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Research Article

Effect of Sewage Sludge Amendment on Non-enzymatic Antioxidants in Vetiver Plants (*Vetiveria zizanioides* L. Nash.)

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Abstract

The study was conducted to evaluate the influence of sewage sludge application on the activity of non-enzymatic antioxidants in vetiver grass such as proline, ascorbic acid, total phenol and total free amino acids. Vetiver plants were grown in poly-ethylene U-V stabilized grow bags (24 X 24 X 40) for a period of three months (90 days), where the soil was treated with different sewage sludge concentrations (0, 25, 50,75 and 100%). After the experimental period, plants were harvested and analysed. The result showed activity of proline, ascorbic acid and total free amino acids was high in roots compared to that of leaves in the experimental plants, while the total phenol content was high in leaves than that in the roots. It was also noted that with an increase in the concentration of sewage sludge application, activity of all the selected antioxidants was also increased significantly ($p < 0.05$). Increased antioxidant activity in plants may be due to various environmental stresses to prevent damages due to ROS formation. Sewage sludge is a mixture of toxic compounds including PCBs, flame retardants, heavy metals, nano-materials, hormones, dangerous pathogens etc., which may induce the antioxidant defence system in vetiver plants to protect them from oxidation due to the generation of ROS. Therefore, the induction of non-enzymatic antioxidants in the present study is considered to be an important protective mechanism to minimize oxidative damage in vetiver plants grown in sewage sludge polluted environment. The study pointed out that the vetiver plants growing in sludge amended area have potentially developed the defence strategy to combat against toxicity induced stress. Therefore, the highly tolerant vetiver plants (Vetiveria zizanioides, L. Nash) may be used for phytoremediation purposes.

Keywords: Antioxidant; Phytoremediation; Sewage Sludge; Vetiver

Introduction

Urbanization is a fantastic idea that is necessary for any nation to develop. It alludes to the idea of developing isolated locations by urbanising them through the construction of infrastructure. All the structures and organisations required for a region's economic development collectively are referred to as infrastructure. Rapid urbanization creates opportunities for economic developments, but it also increases the waste generation.

According to Central Pollution Control Board [1] the decadal assessment revealed an increasing trend in urban population and sewage generation. Since 1971, the urban population of India increased 3 times thereby impacting sewage generation which also increased at a rapid pace. Sewage, or domestic/municipal wastewater, is a type of wastewater that is produced by a community of people. According to the source of generation, characteristics of sewage may also vary. Generally, it is characterised by nutrients (nitrogen and phosphorus); solids (including organic matter); pathogens (including bacteria, viruses and protozoa); helminths (intestinal worms and worm-like parasites); oils and greases; runoff from streets, parking lots and roofs; heavy metals (including mercury, cadmium, lead, chromium, copper) and many toxic chemicals including Polychlorinated biphenyls (PCBs), Polycyclic aromatic hydrocarbons (PAHs), dioxins, furans, pesticides, phenols and chlorinated organics and even heavy metals. In many poor areas of the world, sewage is dumped into local waterways, in the absence of practical alternatives [2]. Sewage sludge is the residual, semi-solid material that is produced as a by-product during sewage treatment after either aerobic or anaerobic digestion processes [3]. As sewage is the waste enriched in water, comprises entirety that is drained into the sewer system and therefore the sewage sludge is the crude form of all these wastes in dry form.

The characteristics of sewage sludge is dependent upon the quality and source of sewage and type of treatment process followed [4]. Due to high nutrient contents (nitrogen, phosphorous and potassium) and high organic matter content sewage sludge may substitute for fertilizer as it improves soil physical, chemical and biological properties and is one of the best option for its disposal [5,6]. Plethora of evidences reported that yield and productivity of a wide variety of crops has been improved by sewage sludge amendment [7-10]. But the toxic metals forming a large component of sludge and as such their excessive use for a longer period increases the metal bioavailability and thus restricts its uses [11]. Plants differ in their abilities to absorb sludge derived metals from the soil. Metal excluders effectively prevent metal from entering their aerial parts over a broad range of metal concentrations in the soil. Metal accumulators actively accumulate metals in their tissues to levels far exceeding those present in the soil or the non-accumulating species growing nearby [12].

