Baker et al., 1988).

5 **Table A. Metal concentrations in the known hyperaccumulator species** [concentration in harvestable material from plants growing in contaminated soils (on dry weight basis)]

Metal	Plant Species	Concentration [mg/kg in shoots]
Cd	<i>Thlaspi caerulescens</i>	1,800
Cu	<i>Ipomea alpina</i>	12,300
Co	<i>Haumaniastrum robertii</i>	10,200
Pb	<i>T. rotundifolium</i>	8,200
15 Mn	<i>Macademia neurophylla</i>	51,800
Ni	<i>Psychotria douarrei</i>	47,500
	<i>Sebertia acuminata</i>	(25% by wt of dried sap)
Zn	<i>T. caerulescens</i>	51,600

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The major limitations of utilizing these hyperaccumulating plant species for phytoremediation are:

- a) Plants such as *Thlaspi* and *Haumaniastrum* are very small, with a very low plant biomass. Although these plants can uptake metals >1% of their dry weight ("DW"), their low biomass limits their ability to uptake large amounts of metal ions. For example, shoots of *T. rotundifolium* can accumulate up to 8200 mg/kg DW of Pb but these plants can only produce 5 to 50 mg of plant dry material during a 5 month growing period. Therefore, these plants would have to be grown over several growth cycles and seasons in order to achieve complete remediation of a site.
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- b) Plants such as *Thlaspi* and *Haumaniastrum* are very small in stature, and therefore are not amenable for harvesting using conventional farm machinery.
- c) Plants such as *Thlaspi* and *Haumaniastrum* have a very slow growth habit (*Thlaspi rotundifolium* has a 5 month growth period). A long growth cycle would result in longer remediation periods.
- d) Tree species such as *Sebertia acuminata* have a longer growth period but due to their tropical origin, they might not be able to over-winter in temperate environments, and hence may not be useful for phytoremediation purposes in North America.
- e) All the hyperaccumulator plant species mentioned above have specific target metal species, which they are capable of accumulating in very large amounts in their plant parts. However, most of the contaminated soil sites have a mixture of metal contaminants. In the presence of such complex metal contaminants, it is very unlikely that these known hyperaccumulators will be able to survive and uptake large levels of the different metal ions. For example, petroleum industries land-farming sites in Sarnia, Ontario, Canada, have a mixture of about 15 different metal ions and organic contaminants in varying concentrations, depending on the location.

Among the hyperaccumulating plant species currently being considered for phytoremediation and which have been characterized in greenhouse and field conditions, the most promising ones are *Thlaspi caerulescens* and plants belonging to the Brassicaceae family.

The limitation of using plants belonging to the Brassicaceae family are:

- a) Most of the currently identified plants are wild relatives of the cultivated crop species *Brassica napus* (canola). Due to the potential for cross-pollination between the wild-relatives and crop species, public acceptance of these plants for phytoremediation is questionable. This problem holds credence considering the potential for evolution of

new weed-like species, which might interfere with current agricultural systems.

b) These plants set seeds readily and might assume weed-like characteristics after repeated growth in contaminated sites.

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c) These plants have relatively larger biomass than *Thlaspi* sp. However, they still do not compare well with plants with denser foliage (larger biomass).

The ability of plants to extract metal ions from soils and accumulate or sequester those metals in their tissues can be tremendously improved by adjusting the pH of the soil and also by the addition of synthetic chelators to the growing media. These two elements increase the release (desorption) of metal ions from soil particles, thereby increasing the availability of those ions to the plant roots, resulting in increased rate of uptake. The limitations of using metal chelators are:

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The addition of chelating agents to metal contaminated soils could bring in new problems regarding health, safety and environmental concerns. Addition of large amounts of chelates will result in rapid solubilization of different metal ions. Some of the metals released will be beneficial to plants and microflora of the soil. However, this will also increase the soil solution concentration of undesirable ions. Moreover, there is a larger risk of releasing large amounts of the solubilized toxic metals in the underground water systems. The use of chelates would increase the bioavailability and uptake of these toxic metals by the natural flora and fauna of the soil, thereby accelerating the spread of these metals in the ecosystems and in the different food chains.

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## **SUMMARY OF THE INVENTION**

The present invention provides a process for remediating a growth medium contaminated with metal ions. More specifically, this invention provides a process for remediating a growth medium contaminated with metal ions using *Pelargonium* sp. plants.

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According to one aspect of the invention, there is provided a method for removing one or more species of metal from a metal contaminated growth medium, comprising growing a *Pelargonium* sp. plant in the growth medium for a time period sufficient for the plant root to uptake and accumulate metal in the root or shoot biomass.

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In a further aspect of the invention, the method for removing one or more species of metal from a metal contaminated growth medium further comprises the extraction of essential aromatic oils or concentration of metals from the root or shoot biomass.

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In another of its aspects, the invention provides a method for removing one or more species of metal from a growth medium (the growth medium is preferably metal contaminated) which comprises growing a *Pelargonium* sp. plant, which has been transformed with a gene sequence encoding for a desirable trait, in the growth medium for a time period sufficient for the plant root to uptake and accumulate metal in the root or shoot biomass.

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These and other embodiments of the present invention are described in greater detail hereinbelow.

## DETAILED DESCRIPTION OF THE INVENTION

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Contamination of cultivable lands with toxic levels of metal ions poses an environmental risk to humans, animals and agriculture. Remediation of such contaminated soils through conventional practices (such as solidification or stabilization techniques, soil flushing, bioremediation, electrokinetics, chemical reduction and oxidation, soil washing, pneumatic fracturing, as well as the process of excavation, retrieval, and off-site disposal) are often expensive, have limited performance potential and are applicable only to small areas. The developed alternative process of the invention is a low-cost phytoremediation system for clean-up of metal-tainted soils using plant species that can hyperaccumulate a wide variety of metal ions. Phytoremediation refers to the effective utilization of certain metal-

hyperaccumulating plant species to uptake, bind, and detoxify environmental contaminants, such as metal ions and organics, through plant-mediated biological, biochemical and physical

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means. Phytoremediation technology also possesses an added advantage because the sites after remediation utilizing this process are still agriculturally viable, while those remediated through conventional practices are often sterile, as most essential nutrients are stripped-off from the soil.

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To date there are only a handful of known hyperaccumulating species. Based on our studies we have identified a plant species, scented geranium (*Pelargonium* sp.) which has shown tremendous potential as an hyperaccumulator of several heavy metal ions.

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The successful use of scented geraniums as hyperaccumulators in phytoremediation was unexpected in view of the research by others in this area. To date, all known hyperaccumulators are seed-propagated. Seed-propagation of the hyperaccumulators allows for ease in seeding the plants in contaminated sites. There are no known hyperaccumulators which are vegetatively (cutting) propagated. Most researchers working in this area, have so far and are still, focusing on identifying hyperaccumulating seed-propagated plants but have never attempted to identify hyperaccumulating vegetatively propagated plants.

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Also, most efforts at identifying hyperaccumulators have focused on identifying and selecting plant species which naturally grow in metal enriched soils, while not much effort was made in recognizing the hyperaccumulating potential of domesticated, economically important, non-food crops/plants. Scented geranium is one such plant species, which is known world-wide for its ornamental characteristics, but has never been considered for its potential in phytoremediation.

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Scented geranium plants grow well in a wide variety of growth media ranging from artificial soil mix (peat, perlite, vermiculite combinations), sand, to petroleum industries land-farming sites which contain mixtures of metal ions and organics. These plants are very hardy and can withstand a wide range of temperature, humidity, photoperiod and watering schedules.

