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Morphological Adaptation of *Vernonia Amygdalina* in a Cadmium-Spiked Soil Influenced by Periodic Soil Wetting with Grey Water

[1] **Ikhajiagbe, B. Omoregie, G. O[2] and Ekhator, P. O[1]**

[1]Environmental Biotechnology and Sustainability Research Group,
Dept. of Plant Biology and Biotechnology, University of Benin, Nigeria
[2]Department of Environmental Management and Toxicology, Fed. Univ.of Petroleum Resources,
E E-mail: omoregie.gloria@fupre.edu.ng

Abstract

The study investigated morphological changes in *Vernonia amygdalina* in a cadmium-spiked soil influenced by periodic soil wetting with grey water. Soil was spiked with cadmium (as cadmium chloride) to obtain a constant concentration of 150 mg/kg. The set up was divided into six sub-treatments of nine replicates each. Each sub-treatment was subjected to periodic wetting with grey water (at 50ml/kg), ranging from daily (D), twice a week (DW), once a week (W), twice a month (WM) and once a month (M). The control soil on the other hand, received regular wetting with tap water (pH 6.46 – 7.02). Subsequently, equal-sized stem cuttings of *Vernonia amygdalina* (20 cm long, 2.5 – 3.0 cm thick) obtained for the study were sown in both treated and control soils. Results obtained at 21 weeks after sowing showed that treated plants had the highest total number of leaves per plant (between 76 and 105), compared to the control (51 leaves). There was also significant leaf chlorosis in the control leaves (60.60%) than in the greywater-wetted plant treatments. No necrotic lesions were observed in any part of the leaves for both control and treated plants. There was significant ($p < 0.05$) change in dry weight of plant shoots, with the daily (D) treated plants weighing more (25.53g) than other treatments (11.56 to 17.25g). However, there were no significant changes in plant root dry weight (1.03 to 3.58g) for all the treatments

Keywords: **Grey water, *Vernonia amygdalina*, Cadmium, Heavy metal, Phytoremediation, Irrigation.**

Introduction

Since the beginning of his existence on earth, man has depended on plants for the fulfilment of his basic needs such as clothing, shelter and especially food. This relationship is bound to become more complex because of the ever-increasing world population (FAO, 2009). To fill man's needs therefore, sustainable agriculture needs to produce enough food for the growing population. However, over the years, the major problems of sustainable agriculture have been pollution of arable soils and depletion of fresh water due to climate change.

Although needed by plants in small quantities, heavy metals such as Cadmium (Cd), Lead (Pb) and Arsenic (As), Zinc (Zn) and Copper (Cu) are the major causes of soil pollution. Cadmium, a highly phytotoxic metal, causes overproduction of reactive oxygen species (ROS) which affects superoxide dismutase (SOD) expression (Rodriguez-Serrano *et al*., 2009). Heavy metals are not usually degradable and are toxic to plants when they are present at high concentrations in their bioavailable forms (Hall, 2002 and Singh *et al.*, 2011). Glass (1999) stated that physical methods of soil cleanup are usually expensive. Therefore, it is imperative to employ cost-effective and ecological friendly methods of reclamation such as phytoremediation.

In both developed and developing countries, a common strategy to ease the demand on freshwater is the reuse of grey water. Grey water is the wastewater from bathroom showers, laundry and kitchen that does not contain faecal matter (Erikkson *et al*., 2002). According to Friedler and Hadari (2006), grey water accounts 50-80% of the total wastewater generated from households. Before grey water can be used for irrigation, important parameters such as its pH, electrical conductivity, total suspended solids, heavy metal composition, faecal coliform, (*Escherichia coli)*, dissolved oxygen, biological and chemical oxygen demands, total nitrogen and phosphorus, need to be considered (Dixon *et al*., 1999; Birks and Hills, 2007 and Erikkson*et al*., 2002). Several studies have shown that grey water has some components which are beneficial to plants. For example, many detergents contain phosphates. Phosphates, in small quantities, are essential for plant growth.

