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Impact of Metals on Secondary Metabolites Production and Plant Morphology in Vetiver Grass (*Chrysopogon zizanioides*)

Funzani A. Melato, Thierry Regnier, Rob I. McCrindle and Ntebogeng S. Mokgalaka*

Department of Chemistry, Tshwane University of Technology, Private Bag X680, Pretoria, 0001, South Africa.

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ABSTRACT

The impact of selected metals on the production of phenolic compounds was investigated in a pot trial experiment. One-month-old vetiver grass (*Chrysopogon zizanioides)* seedlings were exposed to different concentrations (0, 10, 50, 100, and 500 ppm) of As, Cr, Cu, Fe, Ni, Pb and Zn. All the plants except for those treated with As tolerated up to 500 ppm as they did not show any signs of stress such as wilting or necrosis. A significant decrease (>35 %) in the length of the plants treated with As, compared to the control, was observed at 50 ppm which further decreased with increasing As concentration. A serious case of phytotoxicity was observed at 500 ppm As as the plant could not survive. Total soluble phenolics content in vetiver plants increased with increasing concentration of metals in the growth medium. The amount of the cell wall-bound phenolics (2.01 to 5.84 mg GAE g^{-1} DW) was higher than the total soluble phenolics (1.13 to 2.14 mg GAE g^{-1} dry weight DW) and both increased with increasing metal concentrations. Morphological changes associated with metal-induced stress were also examined with a scanning electron microscope which revealed thickened cell walls, loss of cell shape, reduction of intercellular space and the closure of stomata in leaves of metal-exposed plants.

KEYWORDS

Vetiver grass, toxic metals, phytotoxicity, phenolic compounds, morphological changes.

1. Introduction

Environmental pollution has become a major concern which requires immediate attention. Large areas of agricultural land are contaminated with toxins which primarily emanate from human activities such as application of agricultural amendments, emissions, sewage and waste disposal, mining and smelting processes.[1] These processes release toxic metals, which could have adverse effects on humans, plants and the environment.[2] High density and the non-biodegradable nature of metals make them distinguishable from other toxic pollutants that accumulate in living tissues. Certain metals at low concentrations are essential nutrients and take part in redox reactions, electron transfers and other essential metabolic processes in plants;[3] however, high doses those metals can be toxic. Metals with no biological functions can be toxic to plants and microorganisms even at low concentrations.[4]

The adaptive responses of plants to metal contaminated environments include many physiological, molecular, genetic and ecological traits. These traits give certain plants the ability to adapt, detoxify or hyperaccumulate toxic metals.[5] Uptake and accumulation of toxic metals in plant tissues induces major changes in plants at genetic, biochemical and physiological levels, sometimes leading to phytotoxicity.[6] Phytotoxicity can affect the development and health of plants by inhibiting vital processes such as photosynthesis, mitosis and water absorption.

Plants may undergo significant morphological and metabolic changes in response to metal uptake. Many of these changes are believed to be adaptive responses to metal stress.[5,7] Visible symptoms of metal toxicity in plants include the expression of metal-induced alterations at structural and ultrastructural levels. These changes at cell, tissue and organ levels are the results of a direct interaction of the toxic metals with structural components at these sites.[7] Additional consequences of phytotoxicity are enhanced production of reactive oxygen species (ROS) and oxidative damage of important macromolecules including DNA, protens, lipids, chloroplast pigments and enzymes.[8] Plant damage occurs when the ability of antioxidant processes and detoxification mechanisms are lower than the amount of ROS production[3]. Plants produce a wide range of secondary metabolites such as phenolic compounds and polyphenols; however, enhancement of their metabolism can be observed under different environmental factors and stress conditions.[9,10] An increase in phenolic compounds correlated to the increase in activity of enzymes involved in phenolic compounds metabolism was reported by Michalak,[3] suggesting synthesis of phenolics under metal stress. The increase in phenolic content of *Phaseolus vulgaris*[1] with increasing concentrations of Pb in the growth medium was also reported and thought to prevent oxidative damage by scavenging active oxygen species and by breaking the radical chain reactions during lipid per oxidation.