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These plants are not season-dependent and can be grown and harvested several times during a calendar year. The time required for these plants to uptake metal ions is relatively short (uptake data is based on just a 2 week treatment period) and therefore several cycles can be

accommodated in one growing season. These plants are propagated by cuttings and rarely flower, so there is no potential out-crossing, and they do not assume weed-like characteristics. Due to the pleasant but intense lemon-scented leaves with numerous trichomes, these plants are less susceptible to pest outbreak or herbivore damage. The plant has an aesthetic value, and a commercial significance (essential aromatic oils in the biomass) thereby it should promote public acceptance of the technology. The advantages of the process of utilizing plants belonging to the genus *Pelargonium* as candidate plants for phytoremediation are (in comparison to other hyperaccumulator species known to date):

- 10 a) The plants (belonging to the genus *Pelargonium*, especially *Pelargonium* sp. 'Frensham', *Pelargonium* sp. 'Citrosa' and *Pelargonium* sp. 'Beauty Oak') ability to survive on soils contaminated with one or more metal ions. Most known hyperaccumulators have limited potential in phytoremediation, as they are suitable for remediating only specific (individual) metals.
- 15 b) The plants' (belonging to the genus *Pelargonium*, especially *Pelargonium* sp. 'Frensham', *Pelargonium* sp. 'Citrosa' and *Pelargonium* sp. 'Beauty Oak') ability to uptake, translocate and accumulate a wide array of metal ions, such as cadmium, lead, zinc, copper, nickel in the shoot biomass. Most known hyperaccumulators can uptake only one specific metal ion, and therefore are limited in their applicability to remediate soils with complex metal ion mixtures.
- 20 c) The plants (belonging to the genus *Pelargonium*, especially *Pelargonium* sp. 'Frensham', *Pelargonium* sp. 'Citrosa' and *Pelargonium* sp. 'Beauty Oak') possess a very dense foliage (consisting mostly of leaves) for sequestering high levels of metal ions in the above ground parts. The shoot biomass is exceptionally higher than any known hyperaccumulator e.g., *Thlaspi* sp., and plants belonging to *Brassicaceae* family.
- 25 d) The plants (belonging to the genus *Pelargonium*, especially *Pelargonium* sp. 'Frensham', *Pelargonium* sp. 'Citrosa' and *Pelargonium* sp. 'Beauty Oak') have a
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faster and robust growth habit. Most known hyperaccumulators (e.g., *Thlaspi* sp.) have a very slow growth habit. *Pelargonium* sp. 'Frensham' can attain a biomass of greater than 4 kg within 5-6 month growth period.

- 5 e) The plants (belonging to the genus *Pelargonium*, especially *Pelargonium* sp. 'Frensham', *Pelargonium* sp. 'Citrosa' and *Pelargonium* sp. 'Beauty Oak') have an efficient and prolific root system that can efficiently absorb metal ions from the soil or ground water. *Pelargonium* sp. has a prolific root system, which can grow up to 3-4 feet within a 5-6 month growth period.
- 10 f) The plants (belonging to the genus *Pelargonium*, especially *Pelargonium* sp. 'Frensham', *Pelargonium* sp. 'Citrosa' and *Pelargonium* sp. 'Beauty Oak') have the ability to grow in a wide variety of soils with relatively low requirements for water, nutrients and other conditions required to sustain growth and metabolism (they are also
- 15 viable in different types of soil, soil factors, and in adverse environments unlike many other known hyperaccumulators).
- g) The plants (especially *Pelargonium* sp. 'Frensham', *Pelargonium* sp. 'Citrosa' and *Pelargonium* sp. 'Beauty Oak') have the ability to moderately retain senescing and
- 20 dead leaves (without withering) thereby reducing recycling of metal ions to the contaminated soil, (a very distinct characteristic of certain scented geraniums).
- h) The use of harvested plants (especially *Pelargonium* sp. 'Frensham', *Pelargonium* sp. 'Citrosa' and *Pelargonium* sp. 'Beauty Oak' shoot biomass) for extraction of essential
- 25 aromatic oils such as, citronellol, geraniol, iso-methane, geranyl formate etc. No other hyperaccumulator has been shown to have economic return from plants used for phytoremediation.

It is an aspect of the present invention to provide a method for removing one or more

30 species of metal (such as metal in an ionic form, elemental form or organometallic form) from a metal contaminated growth medium, comprising growing a *Pelargonium* sp. plant in the

growth medium for a time period sufficient for the plant root to uptake and accumulate metal in the root or shoot biomass. In a specific embodiment of the invention, the plant is selected from *Pelargonium* sp. 'Frensham', *Pelargonium* sp. 'Citrosa' and *Pelargonium* sp. 'Beauty Oak'. In a more specific embodiment, the plant is selected from *Pelargonium* sp. Frensham and

5 *Pelargonium* sp. 'Beauty Oak'. In the most specific embodiment, the plant is *Pelargonium* sp. 'Frensham'. Other varieties of *Pelargonium* sp. are also useful as hyperaccumulators. A partial list of *Pelargonium* sp. is provided in Table 7. One of ordinary skill in the art can measure the ability of these plants and other *Pelargonium* sp. to hyperaccumulate metals using the methods described in this application.

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In another embodiment of the invention, the growth medium comprises solid medium, semi-solid medium, liquid medium (preferably a hydroponic growth medium) or a combination thereof. In a preferred embodiment, the growth medium comprises soil, sand, sludge, compost or artificial soil mix. In another embodiment of the invention, the growth medium comprises

15 soil, sand, sludge, compost or artificial soil mix which further comprises organic contaminants. The growth medium may also comprise waste water or waste compounds. In specific embodiments, these organic contaminants are selected from the group consisting of petroleum industry by-products and petroleum industry wastes.

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In another embodiment of the present invention, the species of metal to be removed from the contaminated growth medium is any metal likely to be found in metal-contaminated growth medium. In specific embodiments, the metal species is selected from one or more of the group consisting of lead, cadmium, copper, nickel, zinc, antimony, boron, vanadium, chromium, cobalt, manganese, selenium, arsenic, molybdenum, beryllium, barium, mercury,

25 silver, antimony, titanium, thallium, tin, gold, rubidium, strontium, yttrium, technetium, ruthenium, palladium, indium, cesium, uranium, plutonium and cerium. In more specific embodiments, the metal is selected from the group consisting of one or more of the group consisting of lead, cadmium, copper, nickel and zinc. The method is used to remove metal from growth media containing one metal in large quantity or a combination of metals in a

30 variety of concentrations. When the metal contaminate comprises cadmium, it is an embodiment of the invention that the concentration of cadmium accumulated is in the range of

about 200 mg Cd/kg to 30,000 mg Cd/kg (preferably 450 mg Cd/kg to 27,500 mg Cd/kg) dry weight of the plant. When the metal contaminate comprises lead, it is an embodiment of the invention that the concentration of lead accumulated is in the range of 1000 mg Pb/kg to 70,000 mg Pb/kg (preferably 1,300 mg Pb/kg to 66,000 mg Pb/kg) dry weight of the plant.

5 When the metal contaminate comprises copper, it is an embodiment of the invention that the concentration of cadmium accumulated is in the range of about 200 mg Cu/kg to 1,000 mg Cu/kg (preferably 230 mg Cu/kg to 620 mg Cu/kg) dry weight of the plant. When the metal contaminate comprises nickel, it is an embodiment of the invention that the concentration of nickel accumulated is in the range of about 200 mg Ni/kg to 25,000 mg Ni/kg (preferably 400

10 mg Ni/kg to 21,500 mg Ni/kg) dry weight of the plant.