Even in hyper accumulator and hyper tolerant plant species as in other organisms, higher concentrations of toxic chemicals and heavy metals can severely impair central metabolic processes [13,14]. A variety of toxic oxygen species (superoxide, hydrogen peroxide, hydroxyl radicals, etc.) are produced in plants exposed to severe soil conditions and other stresses. Oxidative stress results from an imbalance between the activities of pro-oxidants over antioxidants and might lead to oxidative damage. Sensitive and reliable biomarkers are therefore used to determine if there is evidence of significant contaminant exposures that have exceeded detoxification or compensatory mechanisms and are resulting in adverse effects on physiological and biochemical functions [15].

Therefore, the present study aims to find out the effect of sewage sludge application on the activity of non-enzymatic antioxidants in vetiver grass such as proline, ascorbic acid, total phenol and total free amino acids.

Materials and Methods

Plant materials

The experimental plants used for the present study are the cultivars of Vetiver grass (*Vetiveria zizanioides* L. Nash). The common name of Vetiver grass is Khus grassand in Kerala it is "Ramacham". It is a common aromatic medicinal plant with fast growth and high tolerance (to extreme heat, pH, flood, draught, temperature etc.). The plants were procured from the Aromatic and Medicinal Plants Research Station (Kerala Agricultural University), Odakkali, Ernakulam, Kerala.

Sewage sludge

Thiruvananthapuram, the capital city of Kerala state lies in the southern part of Kerala extending between the latitudes 8° 17' 25" to 8° 51' 46" N and longitudes 76° 40' 25" to 77° 17' 6" E, on the shoreline of Arabian Sea. Muttathara Sewage Treatment Plant comes under the corporation area which is located at 8° 27' 21" to at 8° 28' 3" North latitude and 76° 55' 51" to 76°56' 25" East longitude and about approximately 4 km away from Thiruvananthapuram Central Railway Station and 1 km away from Thiruvananthapuram International Airport. The location map of the study area is shown in figure 1. The raw sewage sludge was collected from the Muttathara Sewage Treatment Plant, it is the left-over solid product of Sewage Treatment.

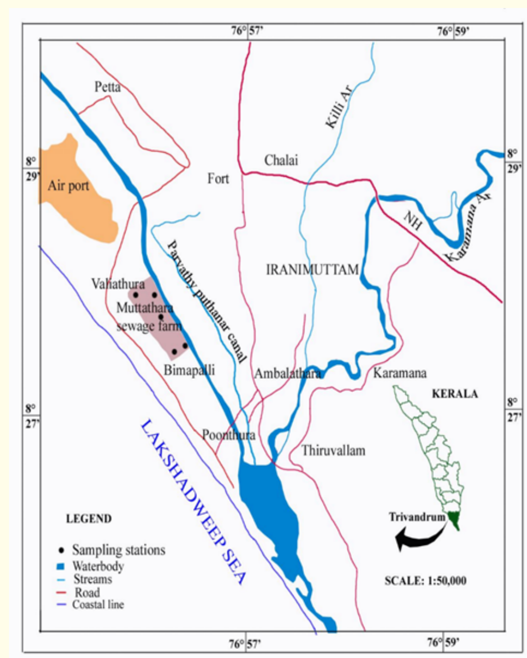

Figure 1: Location map of valiathura sewage farm.

Experimental design

The study was performed in poly-ethylene U-V stabilized grow bags of size 24 X 24 X 40 cm. The collected sewage sludge was further air-dried, crushed to pass a 2 mm sieve, used for the preparation of potting mixture. The air-dried sludge was mixed with garden soil to have five sludge-soil mixtures with sludge representing 0 (Control, C), 25 (T1), 50 (T2), 75 (T3) and 100% (T4) and are filled with 5Kgs of mixture (to get maximum effect of toxic metals and nutrients present in the sewage sludge with in the establishment period of plants) respectively in labelled grow bags and are subjected to heavy metal analysis before starting the experiment. The vetiver plants are removed from the propagating soil and surface sterilized with distilled water to remove any adhering soil and dried in air. Then the tops and roots of the vetiver sprouts were pruned to 10 cm and 5 cm respectively. Two clumps were planted in each grow bag and are maintained under controlled condition for a period of 90 days (3 months to assess the effect of sewage sludge amendment on the establishment period of the plants) for experimental studies. All the plants were watered daily with sufficient amounts (500 mL) of tap water. Replicates were also maintained under controlled natural conditions. After the study period (3 months) the leaf and roots of the plants were collected and analysed for the activity of non-enzymatic antioxidants such as proline, ascorbic acid, total phenol and total free amino acids. The experimental design is shown in table 1.