Phenolic compounds play an important role in protecting plants against biotic and abiotic stress.[10,11] The antioxidant activity of phenolic compounds is mainly due to redox properties. They act as reducing agents, hydrogen donors, singlet oxygen quenchers and have metal chelating potential.[3,12,13] The antioxidant activity of phenolic acids depends on the number of hydroxyl groups in the molecule[12]. Plants utilize different strategies such as exclusion, complexation and cell compartmentation, amongst others, to adapt to metal toxicity.[14]

Metals are taken up through the roots and move to various plant cells including those in the cell walls. Structural and

*To whom correspondence should be addressed. E-mail: mokgalakans@tut.ac.za

ultrastructural studies to establish the extent of morphological changes caused by metal toxicity in plants has been conducted successfully with scanning electron microscopy (SEM).[6,15]

Vetiver grass (*Chrysopogon zizanioides)* is a tall (1–2 m), fast-growing grass with a long (2–3 m), complex root system which can penetrate to the deeper layers of the soil. This deep root system makes the vetiver plant extremely tolerant to drought and very difficult to dislodge when exposed to a strong water flow.[16] Vetiver grass is highly tolerant to hostile soil conditions such as low pH and high salinity.[17] It has been widely used for pollution control, wastewater treatment and many other environmental applications.[18] In this study, to elucidate the mechanism employed by vetiver grass to adapt and tolerate metal-induced stress, the effect of metal toxicity on the production of phenolic compounds, structural and ultrastructural changes caused by accumulation of toxic metals were investigated.

2. Materials and Methods

2.1. Plant Material and Experimental Design

The experiment was conducted in a hothouse under natural conditions in a random block design. One-month-old vetiver grass seedlings used in this experiment were obtained from Hydromulch (Bapsfontein, South Africa). Five different concentrations, *viz.* 0, 10, 50, 100 and 500 ppm of As, Cu, Cr, Fe, Ni, Pb and Zn were prepared from salts and made up in the modified Hoagland's nutrient solution. Approximately 580 g of vermiculite obtained from Hygrotech (Pretoria North, South Africa) was placed in potting bags and used as growth medium. Vermiculite in each potting bag was contaminated with only one metal concentration. Vetiver seedlings of the same age and height were transplanted into the pre-contaminated vermiculite and allowed to grow for four weeks. Each treatment was carried out in triplicate.

At harvest, the growth of each plant was measured from the stem–leaf junction to the apex of the leaf using a measuring tape. The plants were subsequently removed from the soil, washed with tap water, rinsed with 0.1 % HNO_3 to remove any metals that might be on the surface and finally rinsed with distilled water after which they were separated into roots and shoots (above ground biomass). The roots and shoots were then plunged into liquid nitrogen and freeze-dried in a freeze-dryer (Dura Dry μP, FTS Systems, Stone Ridge, USA) for 78 h. The dried leaves were ground, sieved and stored at room temperature until further use. All reagents and solvents were of analytical grade and supplied by Merck Chemicals (Darmstadt, Germany).

2.2. Extraction of Phenolic Compounds

2.2.1. *Total Soluble Phenolics*

Approximately 0.05 g of dried leaves was weighed in triplicate into a 1.5 mL Eppendorf tube and 1 mL of a (4.5: 4.5: 1) dichloromethane: methanol: chloroform mixture was added and vortexed for 1 min. The mixture was then shaken on a Labcon Platform Shaker (Laboratory Marketing Services CC, Maraisburg, South Africa) at 200 rpm for 30 min and centrifuged using a Micro centrifuge (Hemle Labortechnic GmbH, Weingen, Germany) for 3 min at 6000 rpm. The supernatant solution was transferred into a 15 mL centrifuge tube and the extraction was repeated six times. The crude extracts from the six extractions were combined and stored in a fridge at 4 °C for further analysis and the resulting pellet (cell wall) was air dried and stored in a dessicator until further use.

For the determination of the total phenolic content of the extract, the crude extract was reduced by evaporation under nitrogen to 1 mL.

2.2.2. *Isolation of Free Phenolic Acids*

The crude extract was reduced to 4 mL by evaporation under nitrogen and re-adjusted to 5 mL with deionized water. Trifluoroacetic acid (TFA) was added to the solution before partitioning with 5 mL of diethyl ether. The solution was mixed, shaken by hand and the supernatant liquid was removed with a Pasteur pipette into a clean 15 mL centrifuge tube. The extraction was repeated three times and the resultant solution was evaporated to dryness after which 1 mL methanol (80 %) was added to dissolve the free acids.