It is another aspect of the present invention that the method further comprises the step of harvesting one or more parts of the plant selected from the shoot and root biomass. In a specific embodiment, when the shoot biomass is harvested, the shoot biomass comprises a leaf

15 or a stem. In another specific embodiment, when the shoot biomass is harvested, a sufficient portion of the shoot biomass is not harvested to permit continued growth of the plant. It is another embodiment of the present invention that the method further comprises the step of harvesting one or more parts of the plant selected from the shoot and root biomass and extracting essential aromatic oil from said biomass. In specific embodiments, the essential

20 aromatic oil is obtained by distillation. In more specific embodiments, the essential aromatic oil is selected from citronellol, geraniol, iso-methane and geranyl formate. Also, it is another embodiment of the present invention that the method further comprises the step of harvesting one or more parts of the plant selected from the shoot and root biomass and concentrating the metal using any known method for concentrating metals from plant biomass. Specifically, the

25 concentration of the metal may be carried out by air drying, dehydrating, ashing, incineration, smelting, aerobic digestion or anaerobic digestion of the residual oil-extracted shoot biomass.

It is another aspect of the present invention that the method for removing one or more species of metal from a metal contaminated growth medium further comprises growing a

30 *Pelargonium* sp. plant which has been transformed with a gene sequence encoding for a desirable trait. In an embodiment of the invention the *Pelargonium* sp. plant is transformed

with a gene sequence that enhances the plants' ability to uptake metals. In a specific embodiment of the invention, the *Pelargonium* sp. plant is transformed with a gene sequence encoding metallothionein. In another specific embodiment of the invention, the *Pelargonium* sp. plant is transformed with a gene sequence encoding phytochelatin synthase. It is another  
5 embodiment that the *Pelargonium* sp. plant is transformed by the introduction of genes through delivery vehicles such as particle bombardment or *Agrobacterium* vectors or using techniques such as microinjection and electroporation. In a specific embodiment, the *Pelargonium* sp. plant is transformed by an *Agrobacterium* strain.

10 We assess the hyperaccumulation of *Pelargonium* sp. plants. The ability of the plants to uptake different metal ions is investigated by adding different concentrations of each metal ion to the growth medium, and estimating the metal content in harvested roots and shoots. The concentration of the metal ions is increased with each experiment in order to identify the LD50 (concentration at which 50% of the plants exhibit physiological  
15 senescence and death) dose (for the set 2-week treatment period). This experiment identifies the maximum threshold of metal concentration in which the plant can survive and perform remediation processes.

Scented geranium plants are grown on a growth medium containing a mixture of  
20 heavy metals and we assess the plants ability to uptake simultaneously a wide array of metal ions present. This study provides the interaction effects of the different metal ions and how they are selectively taken up by the plant. Other scented geranium plant species along with plants belonging to the *Pelargonium* sp. are grown on similar growth medium containing metal ions either individually or in complex combinations and we assess these plants' ability  
25 to uptake, and translocate metal ions to the shoots. These plants are harvested and used in isolation of essential aromatic oil or concentration of metals. In a preferred variation, the invention relates to a method of obtaining one or more metals from a growth medium.

Plants belonging to the *Pelargonium* sp. are also grown on soils of land-farming sites  
30 (for example land-farming sites), and other contaminated sites (either in the greenhouse or

by direct planting on the site), to show the ability of the said plant to uptake metals from a complex soil medium containing metals as well as organic contaminants.

A preferred mode of utilizing scented geraniums (or plant belonging to the  
5 *Pelargonium* sp.) is to plant well-rooted cuttings (1 month old cuttings) in soil (artificial soil  
mix, soil) which contains one or more of the heavy metal ions. The plants are irrigated  
biweekly with regular tap water (approximate pH range of water being 5-9). The plants do  
not need fertilization if a two-week treatment schedule is followed. Otherwise, plants can be  
fertilized as required. The plants require natural sunlight and normal temperature regimes to  
10 perform essential metabolic activities. The plants should be grown (for a minimum) of 2  
weeks in the growth medium to allow the plant to uptake metal ions and translocate them to  
the shoots. The metal-laden shoot and root biomass, after the two-week or other pre-  
determined treatment period, can be harvested manually or mechanically. The plant material  
(if left behind after harvest of shoots) can be allowed to grow back in the next cycle, or new  
15 cuttings can be planted in the same site. The biomass can be extracted to obtain essential  
aromatic oils and the oil-extracted biomass can be used for metal extraction and recycling or  
disposal. These procedures can be varied by one skilled in the art using known techniques in  
order to use scented geraniums in other soil types. The geraniums may also be grown from  
seeds or other cuttings or plant parts, as known in the art.

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The described process of using plants of the *Pelargonium* sp. for phytoremediation will  
have commercial application in remediating contaminated sites such as those identified by: a)  
the National Priorities List (Superfund sites), b) Resource Conservation and Recovery Act  
(RCRA) Corrective Action sites, c) Underground Storage Tank sites, d) Department of  
25 Defense sites, e) Department of Energy sites, as well as f) other federal, provincial, state and  
private party-owned sites. The primary application will be to clean-up contaminated sites  
associated with abandoned mines, abandoned tailing sites, petroleum industries waste disposal  
sites inclusive of land-farming sites, explosives and unexploded ordinance storage and dump  
sites, sewage sludge dump sites, radioactive and industrial waste-treatment and disposal sites.

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*Pelargonium* sp. plants, or mutants of the plant, developed by mutagenesis with chemical mutagens such as EMS, MMS, ENU, or physical mutagens such as X-ray, gamma-rays; or somaclonal or gametoclonal variants of the said plant(s) selected and developed through tissue culture procedures, spontaneously or induced by mutagens are used in  
5 phytoremediation. These variants and other variants of *Pelargonium* sp. that may be used as hyperaccumulators are within the scope of the invention.

Microorganisms, such as *Pseudomonas* sp., which enhance plant growth or metal availability (through chelating action) may be used by co-habiting with roots in the  
10 rhizosphere. The choice of suitable organisms is made according to considerations known in the art.

Soil chemistry can be regulated to facilitate plant growth, availability of metals (such as entrapped metals) or metal uptake. The plants' hyperaccumulating ability to uptake and  
15 sequester large amounts of metal ions can be enhanced by use of soil treatments or chemicals that make metals in soils more available to the roots of plants, such as metal chelating agents. To enhance the scented geranium plants ability to uptake metal ions from the growth medium, we are utilizing metal-chelating agents such as EDTA, HEDTA, EGTA, DTPA, etc. in the growth medium. Any other suitable chelating agent may also be used in the  
20 methods of this invention. These metal chelators solubilize the metal ions and make them easily available to the roots for easy uptake. Among the chelating agents, EGTA and EDTA have been found to be the most effective chelators at enhancing the accumulation of metal by the plants. EDTA is about 10-20 times less expensive than EGTA, which makes it more attractive for extensive commercial applications in large contaminated sites. We have also  
25 modified the pH of the growth medium and determined the effect of pH on the bioavailability of the different metal species. The interaction of pH and chelating agents on bioavailability of metals and the scented geranium plants ability to uptake those metal ions has been investigated.

30 Genetic transformation technology is used to transform the plant with genes such as the metallothionein gene or phytochelatin synthase gene to enhance uptake and effectively bind

metal in the shoot, through use of site specific promoters. When expressed throughout the plant, the polypeptides produced by these genes bind metals in the root or in the shoot. The polypeptide-metal complex is sequestered in the vacuoles of cells. Sequestration prevents the accumulated metal from interfering with metabolism. The complex cannot be translocated  
5 within the plant. If the polypeptide is expressed in the root, one must harvest the whole plant to gather all of the polypeptide-metal complexes. Preferably the gene is expressed specifically in the shoot so that only the shoot is harvested in order to gather the polypeptide-metal complexes. The remaining plant will then continue to grow and uptake more metal from the contaminated soil.