Table 1: Experimental Design.

Sl. No	Experimental groups	Treatments
1	C (Control)	100% Garden Soil
2	T1 (Test Group 1)	25% Sludge + 75% Garden Soil
3	T2 (Test Group 2)	50% Sludge + 50% Garden Soil
4	T3 (Test Group 3)	75% Sludge + 25% Garden Soil
5	T4 (Test Group 4)	100% Sludge

Methodology

To find out the effect of sewage sludge amendment on non-enzymatic anti-oxidants in vetiver plants, various chemical characteristics as well as heavy metal content of sludge samples were performed by following the standard procedures of [16]. Analysis of different non-enzymatic anti-oxidants such as proline, ascorbic acid, total phenol and total free amino acid content in the leaves and roots of experimental plants were followed by the standard procedures [17]. All the chemicals and biochemicals used for the analysis are of analytical grade.

Heavy metal analysis of the experimental soil samples was analysed by the following standard procedure of Gupta [18]. Di-acid digestion ($HClO_4$ and HF) of soil samples at 200-220^{0}C followed by addition of HNO_3. Diluted and filtered solution was used for the quantitative determination of heavy metals using Atomic Absorption Spectrophotometer (Shimadzu, Model: AA-7000).

For the estimation of proline, plant tissue was homogenized in 3% aqueous sulphosalicylic acid and the filtrate was treated with glacial acetic acid and acid ninhydrin and kept in the boiling water bath for 1hour and then cooled rapidly in ice bath and extracted with toluene and the absorbance of the toluene layer was measured at 520 nm wavelength using a UV-VIS Spectrophotometer (model 118, Systronics, India). From the proline standard curve, concentration in sample was calculated and expressed in mg/g FW.

For ascorbic acid estimation, the plant tissue was homogenized in 4% oxalic acid and the brominated supernatant was made up to 25 mL with 4% oxalic acid. Then 1 mL of brominated sample diluted to 3 mL with distilled water followed by the addition of 1 mL DNPH (2,4 Dinitrophenyl hydrazine reagent) and 1-2 drops of thiourea and mixture was incubated at 37°C for three hours. After incubation, the orange-red ozazone crystals were dissolved in 7 mL of 8% H_2SO_4 and measured the absorbance at 540 nm wavelength in the UV-Vis spectrophotometer (Systronics 118). From the standard curve (L. ascorbic acid) concentration in the sample was calculated and expressed in mg/100g FW.

For the estimation of total phenol, centrifuged the ethanolic extract of the plant tissue at 10,000 rpm for 20 minutes, and the supernatant was evaporated to dryness. Then the residue was dissolved in distilled water and treated with Folin's reagent and Na_2CO_3 solution. Then the mixture was incubated in boiling water bath for a minute, cooled and then measured the absorbance at 650 nm wavelength. Total phenol content in the sample found out from the standard graph (catechol, A.R grade).

For the estimation of Total Free Amino acids, ethanolic extract of the plant sample was centrifuged at 3000 rpm for 5 minutes. Then the filtrate was treated with diluent (water and n-propanol in 1:1 ratio) and incubated the mixture at room temperature for 15 minutes and measured the absorbance at 570 nm using a UV-VIS Spectrophotometer (Systronics 118). Concentration of total free amino acids in the test samples were calculated from the standard curve (L.leucine).

Statistical analysis was carried out using IBM SPSS Statistics 25.0 (X64). To compare the effect of different concentrations of sewage sludge amendment on vetiver plants, one-way ANOVA was used.

Results and Discussion

Physico-chemical characteristics of sewage sludge

Various chemical characteristics and heavy metal content in the sewage sludge samples are tabulated (Table 2). The results show that sludge samples showed slightly acidic pH (5.16 ± 0.8) with low electrical conductivity (2.01 ± 0.6 mS/cm). The chloride content of sludge sample was 3.91 ± 1.2 mg/g. The organic matter content of sewage sludge used for the study was 23.1 ± 1.1%. On a dry weight basis, sewage sludge contains an average of 3.67 ± 0.42% total nitrogen, 2.21 ± 0.35% total phosphorous, 1.76 ± 0.28% sodium, 0.98 ± 0.43% potassium, 1.21 ± 0.33% calcium and 1.46 ± 0.41% Magnesium.