2.2.3. *Isolation of Cell Wall-bound Phenolics*

The dry pellet (cell wall) that remained after the extraction was weighed and transferred into a 15 mL centrifuge tube and dissolved with 1 mL 0.5 M NaOH. The solution was placed in a water bath for 1 h at 96 °C and then cooled down at 4 °C for 40 min. Concentrated HCl (40 μL) was added to the solution followed by 2 mL of diethyl ether. The mixture was shaken by hand and the supernatant liquid was transferred into a clean centrifuge tube. The extraction was repeated three times and the diethyl ether solution was evaporated to dryness. The phenolic compounds extracted were then resuspended with 1 mL of 80 % aqueous methanol. All samples were subsequently stored at 4 °C before being analyzed using the Folin-Ciocalteu reagent.

2.3. Determination of Phenolic Compounds

Phenolic contents were estimated using the Folin-Ciocalteu colorimetric method based on the procedure developed by Singleton and Rossi,[18] using gallic acid as a standard phenolic compound. An aliquot of 5 μL of the extract was transferred into a 96 wells ELISA microplate (Lasec, Centurion, South Africa) and 175 μL of water, 25 μL Folin Ciocalteu phenol reagent and 50 μL $NaHCO_3$ (20 % m/v) were added. The solvent was used as blank. The plate was incubated in an oven at 35–40 °C for 40 min. The absorbance of the resulting blue-coloured solution was measured at 750 nm using a Spectramax190 microplate reader (Molecular Devices, Sunnyvale, USA). Quantitative measurements were based on a standard calibration curve of gallic acid ranging from 40 mg L^{-1} to 200 mg L^{-1} . Phenolic content was expressed as gallic acid equivalence (GAE) in milligrams per gram (mg g^{-1}) of dry material.

2.4. Scanning Electron Microscopy

A Zeiss field emission gun scanning electron microscope (Zeiss Gemini 55 Ultra Plus FEGSEM, Oberkoche, Germany) was used to study morphological changes due to metal-induced stress. Leaves of a control plant and plants treated with 100 ppm Cr and As were examined with a scanning electron microscope (SEM). Cr and As were chosen as they exhibited the highest toxicity effects. The leaf samples were fixed in a 10 % buffered solution of formalin, dehydrated through a series of graded alcohols up to absolute ethanol, dried in the critical point dryer and sputter coated with a thin layer of carbon and gold. The surfaces of the leaves were examined at 20 μm, cross section of the leaves at 100 μm and the stomata at 2 μm.

2.5. Statistical Analysis

To authenticate validity and to evaluate significant differences between the results, the data was subjected to Single Factor ANOVA.

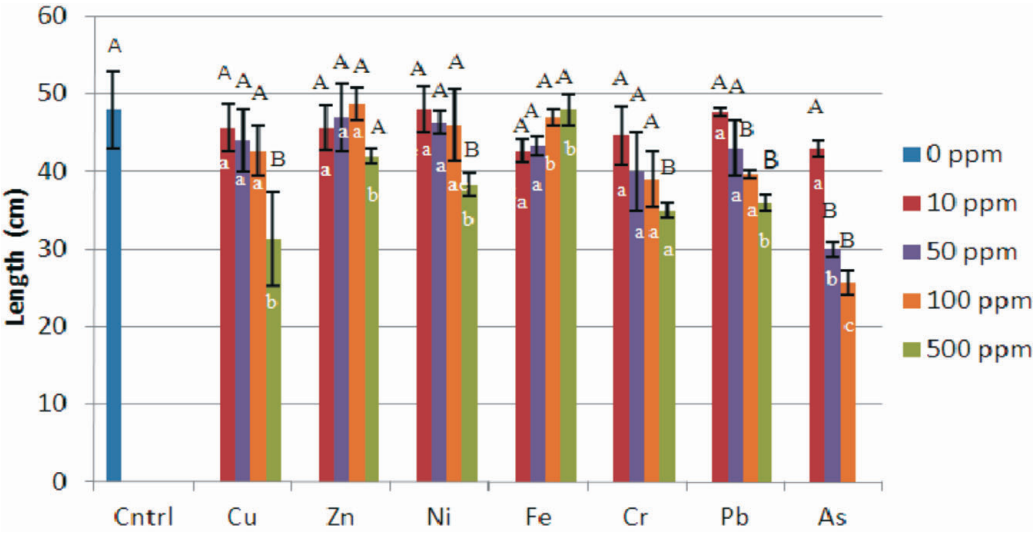

Figure 1 Length of vetiver grass tillers treated with different metal concentrations harvested after four weeks.