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High frequency regeneration and *Agrobacterium*-mediated transformation systems are used to obtain stably transformed scented geraniums containing gene(s) encoding for metallothionein or phytochelatin synthase. The plants may also be transformed with other genes to enhance uptake of metals.

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The invention also relates to *Pelargonium* sp. cells, tissue cultures, plants, seeds or plant parts (such as roots, shoots or cuttings) transformed with recombinant metallothionein gene or phytochelatin synthase gene or a gene that is biologically functional equivalent to the metallothionein gene or the phytochelatin synthase gene and their use in phytoremediation.

20 The invention also relates to methods of making *Pelargonium* sp. including a recombinant metallothionein gene or a phytochelatin synthase gene or a biologically functional equivalent gene, by inserting at least one of the aforementioned genes into a *Pelargonium* sp. cell, tissue culture, plant, seed or plant part. In the preferred embodiment, a plant is generated from the cell, tissue culture, seed or plant part.

25

Biologically functional equivalent nucleotide sequences are DNA and RNA (such as genomic DNA, cDNA, synthetic DNA, and mRNA nucleotide sequences), that encode peptides, polypeptides, and proteins having the same or similar activity as the proteins encoded by the metallothionein gene or the phytochelatin synthase gene. Biologically  
30 functional equivalent nucleotide sequences can encode peptides, polypeptides, and proteins

that contain a region having sequence identity to a region of a metallothionein gene or a phytochelatin synthase gene.

The invention includes *Pelargonium* sp. transformed with biologically functional equivalent nucleotide sequences that encode *conservative* amino acid changes within an amino acid sequence produced by the metallothionein gene or the phytochelatin synthase gene and which produce silent amino acid changes.

The invention includes *Pelargonium* sp. transformed with nucleotide sequences that are biologically functional equivalent to the metallothionein gene or the phytochelatin synthase gene which encode peptides, polypeptides, and proteins having *non-conservative* amino acid substitutions, additions, or deletions but which also retain the same or similar activity as metallothionein gene or the phytochelatin synthase gene. The DNA or RNA can encode fragments or variants of metallothionein gene or the phytochelatin synthase gene. The metallothionein gene or the phytochelatin synthase gene activity of such fragments and variants is identified by assays as known in the art. One skilled in the art could take a fragment including the nucleic acid sequence of the coding region of the gene (or a biologically functional equivalent gene) and insert it in an expression vector. Fragments and variants of the metallothionein gene or the phytochelatin synthase gene encompassed by the present invention should preferably have at least about 40% sequence identity or preferably at least about 60%, at least about 80%, at least about 90% or at least about 95% sequence identity to the naturally occurring gene, or corresponding region or moiety. Most preferably, the fragments have at least 97%, 98% or 99% sequence identity to the naturally occurring gene, or corresponding region. Sequence identity is preferably measured with either the Gap or BestFit programs. BestFit aligns the best segment of similarity between two sequences. Alignments are made using the local homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2:482-489. The algorithm of Needleman and Wunsch (1970 J Mol. Biol. 48:443-453) is used in the Gap program.

In the case of a recombinant gene, the gene would contain suitable transcriptional or translational regulatory elements. Suitable regulatory elements may be derived from a variety of sources, and they may be readily selected by one with ordinary skill in the art. If one were to upregulate the expression of the gene, one would insert the sense sequence and the appropriate promoter into the vehicle. If one were to downregulate the expression of the gene, one would insert the antisense sequence and the appropriate promoter into the vehicle. Genetic engineering and manipulation of plants is described in Rogers et al., 1988 (*Gene Transfer in plants: Production of transformed plants using Ti plasmid vectors, Methods for Plant Molecular Biology*). Other techniques for genetic engineering are known to those skilled in the art.

Examples of regulatory elements include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the vector employed, other genetic elements, such as selectable markers, may be incorporated into the recombinant molecules. The recombinant molecules may be introduced into cells of a *Pelargonium* sp. using delivery vehicles such as particle bombardment or using *Agrobacterium* vectors. They may also be introduced into such cells using physical techniques such as microinjection and electroporation.

An *Agrobacterium*-mediated transformation system may be used to transform Scented geraniums at high frequency (Rogers et al., 1988). The same method will apply to introduce new recombinant metallothionein or phytochelatin synthase genes into geranium plants or cells to obtain transgenic plants with greater heavy-metal accumulating potential. The constructs used to transform the plants preferably contain site-specific promoters. These promoters can regulate and limit the expression of the new genes to specifically targeted tissues. In the phytoremediation context, it is important to increase the capacity of uptake and sequestration of the absorbed metal to the above ground plant parts thereby facilitating its harvest.

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We use procedures and protocols for obtaining transformed cells and subsequently obtaining transformed plants using regeneration systems (through somatic embryogenesis). An efficient and reliable regeneration system (through somatic embryogenesis) is also available for mass propagation of the said plant. Techniques for somatic embryogenesis are described in  
5 KrishnaRaj et al., 1997 (Somatic embryogenesis and *Agrobacterium*-mediated transformation system for scented geraniums (*Pelargonium* sp. 'Frensham'), *Planta*, 201: 434-440; 1997). The effectiveness of the developed regeneration and transformation system was also successfully demonstrated by using the system to incorporate genes which impart resistance to  
10 *Botrytis* infection into scented geraniums, *Pelargonium* sp. 'Frensham' (Bi et al., 1998, Resistance to *Botrytis cinerea* infection in scented geraniums by incorporating a gene *Ace-AMP1* encoding for an antimicrobial protein, *Plant Cell Reports*, in press).

Another aspect of the invention relates to a method of determining whether a test vegetative (cutting) propagated plant is a hyperaccumulator of one or more compounds by  
15 growing the test plant in a growth medium including at least one compound (preferably at least one of the metals described in this application which is present in higher concentration than the metal is typically present in the growth medium) and determining whether the plant hyperaccumulates the compound.

20 The invention also includes the use of a vegetative (cutting) propagated plant as a hyperaccumulator of metal in a growth medium. In another variation, the invention includes a method for removing one or more species of metal from a growth medium comprising growing a vegetative (cutting) propagated plant in the growth medium for a time period sufficient for the plant root to uptake and accumulate metal (preferably in the root or shoot biomass). In the  
25 preferred embodiment of the method, the growth medium is metal contaminated.

The *Pelargonium* sp. can also be planted with hyperaccumulators known in the art and used together in methods of the invention. One skilled in the art could vary growing conditions to optimize growth of more than one type of hyperaccumulator.

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**Experiment 1 - Scented Geranium Plants and Cadmium Uptake (low Cd concentrations)**

Three separate experiments were conducted with replicate cutting propagated plants of scented geraniums to assess their ability to uptake and sequester cadmium in the shoots and roots. In the first experiment, 2 concentration levels of cadmium (Cd1 and Cd2) were selected for treating the scented geraniums [0.6 mg (Cd1) and 2.5 mg (Cd2) of cadmium nitrate dissolved in 250 mL of water per plant; treatments with these concentrations repeated 5 times over a 2 week period; replicate plants were used for each treatment; overall cadmium nitrate fed to each plant being 3 mg (Cd1) and 12.5 mg (Cd2)]. In the second experiment, 2 concentration levels of cadmium (Cd3 and Cd4) were selected for treating the scented geraniums [0.8 mg (Cd3) and 4.2 mg (Cd4) of cadmium nitrate dissolved in 250 mL of water per plant, treatments with these concentrations repeated 4 times in a 2 week period; replicate plants were used for each treatment; overall cadmium nitrate fed to each plant being 3.3 mg (Cd3) and 16.67 mg (Cd4)]. Experiment III was similar to Experiment II except that the plants were watered with alkaline tap water (pH 10.0). In all experiments the plants were harvested after 2 weeks, roots and shoots separated, and dried. Sample preparation consisted of digestion of a known amount of sample in aqua-regia (in both ashed or non-ashed, dried samples), removal of residue by filtration and making up the volume of the filtrate. The samples were analyzed and verified for the concentration of lead by Inductively Coupled Plasma-Emission Spectroscopy (ICP-ES) at Chemisar Laboratories Inc., Guelph, Ontario, Canada and using a Varian Spectra AA - 55 Atomic Absorption Spectrophotometer in our laboratory. The data is presented below in Table 1.

