ing the extent of intra- and inter-specific genetic diversity existing in *N. tabacum* and *N. rustica*. The species and genus-specific AFLP markers identified in this study would be useful in introgression breeding programmes of tobacco.

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Differential accumulation of manganese in three mature tree species (Holoptelia, Cassia, Neem) growing on a mine dump

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Three trees, including *Cassia siamea* (Cassia), *Azadirachta indica* (Neem), *Holoptelia integrifolia* (Holoptelia) belonging to three different families were identified from a manganese mine tailing dump. Manganese content in dump soil and in the stem, green leaves and dry, fallen leaves of the plants was determined. Values were compared with similar samples collected from normal vegetation. Under control condition, manganese content was highest in Cassia. Distribution of metal in samples collected from the dump site revealed that Holoptelia has a special ability to accumulate high amounts of manganese under stress condition followed by Cassia and Neem. There is no literature on metal accumulation in Holoptelia. Mechanism of manganese sequestration in Holoptelia is different from the other two trees growing in the same soil.

Keywords: *Azadirachta indica*, *Cassia siamea*, *Holoptelia integrifolia*, hyperaccumulator, manganese, mine dump.

MANGANESE (Mn) is a trace element found in varying amounts in all tissues and is among the mostly used elements in the industry. It is an essential micronutrient and activator for enzymes involved in tricarboxylic acid cycle. However, Mn is toxic when in excess and consequently it represents an important factor in environment contamination and causes various phytotoxic effects[1].

Phytoremediation is an environmental clean-up strategy in which selected green plants are employed to remove, contain or render environmentally toxic contaminants harmless. This is an emerging biotechnological application and operates on the principles of biogeochemical cycling[2]. This remediation approach is attracting attention from various governments as a cost-effective and environment-friendly green technique to clean-up heavy metal polluted soil using hyperaccumulators[3]. The generation of scientific information on heavy metal-accumulating plants is so extensive that in the last decade a commercial industry has been developed for the application of phytoextraction to restore heavy metal-contaminated sites[4].

Most experimental studies of heavy metal tolerance confirm that populations growing in metal-contaminated

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habitats are different from those growing in clean sites of the same species by possessing genetically based tolerance[5]. Some plants that grow on naturally metal-contaminated soils may adapt and develop to survive and accumulate greater concentration of heavy metals in their shoots than other plant species[6]. High accumulation of manganese uptake in roots, stem and leaves of *Phytolaccia acinosa* populations has been shown[7].

Over the past 10 years, woody plants have been shown to be excellent candidates for phytoremediation, due to rapid growth, high biomass, profuse root apparatus and low impact on the food chain and human health[8,9]. Majority of such work concerns accumulation capacity and biomass production of woody plants as a response to high concentration of pollutants[10]. Phytoremediation using trees provides a potential opportunity to extract or stabilize metals. It involves the use of trees that readily transport targeted metals from soil to plant organs, which allows removal of metal by harvesting from the plant. This process takes longer time but helps in the greening of the land and in reducing pollution[11]. However, there is need to identify trees having the ability to uptake and translocate the metal to the aerial parts. The ability of five woody species, including *Alnus*, *Fraxinus*, *Sorbus*, *Salix* and *Betula* from polluted soil to their above-ground tissues was studied[12]. In a recent study[13], interaction of calcium, with copper and cadmium accumulation in roots and stem of Norway spruce (*Picea abies* L.) has been demonstrated. However, little is known about uptake, accumulation and detoxification of heavy metals, especially about Mn in woody plants[14].

In the present investigation, three naturally growing tree species, including *Cassia siamea* (Cassia), *Azadirachta indica* (Neem) and *Holoptelia integrifolia* (Holoptelia) were identified on a manganese mine dump. An experiment was conducted to generate information on Mn accumulation and distribution in these trees. The data were compared with those generated from samples collected from the trees growing in natural vegetation in contamination-free soil. The dry, fallen leaves of the respective trees were collected from the ground under the trees and were analysed for Mn content.

The manganese mine is located in Gumgaon, Maharashtra. We identified three plants, including Holoptelia, Cassia and Neem growing naturally in the mine dump where sorting of the ore is being carried out (Figures 1 and 2). This was the only Holoptelia growing in the mining area, whereas several plants of Cassia and Neem could be seen growing naturally on the dump. The three plants identified for our study are within an area of approximately 400 sq. m. Leaves and stems (woody twigs) of these trees were collected for metal analysis. The dry leaves from the ground under these trees were also analysed. Similar samples were collected from the trees growing locally in normal soil for control. Soil samples collected from the dump and the normal vegetation were assessed for Mn content. The colour of the soil collected from the normal vegetation and from the dump site was compared.