3. Results and Discussion

3.1. Plant Growth

The growth of plants was estimated using elongation and the results are shown in Fig. 1. All the plants seemed to grow well at all the concentrations as they did not show any sign of stress when compared to the control. The impact of high metal concentrations manifested at 500 ppm which was revealed by a significant decrease in the length of all the plants under similar treatment, even though the plants seemed healthy and did not show any signs of phytotoxicity.[19] However, the lengths of the plants treated with Fe increased with metal concentrations. Plants growing in 50 ppm As contaminated medium showed a significant reduction ($P < 0.05$) in length compared to plants exposed to other metals of the same concentration and the control. A severe case of phytotoxicity was observed with plants treated with As 500 ppm which could not survive. Similar phytotoxicity effects induced by As were reported on the growth of rice,[20] and Indian Mustard (*Brassica juncea*).[18] Furthermore, elevated concentrations of As have been reported to interfere with the metabolic processes that inhibit plant growth and development, which could ultimately lead to death.[20,21]

The ability of vetiver grass to grow and survive in the high levels of metals used in this study, confirms that vetiver grass can tolerate and adapt to metal-induced stress.[22] According to the results of our pot trial, it seems the growth of vetiver can only be inhibited by excessive concentration of As in the soil.

3.2. Soluble and Insoluble (Cell Wall-bound) Phenolics

Total soluble phenolics, free phenolic acids and cell wall-bound phenolics content of all the extracts exhibited a linear correlation with increasing metal concentrations. The concentration of the total soluble phenolic compounds (Fig. 2) ranged from 1.13 to 2.14 mg gallic acid equivalent (GAE) g^{-1} dry weight (DW). The

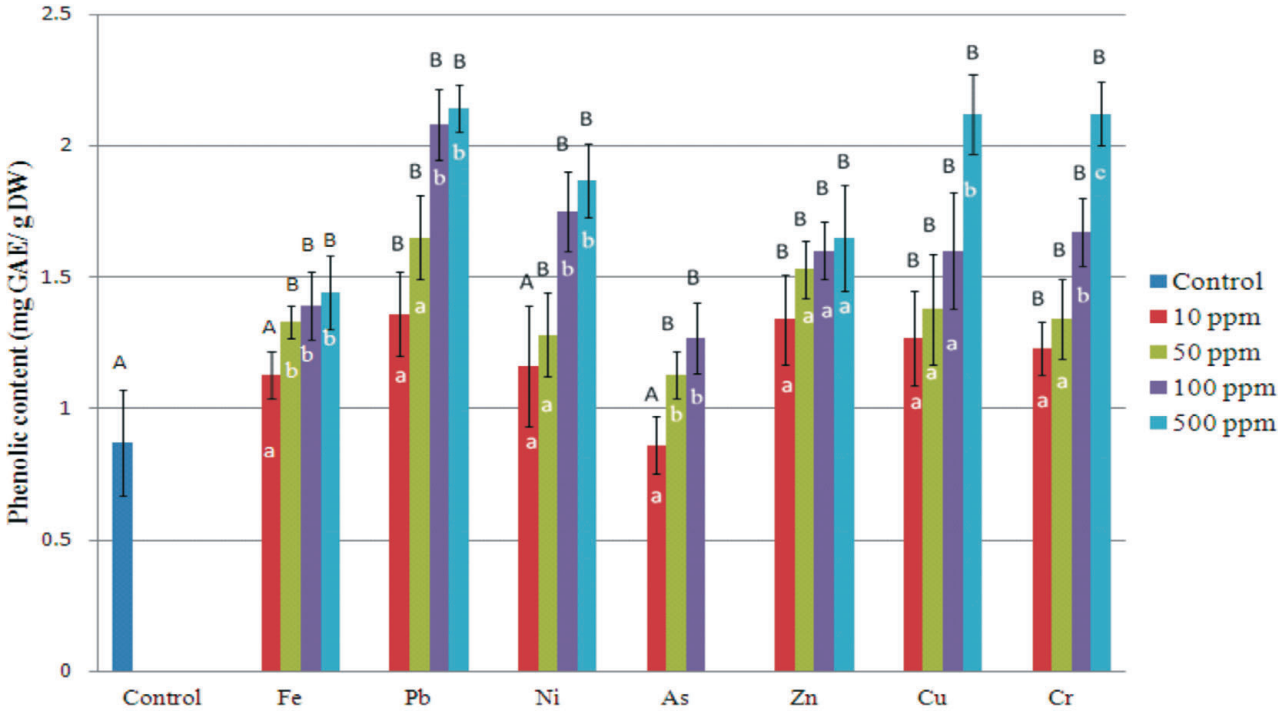

Figure 2 Total soluble phenolics content (as mg gallic acid equivalent per g DW) of vetiver plants exposed to different metals with increasing concentrations.