Sl. No.	Parameters	Value
1	pH	5.16 ± 0.8
2	Electrical Conductivity (mS/cm)	2.01 ± 0.6
3	Chloride (mg/g)	3.91 ± 1.2
4	Organic Matter (%)	23.1 ± 1.1
5	Total Nitrogen (%)	3.67 ± 0.42
6	Total Phosphorous (%)	2.21 ± 0.35
7	Sodium (%)	1.76 ± 0.28
8	Potassium (%)	0.98 ± 0.43
9	Calcium (%)	1.21 ± 0.33
10	Magnesium (%)	1.46 ± 0.41
11	Cadmium (µg/g dry wt.)	3.16 ± 0.03
12	Chromium (µg/g dry wt.)	26.7 ± 0.01
13	Lead (µg/g dry wt.)	34.8 ± 0.95
14	Zinc (µg/g dry wt.)	214.6 ± 1.12
15	Arsenic (µg/g dry wt.)	1.6 ± 0.02
16	Iron (µg/g dry wt.)	12547.6 ± 1.42
17	Copper (µg/g dry wt.)	359.6 ± 1.01

Table 2: Physico-chemical Characteristics of Sewage sludge (Values given are mean ± SD of three replicates).

Heavy metal analysis of sewage sludge revealed that the sludge contained plenty of various toxic heavy metals. Sludge contained 3.16 ± 0.03 µg Cd/g dry wt., 26.7 ± 0.01 µg Cr/g dry wt., 34.8 ± 0.95 µg Pb/g dry wt., 214.6 ± 1.12 µg Zn/g dry wt., 1.6 ± 0.02 µg As/g dry wt., 12547.6 ± 1.42 µg Fe/g dry wt., and 359.6 ± 1.01 µg Cu/g dry wt. The heavy metal content in the sludge is in the order: Fe > Cu > Zn > Pb>Cr > Cd > As.

Appreciably higher concentrations of various nutrients such as nitrogen, phosphorous, sodium, potassium, calcium, and magnesium in the sewage sludge make it suitable for agricultural uses while high concentration of heavy metals, which is a major cause of concern.

Changes in the activity of non-enzymatic anti-oxidants

Assessment of the effect of different concentrations of sewage sludge amendment on the non-enzymatic antioxidant activity in the experimental vetiver plants were carried out and presented in table 3.

Result showed that activity of the selected non-enzymatic antioxidants such as proline, ascorbic acid, and total free amino acid content was high in roots compared to that of leaves in the experimental plants. But in the case of total phenol, concentration was high in leaves than that in the roots. It was also noted that with an increase in the concentration of sewage sludge application, activities of all the selected antioxidants was also increased significantly ($p < 0.05$). It has been understood that the antioxidant activity was minimum in the leaves of control plants and maximum in the roots of T4 group plants.

Experimental Groups	Proline (mg/g FW)		Ascorbic acid (mg/g FW)		Total Phenols (mg/g FW)		Total Free Amino Acids (mg/g FW)	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
C	40.07 ± 0.21^a	42.34 ± 0.11^a	26.37 ± 0.31^a	29.55 ± 0.15^a	13.21 ± 0.01^a	1.89 ± 0.15^a	0.96 ± 0.05^a	2.89 ± 0.13^a
T1	46.23 ± 0.13^b	48.91 ± 0.11^b	27.82 ± 0.15^a	28.96 ± 0.12^a	15.82 ± 0.05^b	4.66 ± 0.05^b	2.41 ± 0.02^b	4.66 ± 0.02^b
T2	48.58 ± 0.12^b	51.63 ± 0.01^b	28.38 ± 0.21^a	31.68 ± 0.05^b	16.37 ± 0.14^b	9.37 ± 0.22^c	4.68 ± 0.15^c	5.71 ± 0.20^b
T3	53.65 ± 0.22^c	55.77 ± 0.11^c	27.64 ± 0.12^a	31.92 ± 0.13^b	19.43 ± 0.02^c	11.21 ± 0.25^d	5.63 ± 0.02^d	7.21 ± 0.25^c
T4	59.61 ± 0.23^d	61.66 ± 0.15^d	29.73 ± 0.25^a	32.76 ± 0.16^b	21.66 ± 0.03^c	15.44 ± 0.01^e	9.22 ± 0.11^e	11.31 ± 0.05^d

Table 3: Changes in the activity of non-enzymatic anti-oxidants.