Dust accumulated on the surface of the leaves and twigs was removed by washing thoroughly with tap water. This was followed by thorough washing of the samples with deionized water. The samples were then dried on a filter paper to eliminate adhering moisture from the surface. The same process was repeated for the control leaves and stem of all three species. Fresh leaves and stem of Holoptelia, Cassia and Neem were taken (1–3 g) in pre-weighed glass beakers of 50 ml volume. Fresh weights of the tissues were determined from the difference in weights. Samples were dried in a oven at 100°C and weighed intermittently until constant weight. Dry weight (DW) per gram of fresh weight (FW) was determined. Dried plant materials were ground in mortar and pestle to a fine powder for Mn analysis.

Manganese estimation was carried out following the method of Shraddha *et al.*[15]. In brief, the dried powder

Figure 1. Trees growing on the manganese mine dump. ***a***, *Holoptelia integrifolia*; ***b***, *Cassia siamea*.

Figure 2. Trees growing on the manganese mine dump. ***a***, *Azadirachta indica*.

(150 mg) of plant tissues was taken in Borosil vials. These were digested with 3 ml of nitric acid and 1 ml of 70% perchloric acid on a hot plate under the hood. Digested sample solution was made to 10 ml volume with deionized water. Mn content in these samples was determined using Atomic Absorption Spectroscopy (Perkin Elmer 1100B). Mn content was calculated in μg per g of dry tissue.

The pH of the soil samples was determined following the method of Sparks *et al.*[16]. In brief, samples collected from the dump site and local vegetation were air-dried at room temperature. Soil samples were taken in glass beakers and after adding distilled water were kept on a shaker for 1 h. On removing from the shaker, these were kept stationary for a period of 1 h for the suspended particles to settle down. The pH of the solution was measured using a pH meter (Model 420A, ORION).

Manganese content in the soil sample was determined using the method described in *Lab Procedures*[17]. Moisture-free samples were ground with mortar and pestle to make a fine powder and the powder was sieved. In brief, 1 g of sieved soil was taken in a test tube and 4 ml of each extracting solution (0.05 N HCl and 0.025 N H_2SO_4) was added and kept on shaker for 15 min. The solution was filtered through Whatman No. 42 filter paper and the volume was made up to 10 ml with extracting solutions. Mn content was determined by Atomic Absorption Spectroscopy.

The aim of the present study was to characterize some of the tree species that naturally colonized in Mn mine tailings. The objective was to assess these plants for their ability to uptake and accumulate Mn in the different organs. The tree species, including Holoptelia, Cassia and Neem, do not appear in the list of the known hyperaccumulators. The Mn hyperaccumulators (>10,000 μg per g) are from the families Apocynaceae, Celastraceae, Clusiaceae, Myrtaceae and Proteaceae[18–20]. In addition, mention may be made of *Eleutherococcus* (formerly *Acanthopanax*) *sciadophylloides* (Araliaceae) from Japan[21], which can accumulate Mn up to 7900 μg per g in leaf dry matter. Some plants that grow on naturally metal-contaminated soils may adapt and develop to survive and accumulate much greater concentrations of heavy metals in their shoots than other plant species[6]. Accumulation of manganese in leaf mesophyll of four tree species, *Gossia bidwillii*, *Virotia neurophylla*, *Macadamia integrifolia* and *Macadamia tetraphylla* has been reported[22].

The pH of the dump soil was 8.84 compared to 7.37 of the control soil and Mn content in the soil of the tailing dump was 1296.33 μg per g. This is approximately 44 times higher than the control soil (Table 1). The colour of the soil from the contaminated site and normal site varied distinctly (Figure 3). Comparison of Mn content (Table 2) in the organs of three tree species of local vegetation in Pune and samples from trees growing on Mn tailing dump, revealed the following.

(i) Manganese content in the samples of leaves and twigs collected from the tailing dump was higher than in the control. This indicates accumulation of Mn in all the plants growing on the tailing dump. Although all the three plants are growing under identical conditions, Mn content in their organs varied. This suggests that the mechanisms of Mn uptake and sequestration in these three plants may be different.