Although irrigation with grey water is beneficial, research has shown some drawbacks of grey water use. It has high salinity which has negative impact on soil structure and permeability, leading to reduced crop yield (Halliwell*et al*., 2001; Oster *et al*., 2001; and Travis *et al*., 2008).Despite these obvious challenges, it is not possible to completely write off grey water reuse in sustainable agriculture particularly given the threat presented by the global water shortage.

In the present study, *Vernonia amygdalina*, popularly called Bitter leaf plant, is grown in Cadmium-spiked soil. Grey water wetting of the plant may present a number of advantages or disadvantages (Halliwell *et al*., 2001; Oster *et al*., 2001; and Travis *et al*., 2008)The inhibitory and phytotoxic effects of Cadmium in soil has also being reported (Hall, 2002 and Singh *et al*., 2011).*Vernonia amygdalina* has been reported to be a highly efficient phytoremediator (Kalagbor *et al*., 2014). The study shall focus on investigating the morphological changes shown by the plant in response to Cadmium toxicity as either ameliorated or aggravated by grey water wetting.

Materials and Methods

Soil Collection and Preparation

Soil at a depth of 0 – 8 cm was collected from a plot at the Botanic Garden of the Department of Plant Biology and Biotechnology, University of Benin, Benin City. The vegetation of the plot was mainly grasses such as *Eleusine indica*, *Panicum maximum* and sedges like *Cyperus iria* and *Kyllinga erecta*. The soil was sun dried to constant weight, and 30 kg of soil was measured into bowls sealed at the bottom to prevent leaching.

Each replicate containing 30 kg soil was spiked with 1.5g cadmium (as cadmium chloride) to a constant concentration of 50mg/kg w/w. This was achieved by dissolution of cadmium chloride in 4 litres of water and then wetting the 30kg-soil with the resulting solution. Before contamination, the water-holding capacity of the soil was determined to be 202.16 ml/kg soil.

Preparation and Application of Grey water

The grey water used for this study was generated by completely dissolving 20 g of branded laundry soap, *Premier* ® completely in 20 litres of water. The resulting solution was used to wash 1.6 kg of dirty clothes gotten from a Laundry House in Ugbowo, Benin City, for 20 minutes. This grey water was prepared and used within 24 hours, otherwise it was discarded.

The frequency of wetting Cd-spiked soil ranged from daily (D), twice a week (DW), once a week (W), twice a month (WM), and once a month (M). The control soil received regular wetting with tap water (pH 6.46 – 7.02). Each bowl received 1.5 litres of water per wetting period. At other times when grey water was not applied to soil, constant water supply was maintained by augmenting with tap water (pH 6.46 – 7.02). This was to ensure that soil moisture was sufficient for plant growth throughout the experiment. Soil moisture was checked using the Soil Moisture Feel Test proposed by USDA (1998). Thus, the treatment with daily wetting (D) was carefully monitored to avoid water-logged soil.

Stem collection and sowing

Equal-sized stem cuttings of *Vernonia amygdalina* (20cm long, 2.5 – 3.0 cm thick) obtained from a fallow land near the University of Benin Senior Staff Quarters, Ugbowo, and were sown in all soil samples. The setup was replicated 9 times.

Physical and chemical analysis

Three months after exposure to the experimental condition, the test plant was observed for the following parameters including plant height, leaf area, primary stem branching, secondary stem branching, root branching and moisture content, as well as length of the longest root. The chlorophyll content index (CCI) of leafs of the test plant were estimated with the aid of a chlorophyll content meter; CCM-200 plus. CCM-200 is a non-destructive chlorophyll content measuring meter. Similarly, foliar proline content was estimated per protein using the methods by Bates *et al*. (1973). The amount of ascorbic acid was estimated using de Pinto *et al*. (1999) method.

Results

Physicochemical parameters of grey water and tap water used for the study are presented on Table 1. Chloride content of the grey water prepared for the study was nearly six times higher (637.8mg/l) than that of tap water (53.98mg/l) (Table 1).

Table 1**: Physicochemical parameters of water types used for the study**.