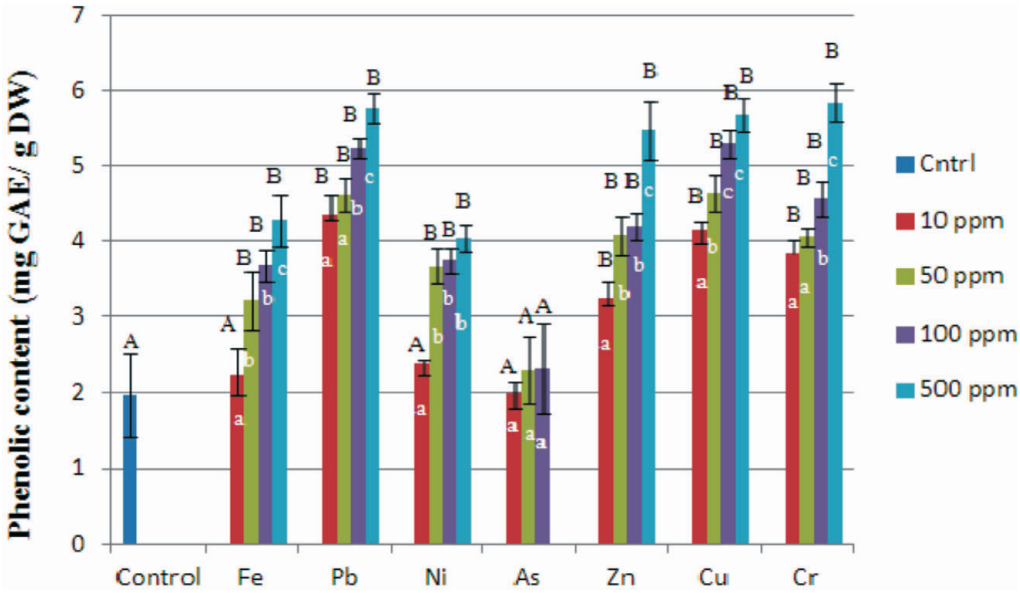

Figure 3 Free phenolic acids content (as mg gallic acid equivalent per g DW) of vetiver plants exposed to different metals in increasing concentrations.

plants treated with Pb 500 ppm, Cr 500 ppm and Cu 500 ppm gave the highest amount of total soluble phenolics which were approximately 2.14 (GAE) g^{-1} dry weight (DW). A similar trend was observed by Hamid[1] who reported an increase in the total phenolics content of *Phaseolus vulgaris* with increasing levels of Pb. Phenolic compounds play a vital role in protecting plants against metal-induced and other forms of stress[10] and hence the enhancement in their metabolism is one of the mechanisms employed by plants to respond to metal-induced toxicity. Since phenolic compounds are reductants, they may scavenge active oxygen species or chelate metals, thus reducing metal toxicity in cells.[23] The total phenolic content of the plants exposed to As exhibited a similar trend that was observed with other metals, increasing with increasing metal concentrations. However, the total phenolics content remained less compared to that of plants in other metal treatments. This is a sign of phytotoxicity induced by As present in plant tissues which inhibit plant growth through interfering with plant metabolism.[24]

Free phenolic acids content of vetiver plants (Fig. 3) exhibited similar trends observed with the total soluble phenolics; however, the content was lower, ranging from 0.47 to 1.75 mg GAE g^{-1} DW. Gorecka *et al.*[9] reported lower concentrations of free phenolic acid content compared to other phenolic acid fractions. No significant differences ($P > 0.05$) were observed in the content of free phenolic acids in vetiver plants treated with As with regard to concentration.