**Experiment 2 - Scented Geranium Plants and Lead Uptake (low Pb concentrations)**

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Two separate experiments were conducted with replicate cutting propagated plants of scented geraniums to assess their ability to uptake and sequester lead in the shoots. In the first experiment, 2 concentration levels of lead (Pb1 and Pb2) were selected for treating the scented geraniums [6.3 mg (Pb1) and 25 mg (Pb2) of lead nitrate dissolved in 250 mL of water per plant; treatments with these concentrations repeated 5 times in a 2 week period; replicate plants were used for each treatment; overall lead nitrate fed to each plant being 31.5 mg (Pb1) and

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125 mg (Pb2)]. In the second experiment, 2 concentration levels of lead (Pb3 and Pb4) were selected for treating the scented geraniums [8.3 mg (Pb3) and 41.7 mg (Pb4) of lead nitrate dissolved in 250 mL of water per plant, treatments with these concentrations repeated 4 times over a 2 week period; replicate plants were used for each treatment; overall lead nitrate fed to each plant being 33.3 mg (Pb3) and 166.7 mg (Pb4)]. Experiment III was similar to Experiment II except that the plants were watered with alkaline tap water (pH 10.0). In all experiments the plants were harvested after 2 weeks, shoots separated, and dried. Sample preparation consisted of digestion of a known amount of sample in aqua-regia (in both ashed or non-ashed, dried samples), removal of residue by filtration and making up the volume of the filtrate. The samples were analyzed and verified for the concentration of lead by Inductively Coupled Plasma-Emission Spectroscopy (ICP-ES) at Chemisar Laboratories Inc., Guelph, Ontario, Canada and using a Varian Spectra AA - 55 Atomic Absorption Spectrophotometer in our laboratory. The data is presented below in Table 1.

15           Based on the results from Experiments 1 and 2 (data presented in Table 1), we have observed that Frensham scented geranium plants uptake and accumulate greater than 450 mg cadmium, and 1,300 mg lead per kg dry weight of shoot, as well as approximately 10 times higher levels of cadmium (than the shoots) in their roots. Plant species that can accumulate greater than 100 mg Cd/kg dry weight of shoot biomass are classified as hyperaccumulators of cadmium (Brown et al., 1994; Journal of Environmental Quality, 23:1151-1157, 1994; and references therein). Similar experiments were also conducted with nickel and copper and we have so far found that scented geraniums can accumulate greater than 230 mg Cu/kg and 400 mg Ni/kg of dry weight of shoot tissue (results summarized in Table 1)

**Table 1. Metal accumulation in shoots and roots of scented geranium (*Pelargonium* sp.) 'Frensham' plants treated with different concentrations of cadmium, lead, copper and nickel in nitrate form. The data points represent mean values of 2 replicate plants in expt. I and 3 replicate plants in expt. II & III). Conc. (mg/l) refer to treatment of plants with the described concentrations during 14 day period.**

Uptake (mg/kg DW of tissue)

Heavy metal	Conc. [mg/l]	Exp I	Heavy metal	Conc. [mg/l]	Exp II	Exp III	Average
<b>Shoots</b>							
CTL		0.0			0.0		
CTL		0.0			0.0		
Cd 1	5.0	16.7	Cd3	10.0	81.0	--	57.2
Cd 2	20.0	97.0	Cd4	50.0	459.5	--	375.6
Pb 1	50.0	34.5	Pb3	100.0	152.5	15.2	67.4
Pb 2	200.0	358.0	Pb4	500.0	1306.9	501.6	722.2
Ni 1	20.0	37.6		40.0	156.0	70.7	96.8
Ni 2	100.0	108.3		200.0	401.1	96.9	254.7
Cu 1	5.0	--		20.0	32.4	17.0	
Cu 2	20.0	18.8		100.0	229.7	13.6	
<b>Root</b>							
Cd 1		542.9	Cd3	10.0		28.2	
Cd 2		3220.0	Cd4	50.0		1987.2	
Pb 1			Pb3	100.0		2202.4	
Pb 2			Pb4	500.0		18777.0	
Ni 1				40.0		267.6	
Ni 2				200.0		1576.5	

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<b>Cu 1</b>	<b>20.0</b>	<b>236.7</b>
<b>Cu 2</b>	<b>100.0</b>	<b>621.2</b>

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### **Experiment 3 - Scented Geranium Plants and Cadmium Uptake (high Cd concentrations)**

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Two separate experiments were conducted with replicate cutting propagated plants of geraniums to assess their ability to uptake and sequester cadmium in the shoots and roots. Five levels of cadmium were selected for treating the scented geranium [6.25 mg (Cd 5), 12.5 mg (Cd 6), 18.75 mg (Cd 7) 25 mg (Cd 8) of cadmium nitrate dissolved in 50 mL water per plant the treatments with these concentrations were repeated daily over a 2 week period; replicates plants were used for each treatment; overall cadmium nitrate fed to each plant being 87.5 mg (Cd 5), 170 mg (Cd 6), 262.5mg (Cd 7) and 300 mg (Cd 8)]. In both experiments the plants were watered with neutral water (pH 7.0). The plants were harvested after 2 weeks; roots and shoots separated, and dried. Sample preparation consisted of digestion of a known amount of sample using a closed teflon vessel method (Topper, 1990). The samples were analyzed using a Varian Spectra AA Atomic Absorption Spectrophotometer in our laboratory. The data is presented below in Table 2.

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### **Experiment 4 - Scented Geranium Plants and Lead Uptake (high Pb concentrations)**

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Two separate experiments were conducted with replicate cutting propagated plants of geraniums to assess their ability to uptake and sequester lead in the shoots and roots. Five levels of lead were selected for treating the scented geranium [25 mg (Pb 5), 37.5 mg (P6), 50 (Pb 7), 62.5 (Pb 8) of lead nitrate dissolved in 50 mL water per plant; treatments with these concentrations were repeated daily over a 2 week period; replicates plants were used for each treatment; overall lead nitrate fed to each plant being 350 (Pb5), 525 mg (Pb 6), 1162 mg (Pb 7) and 875 mg (Pb 8)]. In both experiments the plants were watered with neutral water (pH 7.0). The plants were harvested after 2 weeks; roots and shoots separated, and dried. Sample preparation consisted of digestion of a known amount of sample

following a closed teflon vessel method (Topper, 1990). The samples were analyzed using a Varian Spectra AA Atomic Absorption Spectrophotometer in our laboratory. The data is presented below in Table 2.

5 **Experiment 5 - Scented Geranium Plants and Nickel Uptake (high Ni concentrations)**

Two separate experiments were conducted with replicate cutting propagated plants of geraniums to assess their ability to uptake and sequester nickel in the shoots and roots. Five levels of nickel were selected for treating the scented geranium [6.25 mg (Ni 5), 12.5 mg (Ni 6), 18.75 mg (Ni 7) 25 (Ni 8) of nickel nitrate dissolved in 50 mL water per plant; treatments with these concentrations were repeated daily over a 2 week period; replicate plants were used for each treatment; overall nickel nitrate fed to each plant being 87.5 mg (Ni 5), 170 mg (Ni 6), 262.5 mg (Ni 7) and 350 mg (Ni 8)]. In both experiments the plants were watered with neutral water (pH 7.0). The plants were harvested after 2 weeks; roots and shoots separated, and dried. Sample preparation consisted of digestion of a known amount of sample following a closed teflon vessel method (Topper, 1990). The samples were analyzed using a Varian Spectra AA Atomic Absorption Spectrophotometer in our laboratory. The data is presented in Table 2.