Parameters	**Tap Water**	**Grey water**
TSS (mg/l)	1.00	4.04
Dissolved Oxygen (mg/l)	0.96	1.52
Biochemical Oxygen Demand(mg/l)	0.24	1.36
Chloride (mg/l)	53.98	639.80
Phosphate (mg/l)	0.02	4.62
Nitrate (mg/l)	0.09	1.30

Plant morphological development monitored from the first to the eleventh weeks showed progressive increase in plant height of control plants from 2.63cm at 2WAS to 22.03cm at 11WAS, compared to DW which progressed from 1.93cm to 29.27cm during the same period (Fig. 1). Comparatively however, plants showed almost similar growth pattern.

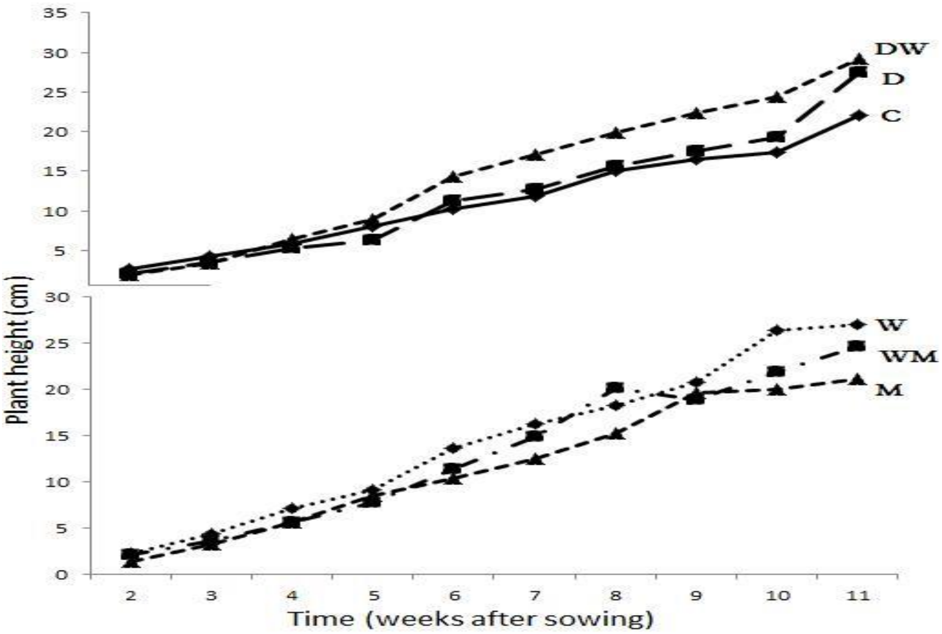

Fig.1: Plant height of *Vernonia amygdalina* sown in Cd-contaminated soil and wetted with greywater. C control, D daily wetting, DW wetting twice a week; W weekly wetting, WM wetting twice a month; M monthly wetting. WAS weeks after sowing.

Chlorophyll content of test plant under experimental conditions revealed a significant development in chlorophyll content index from the first to the third month for all plant treatments (Fig. 2). During the first month, chlorophyll content ranged from 14.66 to 23.73 CCI compared to a range of 34.33 to 48.13 CCI during the third month.