Proline is a heterocyclic amino acid found abundantly in basic proteins. Accumulation of free proline is also an indicator of heavy metal induced stress tolerance to protect the plant from oxidation. It functions as an osmolyte due to its antioxidant and osmoprotective properties, aids in the restoration of pigments and stress tolerance through osmoregulation and stabilization of protein synthesis, macromolecules and organelles, protects enzymes from denaturation, and also provides nitrogen and energy for recovery growth [19]. Higher proline content in the roots may be due to higher heavy metal induced stress, as the roots of the plants are in close contact with heavy metals.

Ascorbic acid is considered as a powerful antioxidant because of its ability to donate electrons in several enzymatic and non-enzymatic reactions. They are involved in the scavenging system since the reaction consumes molecular oxygen, rather than hydrogen peroxide. Elevated concentrations of ascorbic acid on sewage sludge amendment may be due to heavy metal stress in plants suggesting that it plays an important role in detoxifying the ROS generated under heavy metal stress with scavenging of H_2O_2, a reaction catalysed by APX. Ascorbic acid protects critical macromolecules from oxidative damage [20]. According to Sinha., *et al*. [21] the important antioxidants such as cysteine, proline, ascorbic acid and non-protein thiol (sulfhydryl) have a very significant role in detoxification of toxic metal ions. Singh., *et al*. [22] reported that ascorbate is a ubiquitous soluble antioxidant in photosynthetic organisms and the most important reducing substrate for H_2O_2 detoxification. Antioxidative defence system provides a rationale for its use as a stress marker.

Increasing the biosynthesis of phenolic compounds in plants subjected to heavy metal stress helps to protect plants from oxidative stress [23,24]. Phenolic compounds are considered beneficial antioxidants that can reduce the toxic effects of heavy metals by raising plant tolerance and scavenge ROS in plants exposed to stress factors [25-27]. Therefore, the concentration of phenols in plant tissues is a good indicator to predict the range of tolerance to the stress factors that occur in plants [28].

The changes in the concentration of total Free Amino Acids indicated that they are involved in the responses to heavy metal stress. Free amino acids (AA) play an important role in the detoxification of toxic elements [29]. According to Zhu., *et al*. [30] greater accumulation of free amino acids in heavy metal stress is an indication of stress tolerance to neutralize the impacts of ROS.

Redox metabolism abnormalities brought on by heavy metal toxicity in plants result in oxidative damage, which is defined by the increased formation of reactive oxygen species (ROS). Plants have evolved biological detoxification and defence mechanisms that prevent the cellular components from being oxidised to reduce the harmful effects of ROS. Ascorbic acid (AA), phenolic compounds, amino acids, and proline, which play the most important and effective roles in detoxifying ROS, are included in the antioxidant defence activities of plants. Changes in their activity are frequently used to predict metal tolerance [31].

Antioxidants play a protective function in stress conditions by neutralizing radicals before they damage cells. One of the early plant responses to heavy metal stress is the production of ROS in chloroplasts, mitochondria, and peroxisomes and it damages the physiological and biochemical pathways, resulting in decreased cell membrane stability, photosynthesis efficiency, hormonal and nutrient imbalances, inhibition of DNA replication, gene expression and cell division [25,32,33]. According to Swapna., *et al*. [34] even in hyper accumulator and hyper tolerant plant species as in other organisms, higher concentrations of toxic chemicals and heavy metals can severely impair central metabolic processes. So, the study proved that use of sewage sludge as an amendment increased the non-enzymatic anti-oxidant activities in exposed vetiver plants. This is a sign of ROS generation brought on by dangerous heavy metals, as well as plants' capacity to detoxify these ROS and their tolerance to stress.

Conclusion

The study suggest that non-enzymatic antioxidant activity can be used as a biomarker of heavy metal toxicity in vetiver plants and the vetiver plants growing in sludge-amended area have potentially developed efficient defense strategy to combat excess ROS as a result of heavy metal-induced oxidative stress. Results strongly suggest that vetiver plants can tolerate higher levels of heavy metal toxicity and therefore, the highly tolerant vetiver plants (*Vetiveria zizanioides*, L. Nash) may be used effectively for phytoremediation purposes.

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Conflicts of Interest

The authors declare no conflict of interest.

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