20 Based on the results from Experiments 3-5 (data presented in Table 2), we have observed that Frensham scented geranium plants can uptake and accumulate greater than 456 mg of cadmium, 3005 mg of lead, or 1195 mg of nickel per kg dry weight of the shoot tissue, as well as 27,043 mg of cadmium, 60,986 mg of lead, or 21,141 mg of nickel per kg dry weight of the root tissue.

**Table 2. Metal accumulation in shoots and roots of scented geranium (*Pelargonium* sp. 'Frensham') plants treated with different concentrations of cadmium, lead, and nickel in nitrate form. The shoot and root uptake levels represent average of 2 independent experiments. Concentrations (mg/L) refer to treatment of plants with the described concentrations during 14 day period.**

<b>Heavy Metal</b>	<b>Concentration mg/L</b>	<b>Shoot Uptake mg/kg DW</b>	<b>Root Uptake mg/kg DW</b>
CTL	0	0	0
CTL	0	0	0
Cd 5	250	42.38879	1716.463
Cd 6	500	128.8529	5492.272
Cd 7	750	778.42	27043.57
Cd 8	1000	456.9888	5125.175
Pb 5	1000	639.1571	38441.85
Pb 6	1500	520.7667	36400.92
Pb 8	2000	2228.733	60986.25
Pb 9	2500	3005.473	40694.12
Ni 5	250	288.872	14998.9
Ni 6	500	395.0483	8086.088
Ni 7	750	508.051	12120.64
Ni 8	1000	1195.106	21141.78

### **Experiment 6 - Scented Geranium Plants and Metal Uptake from Mixture of Metal Contaminants (Cadmium, Lead and Nickel)**

Two experiments were conducted with replicate cutting propagated plants of  
5 geraniums to assess their ability to uptake and sequester metals in the shoots and roots, when  
treated with a mixture of cadmium, lead, nickel. In the first experiment, a solution  
containing 0.8 mg of  $\text{CdNO}_3$  + 8.3 mg of  $\text{PbNO}_3$  + 5 mg of  $\text{NiNO}_3$  was used for treating the  
scented geraniums plants; the metals were provided as cadmium, lead and nickel nitrates  
dissolved in 250 mL water per plant, applied daily over a 10 days period. In the second  
10 experiment, a solution containing higher concentrations of metal mixture (provided as  
nitrates) was used for treatment, viz., 3.12 mg of  $\text{CdNO}_3$  + 31.25 mg of  $\text{PbNO}_3$  + 12.5 mg of  
 $\text{NiNO}_3$  treatments with these concentrations were repeated daily over a 10 day period;  
replicates plants were used for each treatment. The overall cadmium, lead and nickel nitrate  
fed to each plant being for the first experiment: 8 mg Cd, 83 mg Pb, and 50 mg Ni. For the  
15 second experiment the total amount fed was 31.2 mg Cd, 312.5 mg Pb and 125 mg Ni. In  
both experiments the plants were watered with neutral water (pH 7.0). The plants were  
harvested after 10 days; roots and shoots separated, and dried. Sample preparation consisted  
of digestion of a known amount of sample following a closed teflon vessel method (Topper,  
1990). The samples were analyzed using a Varian Spectra AA Atomic Absorption  
20 Spectrophotometer in our laboratory. The data is presented in Table 3.

Based on the results from this experiment (data presented in Table 3), we have  
observed that scented geranium plants are capable of tolerating a complex mixture of toxic  
metals and can accumulate a wide variety of metals simultaneously. The treated plants  
25 accumulated greater than 207 mg Cd + 206 mg Pb + 451.5 mg Ni per kg dry weight of the  
shoot tissue, as well as 1,111 mg Cd + 27,803.5 mg Pb + 1,385 mg Ni per kg dry weight of  
the root tissue.

**Table 3. Metal uptake in Frensham scented geranium plants treated with a mixture of heavy metals (metal mixture composed of lead nitrate, cadmium nitrate and nickel nitrate at different concentrations; Cd3+Pb3+Ni3 or Cd9+Pb9+Ni9 combinations). The metal uptake values provided are average of 2 plants per treatment. Concentrations (mg/L) refer to treatment of plants with the described concentrations during 10 day period.**

<b>Metal</b>	<b>Concentration mg/L</b>	<b>Metal uptake (Expt. I) mg/kg DW</b>	<b>Metal</b>	<b>Concentration mg/L</b>	<b>Metal uptake (Expt. II) mg/kg DW</b>	<b>Average</b>
<b>Shoots</b>						
<b>Cd 3</b>	<b>10</b>	<b>153</b>	<b>Cd 9</b>	<b>25</b>	<b>261</b>	<b>207</b>
<b>Pb 3</b>	<b>100</b>	<b>150</b>	<b>Pb 9</b>	<b>250</b>	<b>262</b>	<b>206</b>
<b>Ni 3</b>	<b>40</b>	<b>328</b>	<b>Ni 9</b>	<b>100</b>	<b>575</b>	<b>451.5</b>
<b>Roots</b>						
<b>Cd 3</b>	<b>10</b>	<b>913</b>	<b>Cd 9</b>	<b>25</b>	<b>1309</b>	<b>1111</b>
<b>Pb 3</b>	<b>100</b>	<b>9760</b>	<b>Pb 9</b>	<b>250</b>	<b>45847</b>	<b>27803.5</b>
<b>Ni 3</b>	<b>40</b>	<b>1344</b>	<b>Ni 9</b>	<b>100</b>	<b>1427</b>	<b>1385.5</b>

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**Experiment 7 - Scented Geranium Plants Tolerance to Cadmium, Lead and Nickel (high Cd, Pb and Ni concentrations)**

Two separate experiments were conducted with replicate cutting propagated plants of Frensham scented geraniums to assess their ability to tolerate varying levels of cadmium,

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lead and nickel. Five levels of cadmium, lead and nickel were selected for treating the scented geranium: [0, 6.25, 12.5, 18.75, 25 mg of cadmium nitrate; or 0, 25, 37.5, 50, 62.5 mg of lead nitrate; or 0, 6.25, 12.5, 18.75, 25 mg of nickel nitrate dissolved in 50 mL water per plant]. The ability of scented geranium to tolerate excess metal ions, was ascertained by comparing its chlorophyll *a* fluorescence kinetics to two well established hyperaccumulators namely indian mustard (*Brassica juncea*) and sunflower (*Helianthus annuus*). The fluorescence measurements were made on 3 leaf samples, for a total of 9 measurements per treatment per experiment in scented geranium and 2 leaf samples, for a total of 4 measurements per treatment for each indian mustard and sunflower. The Fv/Fm ratio was recorded on the same leaves on alternate days over the 14-day treatment period for scented geranium and up to 7 and 5 days for indian mustard and sunflower, respectively, with a portable fluorometer (Plant Efficiency Analyzer, Hansatech, England). The data is presented below in Table 4.