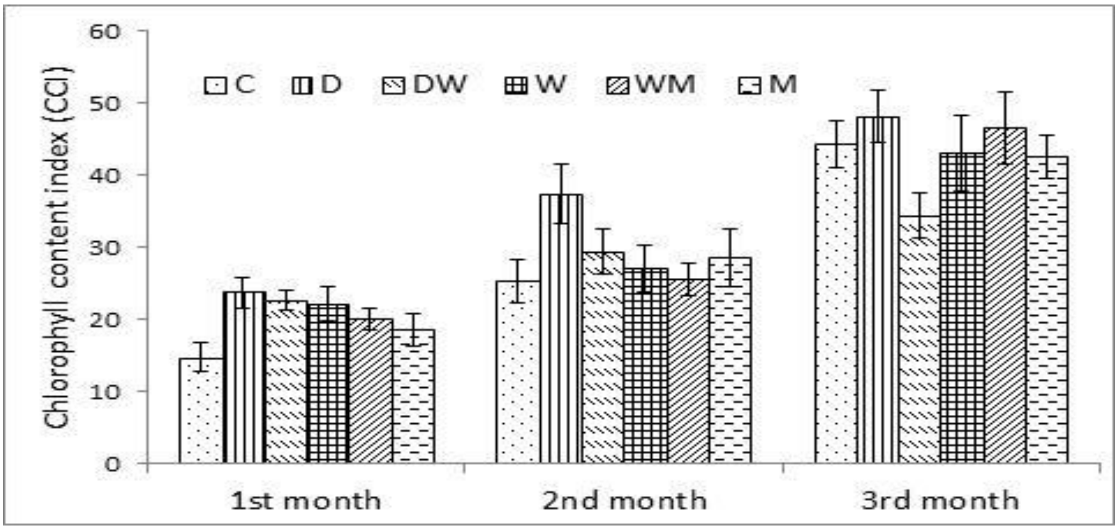

Fig. 2: Chlorophyll content of *Vernonia amygdalina* sown in Cd-contaminated soil and wetted with greywater. C control, D daily wetting, DW wetting twice a week; W weekly wetting, WM wetting twice a month; M monthly wetting.

Some growth parameters of the test plant have been presented on Table 2. Leaf area at 6 WAS ranged from 18.43cm^2 in the control to 39.13 cm^2 in the plant exposed to weekly wetting (W). There was no significant change in the number of primary stem branching (4.10 to 4.70). However, there were more significant secondary branching in DW, WM and W (12.20 to 15.50) compared to D (4.4) and the control (9.30). Length of the longest root was significantly lowest in WM (30.02cm) compared to the range of values of 40.1 to 51.65cm in the other treatments.

Table 2**: Some plant growth parameters *Vernonia amygdalina* sown in Cd-contaminated soil and wetted with greywater.**

Treatment	Leaf area (cm2) at 6 WAS	Primary stem branching	Secondary stem branching	Leaf chlorophyll (CCI) at 8 WAS	Primary root branching at 12 WAS	Secondary root branching at 12 WAS	Length of the longest root (cm) at 12 WAS
C	18.43^{c}	4.30^{a}	9.30^{b}	25.26^{b}	35.32^{bc}	120.94^{b}	45.02^{a}
D	24.40^{bc}	4.10^{a}	4.40^{c}	37.40^{a}	23.33^{c}	220.22^{a}	40.01^{a}
DW	36.46^{ab}	4.50^{a}	12.20^{ab}	29.40^{ab}	30.32^{bc}	102.86^{b}	51.65^{a}
W	39.13^{a}	4.20^{a}	9.20^{b}	27.06^{ab}	70.67^{a}	38.45^{b}	42.64^{ab}
WM	37.36^{a}	4.20^{a}	15.50^{a}	25.50^{b}	42.44^{b}	145.34^{b}	30.02^{b}
M	19.20^{c}	4.70^{a}	12.30^{ab}	28.56^{ab}	32.65^{bc}	135.43^{b}	49.34^{a}

C control, D daily wetting, DW wetting twice a week; W weekly wetting, WM wetting twice a month; M monthly wetting. WAS weeks after sowing. Means with alphabetical superscripts on the same column do not differ significantly ($p > 0.05$) from the other.

There was significant change in dry weight of shoots with plants in D showing significantly higher value (25.53g) compared to other treatments (11.56 to 17.25g). However, there were no significant changes in root dry weight (1.03 to 3.58g) (Table 3). Root to shoot ratio was significantly higher in D (14.21) and WM (11.22) compared to 4.27 in the control. Whereas, mean moisture content for entire plants ranged from 49.45% in DW to 81.69% in W.