The content of cell wall-bound phenolics was significantly higher ($P > 0.05$) than for total soluble phenolics, ranging from 2.01 to 5.84 mg GAE g^{-1} DW (Fig. 4). Similarly, the concentration of cell wall-bound phenolics increased with increasing concentrations for all the metals studied. Similar trends were reported by Hamid *et al.*,[1] who indicated that increasing levels of toxic Pb

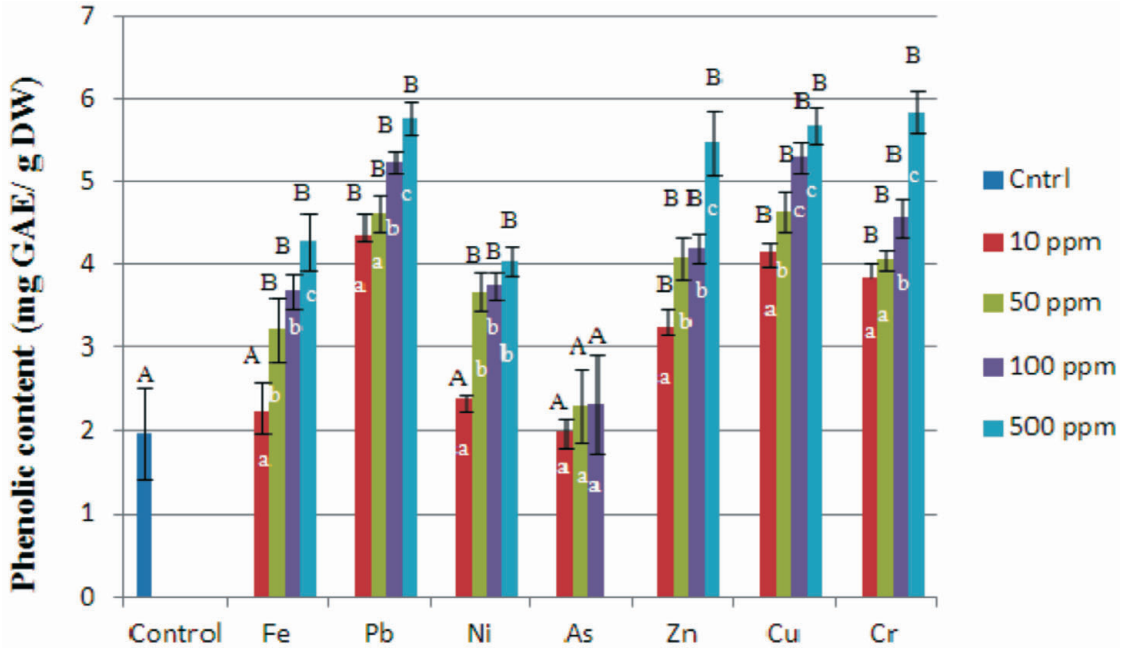

Figure 4 Cell wall-bound phenolics content (as mg gallic acid equivalent per g DW) of vetiver plants exposed to different metals with increasing concentrations.

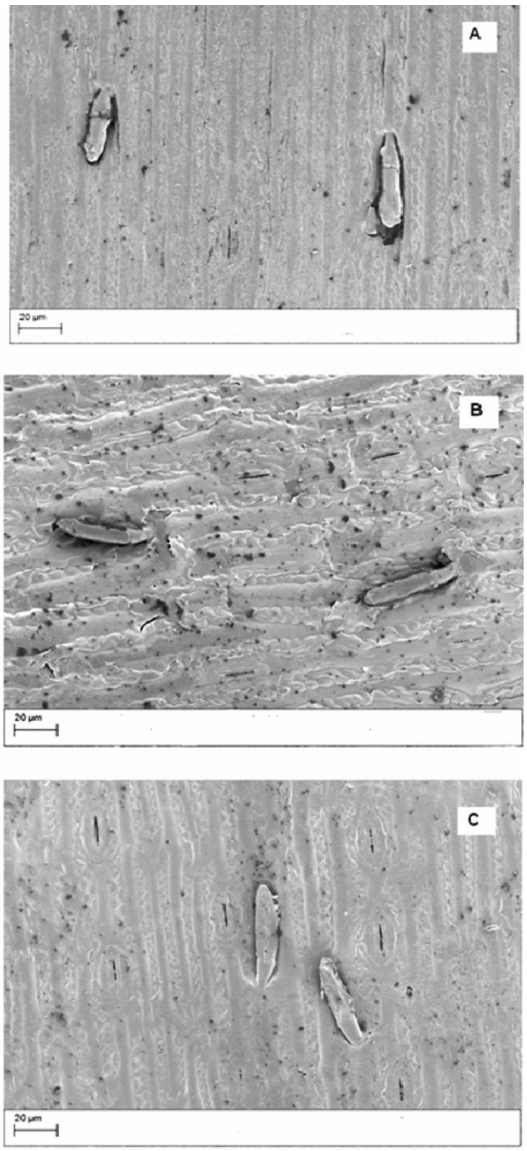

Figure 5 SEM micrographs showing epidermal cells on the top surface of the leaves. Control vetiver plant (**A**) and vetiver plants exposed to Cr (**B**) and As (**C**).