(ii) In the leaf tissues Mn content was significantly higher compared to the twigs irrespective of location of the plant, thereby maintaining a gradient between these two organs. This is possibly due to deposition of Mn in the leaves. The three trees under study have been growing in the high Mn-containing medium for several years, but Mn is not evenly distributed in the organs. There is a need to conduct detailed studies to understand the process of transfer of Mn from the twigs to the leaves to maintain the gradient.

(iii) Among the samples collected from normal vegetation, Mn content varied in the three species (Table 2). Mn content in this soil was 29.27 ± 1.55 μg per g. Cassia showed highest Mn content followed by Neem and Holoptelia in the leaves and twigs respectively. The values are several fold higher than the concentration of the metal in the soil. Further studies are needed to determine if these are the required concentrations in the organs for their normal functioning. The varying amounts of Mn in the organs of the three plants under normal and identical conditions indicate that the ability of a plant to uptake and accumulate Mn differs from species to species.

(iv) Among the three trees in the dump site, Mn content was highest in the tissues of Holoptelia (Table 2). This was

Table 1. Manganese content and pH in soil of dump site and normal vegetation

Soil	Mn (μg per g)[†]	pH
NCL (control)	29.27 ± 1.55	7.37
Mn tailing dump	1296.33 ± 102.89	8.84
NCL : Mn dump	Ratio is 1 : 44	
t-test	S1%	

[†]Mean of three repeats.

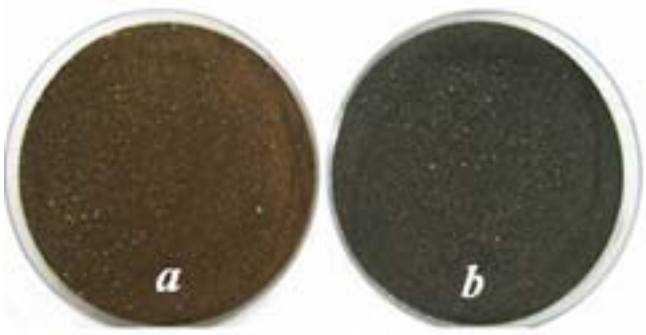

Figure 3. ***a***, Sample of normal soil; ***b***, Sample of soil from Mn mine dump.

Table 2. Distribution of manganese (μg per g) in leaf and stem of Holoptelia, Cassia and Neem

Tree		Leaf (A)†	Stem (B)†	*t*-test (A and B)	Dry fallen leaves†
Holoptelia	Control (a)	168.59 ± 57.20 (11)	98.78 ± 41.70 (6)	S-5%	143.12 ± 8.50 (6)
	Dump (b)	1744.06 ± 539.46 (11)	1248.15 ± 268.3 (9)	S-5%	2682.19 ± 94.11 (6)
	t-test (a and b)	S-1%	S-1%		S-1%
Cassia	Control (a)	437.56 ± 144.9 (9)	248.88 ± 13.34 (3)	S-1%	185.58 ± 32.1 (6)
	Dump (b)	1199.35 ± 296.9 (9)	642.96 ± 123.52 (3)	S-1%	2852.50 ± 131.7 (6)
	t-test (a and b)	S-1%	S-5%		S-1%
Neem	Control (a)	286.86 ± 122.6 (9)	174.78 ± 20.04 (3)	NS	139.36 ± 15.2 (6)
	Dump (b)	726.60 ± 177.1(9)	626.66 ± 113.40 (3)	NS	2513.30 ± 127.7 (6)
	t-test (a and b)	S-1%	S-5%		S-1%

†Figures in parenthesis indicate number of replicates.

followed by Cassia and Neem. In Holoptelia, the ratio of Mn in leaf samples of control and mine dump respectively, was approximately 1 : 8. In twigs, the ratio was 1 : 13. It appears that under Mn stress condition the uptake of Mn by Holoptelia is more. The optimum capacity of this plant to uptake and accumulate Mn needs to be determined by designing appropriate experiments.

(v) Uptake of Mn by Cassia in the dump site was more (1199.35 μg per g) as compared to the level of Mn in control (437.5 μg per g), but it was not as high as in Holoptelia. However, the ratio of Mn content between the control and dump site soil sample was 1 : 44 (Table 1), whereas the ratio between the control and dump site samples of both leaves and twigs of Cassia was only 1 : 3. Thus the amount of Mn accumulated in the two organs of Cassia may be the optimum for this plant. The mechanism of Mn tolerance in this plant is possibly different from that in Holoptelia.