Table 3**: Shoot and root weight parameters of *Vernonia amygdalina* sown in Cd-contaminated soil and wetted with greywater.**

Treatment	**Wet wt. (g)**		**Dry wt. (g)**		**Shoot-root ratio (per dry wt.)**	**Percentage moisture (%)**		**Mean (%)**
	Shoot	**Root**	**Shoot**	**Root**		**Shoot**	**Root**	
C	46.23^{bc}	13.41^{a}	13.06^{b}	3.06^{a}	4.27^{c}	71.75^{a}	77.18^{a}	74.47
D	76.43^{a}	03.86^{b}	25.53^{a}	1.76^{a}	14.51^{a}	66.60^{ab}	54.40^{b}	60.50
DW	25.60^{c}	06.90^{b}	12.60^{b}	3.58^{a}	3.52^{c}	50.78^{b}	48.12^{b}	49.45
W	59.66^{ab}	12.03^{a}	12.03^{b}	1.98^{a}	6.06^{bc}	79.84^{a}	83.54^{a}	81.69
WM	30.81^{c}	04.15^{b}	11.56^{b}	1.03^{a}	11.22^{ab}	62.48^{ab}	75.18^{a}	68.83
M	41.73^{bc}	07.20^{ab}	17.25^{b}	3.25^{a}	5.31^{c}	58.66^{b}	54.86^{b}	56.76

C control, D daily wetting, DW wetting twice a week; W weekly wetting, WM wetting twice a month; M monthly wetting. WAS weeks after sowing. Means with alphabetical superscripts on the same column do not differ significantly ($p > 0.05$) from the other.

Morphological parameters as observed on the field showed that the replicates in the control almost had equal heights during the experiment (Table 4). Chlorosis was significantly in the greywater wetted plant treatments especially in M but the highest frequency of chlorotic leaves were recorded in the control. There were no reports of leaf folding in DW, W and M treatments. The D treatment had 40% of leaves looking burnt on the tip with more than 50% of entire leaf surface folded inwards. The soil in some treatments (DW and WM) appeared slightly white at the start of the experiment but later began to look densely white close to the termination of the experiment. In the D treatment however, the soil surface always appeared dry and hard but was moist within. The leaves of some treatments (DW and WM) always looked healthy but white spots were observed on the leaves of the control.

Table 4: **Morphological observations *Vernonia amygdalina* sown in Cd-contaminated soil and wetted with greywater.**

Treatments	Observations
Control	Most leaves were chlorotic at the most frequent rate. Foliar chlorosis began from the tip of the leaf down to entire leaf surface. Had the lowest frequency of leaf senescence. White spots were observed on the leaves.
Daily wetted plants	The surface soil was always appearing hard/ dry but moist within. The soil was looking whitish at the middle of the work but close to termination period, the soil began to look dark/ black. 40% of the leaves were folding from the tip of the leaves down to the middle of the leaf. Same 40% were also looking burnt on the tips of the leaves. More than 50% of entire leaf surface was folded inwards. The leaves were very dense and thick.
Plants wetted twice a week	None of the leaves were necrotic. Leaf folding was not observed. 24% of the leaves senesced. White spots were observed on the leaves. The leaves looked healthy apart from a number of leaves that were chlorotic. The surface soil was relatively hard to touch but not as hard as the D-treatment soil. The soil was looking lightly whitish at the middle of the work but close to termination period, the soil began to look whitish.
Plants wetted weekly	20% of the leaves were chlorotic. No necrotic leaves were observed. No leaf folding was observed. 22% of the leaves senesced.
Plants wetted twice a month	26% of the leaves were chlorotic. None of the leaves were necrotic. 21% of the leaves were folded inwards. Total senesced leaves were 32%. The leaves looked healthy. The soil was looking lightly whitish at the middle of the work but close to termination period, the soil began to look whitish. White spots were observed on the leaves.
Plants wetted twice monthly	Had 40% chlorosis. No necrosis was observed. There was no leaf folding. 19% leaf senescence was observed.