Based on the results from these experiments (data presented in Table 4), we have observed that Frensham scented geraniums are highly tolerant to cadmium, lead and nickel compared to both indian mustard and sunflower. This was evident from the Fv/Fm ratio, a non-destructive indicator of the metabolic status of plants. The Fv/Fm ratio decreased from 0.82 to 0.43 (after 14 days) in scented geraniums, compared to a decline in the ratio from 0.80 to 0.02 (within 7 days) for indian mustard and 0.81 to 0.03 (within 5 days) for sunflower. These results confirmed that scented geraniums are metabolically active, while indian mustard and sunflower were physiologically restricted at the highest metal levels tested, as an Fv/Fm ratio of less than 0.3 is indicative of senescing and dead tissue.

**Table 4. Chlorophyll *a* fluorescence (Fv/Fm ratio) in Frensham scented geraniums, indian mustard and sunflower plants treated with cadmium, lead and nickel for a 14, 7 and 5 days, respectively. (The Fv/Fm ratio are average of 9 measurements for scented geranium and 4 measurements each for indian mustard and sunflower, recorded on alternate days on the same leaves. Ratio was not collected for indian mustard and sunflower beyond 7 and 5 days, respectively because these plants were physiologically dead after these periods)**

Cd (1000mg/L)	Fv/Fm ratio		
	Scented geraniums	Indian mustard	Sunflower
Day 1	0.804	0.794625	0.8165
Day 3	0.769	0.580125	0.252475
Day 5	0.738	0.089875	0.026
Day 7	0.640	0.032	
Day 9	0.623		
Day 11	0.579		
Day 13	0.553		
Day 15	0.482		
Pb (2500mg/L)			
Day 1	0.824	0.806625	0.808125
Day 3	0.813	0.523625	0.225625
Day 5	0.801	0.322	0.031
Day 7	0.758	0.03725	
Day 9	0.673		
Day 11	0.656		
Day 13	0.591		
Day 15	0.500		
Ni (1000mg/L)			
Day 1	0.825	0.797375	0.82075
Day 3	0.774	0.348875	0.296375

<b>Day 5</b>	<b>0.754</b>	<b>0.060875</b>	<b>0.02625</b>
<b>Day 7</b>	<b>0.741</b>	<b>0.02125</b>	
<b>Day 9</b>	<b>0.663</b>		
<b>Day 11</b>	<b>0.590</b>		
<b>Day 13</b>	<b>0.523</b>		
<b>Day 15</b>	<b>0.429</b>		

### Experiment 8 - *Pelargonium* sp. Grown in Various Media

Different plant growth media, such as different soil types, artificial soil mix, and hydroponics which contain one or more of the metal ions, can be used for growth of scented geraniums. Scented geraniums can be used for both phytoextraction and rhizofiltration processes, based on the growth medium type. In case of soil or artificial soil mixes, the process would be phytoextraction, while using a hydroponic growth medium the process would be a rhizofiltration process. We have successfully grown scented geranium plants in both the soil and the hydroponic systems without any differences in their growth habit, and their metal uptake potential.

Citrosa scented geranium and Frensham scented geranium (*Pelargonium* sp. 'Citrosa' and *Pelargonium* sp 'Frensham') plants were planted directly in soils contaminated with low concentrations of lead and copper (the soil also contained hydrocarbon contamination). The levels of contamination were 167.12 ppm of copper and 232.8 ppm of lead, and approximately 40,000 ppm of hydrocarbons. Plants (12 replicates) were harvested on a weekly basis after 2, 3, 4, 5 weeks of growth in the contaminated soils; roots and shoots separated, washed and dried. Sample preparation consisted of digestion of a known amount of sample in a closed teflon vessel method (Topper, 1990). The samples were analyzed using a Varian Spectra AA Atomic Absorption Spectrophotometer in our laboratory.

Based on the results from this experiment (data presented in Table 5), we have observed that scented geranium plants (Frensham and Citrosa scented geraniums) can tolerate a mixture of contamination (heavy metals and hydrocarbons) and they can accumulate greater than 36 mg of copper, 23 mg of lead per kg dry weight of shoot, as well as greater than 35 mg of copper, and 14.9 mg of lead per kg dry weight of the root tissue, under field conditions. Both types of scented geraniums accumulated metal ions (lead and copper) in a linear fashion (metal content in root and shoot increasing with increasing duration of growth in the contaminated soil) even after 5 weeks of growth.

**Table 5. Metal uptake by scented geranium plants (*Pelargonium* sp. 'Frensham') grown on contaminated soil containing low-concentrations of lead and copper. The results are average of 12 replicate plants each week starting at week 2 after planting.**

**Scented geranium shoots**

<b>Time (weeks)</b>	<b>Copper (mg/kg DW)</b>	<b>Lead (mg/kg DW)</b>
<b>Week 2</b>	<b>19.8</b>	<b>12.8</b>
<b>Week 3</b>	<b>20.99</b>	<b>14.00</b>
<b>Week 4</b>	<b>38.66</b>	<b>16.73</b>
<b>Week 5</b>	<b>36.04</b>	<b>23.67</b>

**Scented geranium roots**

<b>Time (weeks)</b>	<b>Copper (mg/kg)</b>	<b>Lead (mg/kg)</b>
<b>Week 2</b>	<b>12.9</b>	<b>9.43</b>
<b>Week 3</b>	<b>28.36</b>	<b>9.41</b>
<b>Week 4</b>	<b>35.94</b>	<b>14.42</b>
<b>Week5</b>	<b>34.36</b>	<b>14.9</b>

### **Experiment 9: Beauty Oak Scented Geranium Plants and Metal Uptake from Mixture of Metal Contaminants (Lead, Cadmium and Nickel)**

An experiment was conducted with replicate cutting-propagated plants of Beauty Oak  
5 scented geraniums to assess their ability to uptake and sequester metals in the shoots and  
roots, when treated with lead, cadmium and nickel, alone and in combination. Solutions  
containing 50.0 mg of  $\text{Pb}(\text{NO}_3)_2$  + 10.0 mg of  $\text{Cd}(\text{NO}_3)_2$ , and 10.0 mg of  $\text{Ni}(\text{NO}_3)_2$  were used  
alone and combined for treating the scented geraniums plants; the metals were provided as  
lead, cadmium and nickel nitrates dissolved in 100 ml of half-strength Hoagland's fertilizer  
10 solution (lacking the micronutrients) per plant, applied as needed over a 14 day period.  
Replicate plants were used for each treatment. The total lead, cadmium and nickel nitrate  
fed to each plant over the whole treatment period were 350 mg Pb, 70 mg Cd, and 70 mg Ni.

The plants were harvested after 10 days; roots and shoots were separated and dried.  
15 Sample preparation consisted of digestion of a known amount of sample following a closed  
Teflon vessel method (Topper, 1990). The samples were analyzed using a Varian Spectra  
AA Atomic Absorption Spectrophotometer in our laboratory.

Based on this experiment and data presented in Table 6, we have observed that  
20 another variety of *Pelargonium Sp.*, Beauty Oak scented geranium plants are capable of  
tolerating individual as well as a complex mixture of toxic metals and can accumulate a wide  
variety of metals simultaneously. The treated plants accumulated greater than 65379 mg Pb,  
3948 mg Cd and 7350 mg Ni per kg dry weight of root tissue when metals were applied  
separately, and 68018 mg Pb + 48 mg Cd + 118 mg Ni per kg dry weight of root tissue when  
25 metals were applied in combination.

We also assess other varieties of *Pelargonium sp.* for their ability to hyperaccumulate  
metals (Table 7).