(vi) In Neem leaf and twig samples from the control soil, Mn content was higher (Table 2) than in Holoptelia, whereas the amount in the organs of plants in the dump site was less than in Holoptelia. We presume that the amount of Mn detected in the organs of plants in normal soil is that required for their normal functioning and growth. In Mn-rich soil, uptake of the metal in each plant is according to its own specific ability.

(vii) Comparison of data generated from the three trees growing on the dump site (Table 2) suggests that Holoptelia has a special ability to accumulate higher amounts of Mn under stress condition. Till date there is no report on metal accumulation in this species. Further studies will reveal more information on its optimum ability to accumulate Mn and also other metals.

(viii) The metal contents in the mature fallen leaves of all three trees were estimated (Table 2). In the fallen leaves collected from trees growing in normal soil, Mn content was less than in their green counterparts. This observation cannot be explained with the present knowledge. It is assumed that the amount of Mn required by the plant organ for its normal function is taken up by the plants and is maintained. The cellular activities diminish gradually with aging prior to abscission. Presumably, demand for Mn as co-factor for cellular processes is reduced in the maturing tissues, causing mobilization and transfer of the metal to the more active organs, thereby resulting in reduction of Mn in the dry, fallen leaves. Foliar Mn sequestration in *Gossia bidwillii* (Myrtaceae), a species discovered relatively recently to be Mn-hyperaccumulating[23], has been shown to occur in the photosynthetic tissues[22]. A possible association of reduction in Mn in aging leaves with reduction in photosynthetic activity requires further investigations. The pattern of Mn content in dry leaves of the three species was similar to those in green leaves; Cassia showing the highest amount followed by Holoptelia and Neem respectively.

(ix) Unlike the dry leaves of plants from normal soil, in dry fallen leaves of the dump site Mn content was higher than in the green counterparts. In view of our assumption regarding reduced Mn in matured leaves of control plant, in the dump site there is unlimited supply of Mn from the soil. Thus, Mn from the aging leaves need not move to the more active parts, leaving the Mn content unaltered in the dry leaves. Due to optimum accumulation of Mn in leaves prior to abscission, Mn content is higher in the dry leaves than in the green leaves. Abscission of the leaves with high Mn may be the mechanism of these plants to eliminate excess Mn from the system. In Cassia, the ratio of Mn content between control and dump site fallen leaves was approximately 1 : 15 (Table 2). In Holoptelia, the ratio was approximately 1 : 19 times in fallen leaf samples (Table 2). In Neem, it was approximately 1 : 18. When all the three values are compared, they are almost similar.

All plants take up metals to varying degrees from the substrates in which they are rooted[24]. The flora of metal-contaminated sites is typically impoverished in comparison with that of the surrounding vegetation and populations of plants growing there are often genetically distinct from those of the same species in the adjacent location with soil of low heavy-metal content[25]. Moreover, the level of tolerance developed can often be related to the amount of metal in the soil[26].

There is evidence from natural establishment of trees on contaminated sites that some types of trees can survive under such adverse conditions, e.g. *Salix* (willow), *Betula*

(Birch), *Populus* (Poplar), *Alnus* (Alder) and *Acer* (Sycamore). The main characteristics of trees that make them suitable for phytoremediation is their large biomass, both above and below ground level. Physical phytostabilization can be readily achieved, and is often the main benefit of using trees on such sites. Vegetation of tree species helps in decreasing the risk of soil, water and wind erosion. Phytoremediation and especially the use of trees is an emerging and developing technology, and this has grown rapidly in recent years[27]. From the present study it may be concluded that accumulation of Mn in the three tree species and in their different parts varies. This could be due to differences in the mechanisms of uptake and sequestration of this metal in different plant systems[28]. These three trees are from different families. However, all three plants have the ability to thrive on Mn-rich dump. This common characteristic makes them suitable for restoration of Mn-contaminated sites, but all three may not be suitable for phytoremediation. The aim in a phytoremediation programme is to reduce toxic metal from the soil. Among the three trees tested, Holoptelia has the ability to take up more metal. Thus this plant is more suitable for removal of Mn from the soil. Further studies using higher concentrations of Mn need to be carried out to determine whether Holoptelia is a hyperaccumulator. The differential accumulation of Mn in the three tree species demands further studies to determine the mechanism of Mn tolerance in them.

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