\Proline activities in the leaves of the test plant revealed heightened activities only in the control and D compared to significant low proline concentration in DW, W, WM and M (0.21 to 0.034mg/protein) (Fig. 3). There was no significant difference in ascorbate activities in both treated and control plants ($P > 0.05$). Correlation between any two parameters tested during the study at 12 weeks after sowing was not significant at the 0.05 level (2-tailed) (Table 5). The implication is that one parameter may not necessarily affect the other. An attempt was made to establish a regressional model between plant height and leaf area (Fig. 4). The model was presented as $y = 0.24x + 18.42$, where y is plant height, and x the leaf area. However, with a low linear squared coefficient of correlation (0.482), the regressional model was considered only fairly reliable.

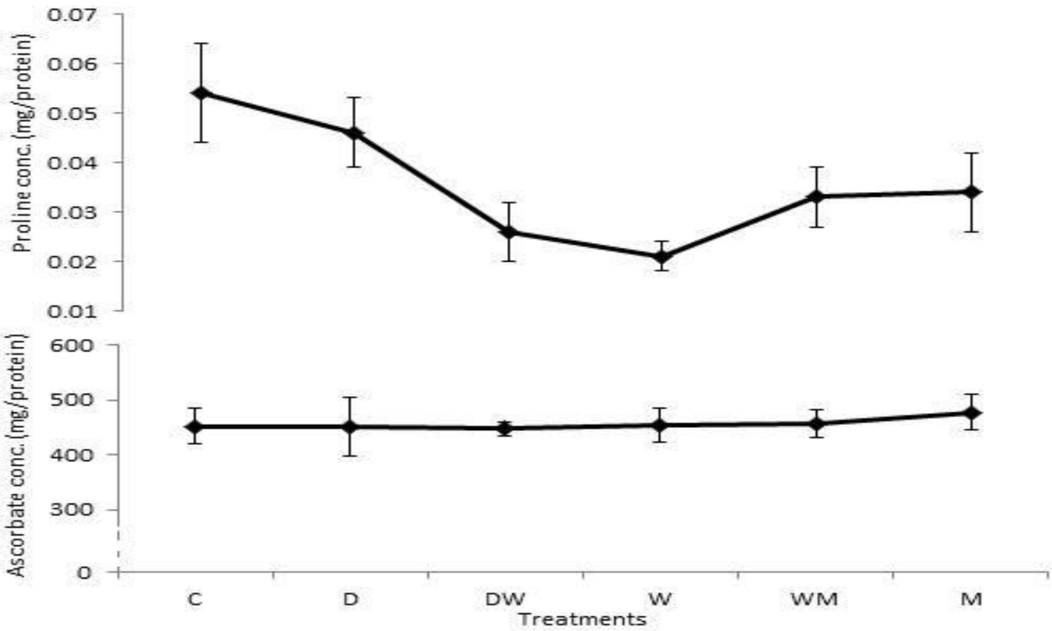

Fig. 3: Proline and ascorbate activity of leaves of *Vernonia amygdalina* plant sown in Cd-contaminated soil and wetted with greywater. C control, D daily wetting, DW wetting twice a week; W weekly wetting, WM wetting twice a month; M monthly wetting.

Table 5**: Correlationship between parameters tested during the study at 12 weeks after sowing test plants**

Parameters	**Plant height**	**Leaf area**	**Root length**	**Shoot-root ratio**	**Proline**	**Ascorbate**
Plant height	1					
Leaf area	0.694	1				
Root length	0.017	-0.319	1			
Shoot-root ratio	0.206	0.049	-0.732	1		
Proline	-0.461	-0.793	-0.101	0.246	1	
Ascorbate	-0.701	-0.434	0.154	-0.105	-0.087	1