**Table 6. Metal accumulation in shoots and roots of Beauty Oak scented geranium plants treated with concentrations of cadmium, lead, and nickel (in nitrate form) alone and in combination. The shoot and root uptake levels represent an average of 4 replicates per treatment. Concentrations (mg/L) refer to treatment of plants with the described concentrations over a 14 day period. (DW = Dry weight of tissue)**

<b>Heavy Metal</b>	<b>Concentration (mg/L)</b>	<b>Shoot Uptake (mg/kg DW)</b>	<b>Root Uptake (mg/kg DW)</b>
<b>Control</b>	<b>0</b>	<b>0</b>	<b>0</b>
<b>Pb</b>	<b>500</b>	<b>209.4</b>	<b>65379.9</b>
<b>Cd</b>	<b>100</b>	<b>17.1</b>	<b>3948.1</b>
<b>Ni</b>	<b>100</b>	<b>81.7</b>	<b>7350.2</b>
<b>Pb + Cd + Ni</b>	<b>500 + 100 + 100</b>	<b>51.0 + 4.0 + 57.0</b>	<b>68018.1 + 48.9 + 118.4</b>

**Table 7 - Pelargonium sp. Plant Patents**

Patent No.	Variety Name	Patent No.	Variety Name	Patent No.	Variety Name
USPP007656	315-(83-48-1)	USPP009534	BFP-825 Salmon Rose	USPP008523	Designer Salmon
USPP007620	Flair	USPP009535	BFP-901 Bright Red	USPP008522	Designer Coral
USPP007538	Fantasy	USPP009237	Klehis	USPP008382	Klepelwa
USPP007467	Allure	USPP009231	Pink Pearl	USPP008296	BSR-100B Dark Salmon cultivar
USPP007387	Majestic	USPP009228	BFP-445 Salmon	USPP008288	BSR-233 Bright Coral cultivar
USPP007343	Crystal	USPP009230	BFP-285 Pink Parfait	USPP008287	BSR-284 Pink cultivar
USPP010396	Pink Passion	USPP009229	Starburst Red	USPP008286	BSR-194 Cherry cultivar
USPP010139	Sunrise	USPP009217	BFP-721 Bright Lilac	USPP008285	BSR-232 Light Scarlet cultivar
USPP010399	BFP-1328 Red	USPP009219	BFP-420 Bright Red	USPP008284	BSR-177 White cultivar
USPP010395	Purple Rose	USPP009218	Pink Heart	USPP008089	Jubilee
USPP010398	BFP-484 White	USPP009215	Klespri	USPP007627	208(81-344-3)
USPP010397	BFP-1409 Light Salmon	USPP009216	Kleirro	USPP007576	Centennial
USPP009596	BFP-790 Pink Parfait	USPP009204	Klegoes	USPP007351	821-(82-116-13)
USPP009589	BFP-864 Bright Lavender	USPP009206	Klevette	USPP006761	Serenade
USPP009590	BFP-873 Bright Red	USPP009205	Klemiga	USPP006717	Feeling
USPP009580	BFP-837 Scarlet	USPP008669	Designer Scarlet	USPP010606	Guilan
USPP009560	BFP-817 Light Salmon	USPP008552	Designer Light Pink	USPP009287	L'amour
USPP009551	BFP-788 Bright Scarlet	USPP008521	Showcase Red	USPP004785	Geranium plant
USPP009552	Lilac Chiffon	USPP008525	Designer Hot Pink	USPP004778	Geranium plant
USPP009544	BFP-838 Dark Red	USPP008524	Designer Rose	/	/

Based on the findings reported here, along with an extensive comparison with published literature on other hyperaccumulators, we have concluded that Scented geraniums (*Pelargonium* sp.) are hyperaccumulators of heavy metals (lead, cadmium, copper, nickel, chromium etc.) and can be used for phytoremediation of metal-tainted soils. The data obtained so far utilizing this process satisfies the EPA's (US Environmental Protection Agency) set goal for metal hyperaccumulators, which is "... absorb, translocate and tolerate levels of metals in the 0.1 to 1.0 percent range....".

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All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety. This application claims priority from U.S. application no. 60/064,238, which is incorporated by reference in its entirety.

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The present invention has been described in terms of particular embodiments found or proposed by the present inventors to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. All such modifications are intended to be included within the scope of the appended claims.

**WE CLAIM:**

1. A method for removing one or more species of metal from a growth medium, comprising growing a *Pelargonium* sp. plant in the growth medium for a time period sufficient for the plant root to uptake and accumulate metal in the root or shoot biomass.
2. A method according to claim 1, wherein the *Pelargonium* sp. plant is selected from *Pelargonium* sp. 'Frensham', *Pelargonium* sp 'Citrosa' and *Pelargonium* sp 'Beauty Oak'.
3. A method according to claim 2, wherein the *Pelargonium* sp. plant is selected from *Pelargonium* sp. 'Frensham', and *Pelargonium* sp 'Beauty Oak'.
4. A method according to claim 3, wherein the *Pelargonium* sp. plant is *Pelargonium* sp. 'Frensham'.
5. A method according to claim 1, wherein the growth medium comprises solid medium, semi-solid medium, liquid medium or a combination thereof.
6. A method according to claim 5, wherein the growth medium comprises soil, sand, sludge, compost, or artificial soil mix.
7. A method according to claim 1, wherein the metal is selected from one or more members of the group consisting of lead, cadmium, copper, nickel and zinc.
8. A method according to claim 1, wherein the metal is selected from one or more members of the group consisting of antimony, boron, vanadium, chromium, cobalt, manganese, selenium, arsenic, molybdenum, beryllium, barium, mercury, silver and antimony.
9. A method according to claim 1, wherein the metal is selected from one or more members of the group consisting of titanium, thallium, tin, gold, rubidium, strontium, yttrium, technicium, ruthenium, palladium, indium, cesium, uranium, plutonium and cerium.
10. A method according to claim 5, wherein the growth medium comprises organic contaminants selected from a group consisting of petroleum industry by-products and petroleum industry wastes.
11. A method according to claim 1, wherein the metal comprises cadmium accumulated at

- a concentration of about 450 mg Cd/kg to 27,500 mg Cd/kg dry weight of the plant.
12. A method according to claim 1, wherein the metal comprises lead accumulated at a concentration of about 1,300 mg Pb/kg to 70,000 mg Pb/kg dry weight of the plant.
  13. A method according to claim 1, wherein the metal comprises nickel accumulated at a concentration of about 400 mg Ni/kg to 21,500 mg Ni/kg dry weight of the plant.
  14. The method of claim 1, wherein one or more parts of the plant selected from the shoot and root biomass is harvested
  15. A method according to claim 14, wherein the shoot biomass comprises a leaf or a stem.
  16. A method according to claim 14, wherein a sufficient portion of the shoot biomass is not harvested to permit continued plant growth.
  17. A method according to claim 1, wherein one or more parts of the plant selected from the root or shoot biomass is harvested and essential aromatic oil is extracted from the root or shoot biomass.
  18. A method according to claim 17, wherein essential aromatic oil is obtained by distillation.
  19. A method according to claim 18, wherein the essential aromatic oil is selected from a group consisting of citronellol, geraniol, iso-methane and geranyl formate.
  20. The method of claim 1, wherein one or more parts of the plant selected from the root and shoot biomass is harvested and metal in the root or shoot biomass is concentrated.
  21. A method according to claim 20, wherein concentration of metal is carried out by a method selected from air drying, dehydrating, ashing, incineration, smelting, aerobic digestion and anaerobic digestion of the residual oil-extracted shoot biomass
  22. The method of claim 1, wherein *Pelargonium* sp. is transformed with a gene sequence which enhances the plants' ability to uptake metals.
  23. The *Pelargonium* sp. of claim 22, transformed by an *Agrobacterium* strain.
  24. A *Pelargonium* sp. cell, tissue culture, plant, seed or plant part including a recombinant metallothionein gene or phytochelatin synthase gene or a gene that is biologically functionally equivalent to the metallothionein gene or the phytochelatin synthase gene.