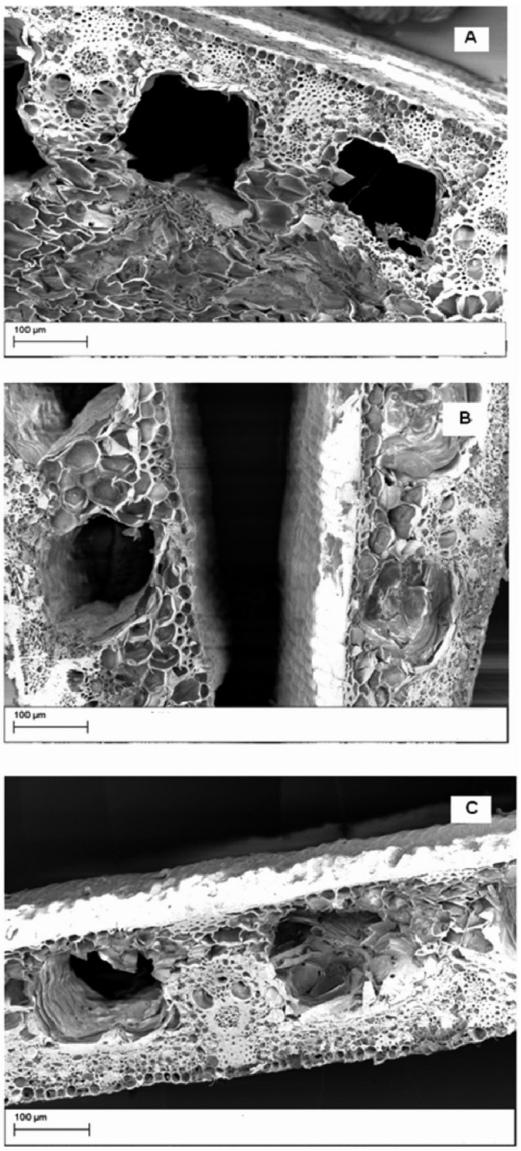

Figure 6 SEM micrographs showing cell walls on the transverse section of the leaves. Control vetiver plant (**A**) and vetiver plants exposed to Cr (**B**) and As (**C**).

markedly increased the phenolic content in cell walls of *Phaseolus vulgaris.* Connan and Stengel[25] also reported similar observations of increased levels of cell-wall bound phenolics under elevated Cu concentrations in brown algae.

No significant differences ($P > 0.05$) were observed in the cell wall-bound phenolic content of plants growing in As (Fig. 4) with respect to concentration and their content remained relatively low, ranging from 2.01 to 2.33 mg GAE g^{-1} DW, compared to plants in other treatments. Plants exposed to 500 ppm Pb, 500 ppm Cu and 500 ppm Cr (Fig. 4) produced the highest amounts of cell wall-bound phenolics, 5.76, 5,67 and 5.84 mg GAE g^{-1} DW, respectively. High amounts of cell wall-bound phenolics is an indication that a large amount of phenolics acids are bound to the cell wall. Plants employ different mechanisms to detoxify metals or to increase their tolerance to metal concentration. These include sequestration of metals in extracellular matrix of protective tissue[26] and metal binding onto cell wall compounds.[14,27]

3.3. Scanning Electron Microscopy

Plants exposed to metals can undergo significant morphological and metabolic changes at structural and ultrastructural levels in response to metal-induced stress.[7,26] In this study, scanning electron micrographs of the top surface of the plant exposed to Cr (Fig. 5B) showed more compaction of epidermal cells with associated thickened cell walls compared those treated with As (Fig. 5C) and the control (Fig. 5A). Compared to the control, the cell wall of the plant exposed to As (Fig. 5C) was thicker but thinner than that of the plant growing in Cr.