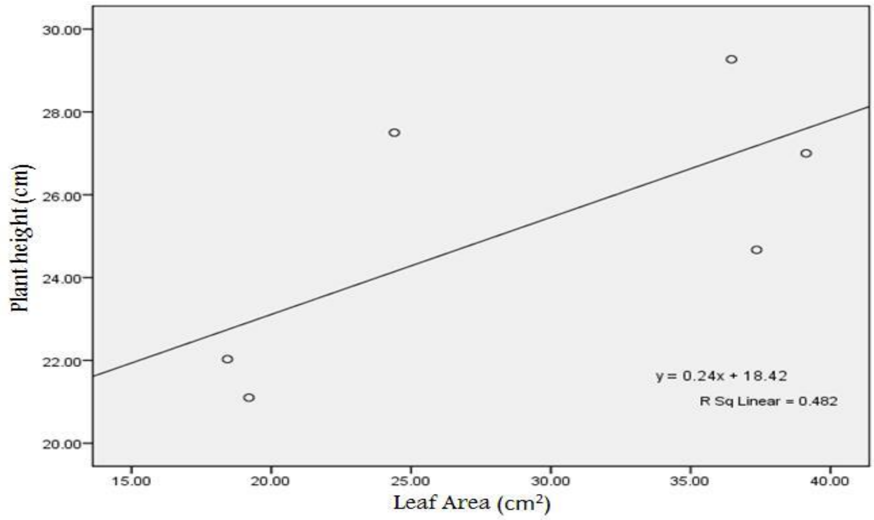

Fig. 4: Scatter plot and regressional model establishing relationship between leaf area and plant height

Discussion

The results indicate improved morphological characteristics of *Vernonia amygdalina* under the experimental conditions upon wetting with grey water. For example, in spite of the imposed challenge of Cd phytotoxicity, there was significant increase in the production of leaves at the 12th week after sowing (12 WAS) in the soil wetted twice a month (WM), compared to the control soil (C) which never received grey water.

Leaf chlorosis and senescence was greatly reduced in grey water-treated plants compared to the control which had high incidence of chlorosis. Although chloride toxicity possibly due to chlorides in the cadmium salt (CdCl) used or grey water was evident, the nitrogen in phosphorus in grey water contributed to reduced chlorosis in treated plants (Anonymous, 1999).

Root growth also improved upon wetting with grey water. Grey water has a rich supply of Phosphorous (as phosphates, PO_4^{3-}), which originates from wash and dish-washing powder where it is used for softening the water. It also contains potassium ions (K^+), and nitrogen (as nitrates NO_3^-), making it a good nutrient or fertilizer source for irrigation. The development of a plant's shoot is closely related to the morphology and physiology of its root, as this affects the pattern of nutrient transfer and plant performance under water stress (DoVale *et al*., 2015; Lynch, 2014 and Lynch *et al*., 2014).

Some important features of highly successful rooting systems include prevalence of lateral or branched roots, root size and rooting depth. Several authors have associated increased in lateral root density with greater uptake of water and nutrients (Robbins and Dinneny, 2015; Sun *et al*, 2015; Tian *et al*, 2014). Root size and depth is also essential for accessing stored water and soluble nutrients (e.g. N) that are easily leached into deeper soil layers (Wasson *et al*., 2012).

Proline accumulation is an indication of osmotic, salinity (Kavi *et al*., 2005) temperature (Naidu *et al*., 1991) and heavy metal stress in plants (Sharma and Dietz, 2006). Besides its role as an excellent osmolyte, proline also acts as a metal chelator, an antioxidative defense molecule and a signaling molecule. The amount proline accumulated in grey water-treated plants was less than in that in the control plants.

Ascorbate activity was insignificant in the leaves of all the treatments although high in concentration (451.72 to 477.24 mg/protein). According to Pastori *et al*. (2000), various stresses lead to an increase in the antioxidant activity of plant leaves. Cadmium, a non-redox active metal, indirectly inflicts oxidative stress by several mechanisms such as glutathione depletion, binding to the sulfhydryl groups in proteins (Valko *et al*., 2005), inhibition of antioxidative enzymes, or induction of various ROS-producing enzymes like NADPH oxidases (Bielen *et al*., 2013). The non-increase of ascorbate in the leaves meant that the concentration or accumulation was in no way influenced by grey water accumulation unlike proline.

Conclusion

The phytotoxic effects of Cd has been corroborated as earlier reported in literature, however, the application of grey water, to a very large extent, reduced the phytotoxic effects on the test plant. Further research is however required to further elucidate the mechanisms of action of grey water in Cd-spiked soils,

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