The transverse section of plants treated with Cr (Fig. 6B) and As (Fig. 6C) showed greater loss of cell shape and decrease in intercellular spaces in comparison with the control (Fig. 6A). Sridhar[28] observed similar anatomical changes in the SEM micrographs of cross sections of barley (*Hordeum vulgare*) treated with Zn. Cell deformation was attributed to Zn accumulation in plant cells.

The SEM micrographs of the stomata on the leaf skin layer of the plants treated with Cr and As are shown in Fig. 7B and Fig. 7C, respectively, and for the control in Fig. 7A. The stomata on the leaves of the plants treated with Cr and As appeared slightly smaller and closed compared to the control. The closure of stomata in the leaves of plants exposed to high metal concen-

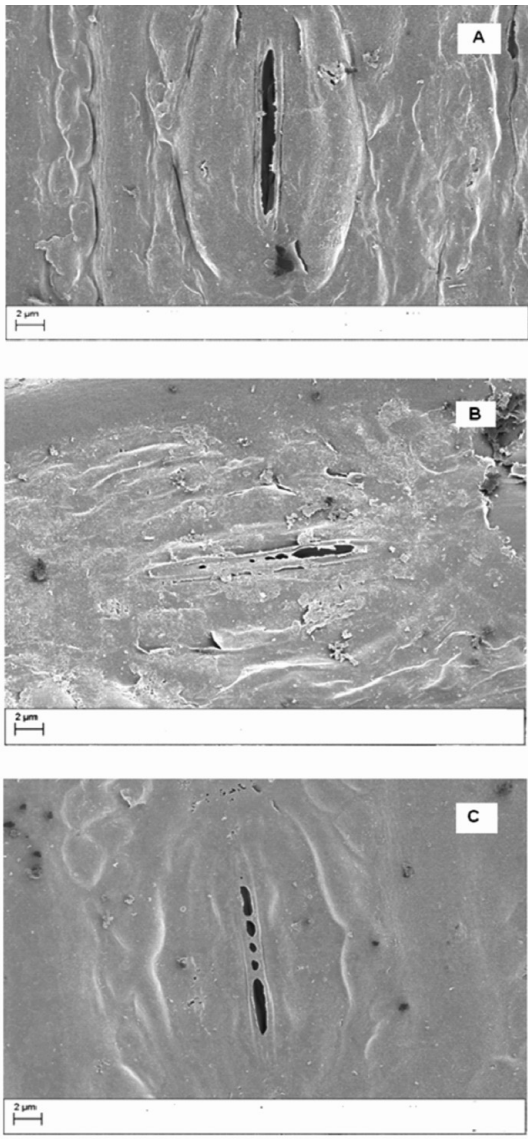

Figure 7 SEM micrographs showing stomatal cells on the top surface of the leaves. Control vetiver plant (**A**) and vetiver plants exposed to Cr (**B**) and As (**C**).

tration are in good agreement with a previous report[7] showing the closure of stomata in the leaves of *Helianthus annuus* L. growing on soil amended with tannery sludge containing high concentration of metals. In addition, stomata closure due to Cd-induced stress was reported by Sandalio *et al.*[6] in pea leaves. The main reason behind the stomatal closure by plants under metal-induced stress is that plants close the stomata as a strategy to prevent water loss through transpiration. In the presence of metals, the rate of translocation of water and mineral solutes is disturbed and thus affects normal biochemical and growth processes.[6,7] The plant regulates the stomatal aperture so that the water loss is not exacerbated during adaptation to metal-induced stress.

4. Conclusion

The ability of vetiver grass to survive high metal concentration levels is an indication that it can tolerate and adapt to harsh and inhospitable conditions. Its growth can only be significantly hindered by high concentrations (>50 ppm) of As in the growth medium. Phenolic compounds, due to their antioxidative and metal chelating properties play a vital role in protecting plants against metal-induced stress. A linear correlation was shown between metal concentrations and the content of total soluble phenolics, free phenolic acids and cell wall-bound phenolics. This confirms that higher metal concentrations in the growth medium enhance the production of phenolic compounds in vetiver grass. Increased amount of cell wall-bound phenolics signifies that vetiver binds metals onto the cell wall as a mechanism for detoxification. These findings were corroborated by the thickened cell wall revealed by SEM studies. The findings of this study qualifies vetiver grass as a potential candidate for phytoremediation of soils contaminated with high levels of metals.

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