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Variation in antioxidant potential of *Vigna unguiculata* grown in pure and amended soil

Zohaib Saeed[1,2], Shahid Iqbal[1], Umer Younas[1], Muhammad Pervaiz[2,3], Syed M. Bukhari[4], Asma Zaidi[4]*

[1]Department of Chemistry, University of Sargodha, Sargodha, Pakistan

[2]Department of Chemistry, Government College University, Lahore, Pakistan

[3]Department of Chemistry, The University of Lahore, Lahore, Pakistan

[4]Department of Chemistry, COMSATS University Islamabad, Abbottabad Campus, 22060, KPK, Pakistan

**Corresponding author: majidbukhari@cuiatd.edu.pk*

Abstract

The variation in antioxidant capacity of Vigna unguiculata was determined by carrying out a pot experiment in pure and amended soil. The soil amendments were done with various percentages of sewage sludge, and the suitable percentage of sewage sludge in the soil was assessed for agricultural purpose. *V. unguiculata* was grown in separate pots with varying soil amendments, including control soil, 20%, 40%, 60% and 80% sewage sludge proportion in the soil and with pure sewage sludge. The contents of grown plants were extracted by solvent extraction, and all extracted samples were subjected to analyses in order to determine the growth parameters and antioxidant capacity variations among them as a factor of soil amendments. The antioxidant contents increased in a linear fashion in all plant parts when grown in soil amendments possessing 20%, 40% and 60% sewage sludge proportion. However, in the cases of the plants grown in 80% sewage sludge soil amendment and in pure sewage sludge, the antioxidant contents decreased considerably. This can be attributed to the enhanced oxidative stress caused by higher concentrations of metal ions present with in the sewage sludge. Plants showed significant improvement in antioxidant potential assessed by a DPPH assay, ABTS+ scavenging activity and lipid peroxidation method up to soil amendments with 60% sewage sludge proportion. On the other hand, the antioxidant ability showed a decreasing trend at higher proportions of sewage sludge in the soil.

Keywords: Agriculture; antioxidant activity; contents in plant; sewage sludge; soil.

1. Introduction

The rising global population requires more goods and resources, which require more industrialization to meet the needs. The downside of this rapid industrialization is that the industries produce hazardous waste products and generally discharge them into local water channels such as streams, canals and rivers. All of this waste can be regarded as sewage sludge. The sewage sludge is the residual material which is produced as byproduct during sewage treatment of

industries and municipal water. It exists as a semi solid substance. One of many challenges for environmental scientists is to safely dispose of this unwanted sewage sludge (Singh *et al*., 2011). Currently in Pakistan, the sludge is dumped, burned, put in landfills or disposed of in water channels. Putting the sewage sludge in landfills is expensive whereas the burning and water release is a serious health hazard for human beings and wildlife. Therefore, scientists are developing comparably efficient and economical means to dispose of sewage sludge (Marti *et al*., 2009). Many researchers have experimented with mixing sewage sludge with soil in different proportions. The mixture is then applied on agricultural fields in order to observe whether it helps plants grow, its possible antioxidant potential on plants, and to identify any impacts it may have on soil nutritional capabilities. It is possible to significantly protect agricultural soil by adding sewage sludge to it. Its properties and antioxidant capabilities are also improved (Tewari *et al*., 2008).

Several important nutrients are present in sewage wastes including organic matter, nitrogen, phosphorous, nutritional microorganisms and trace metals (Antolin *et al*., 2005). These nutrients play a vital role in improving soil fertility. The application of sewage sludge in agriculture generally results in vital organic compounds and nutrients being recycled. However, the sewage sludge should be mixed with soil in limited quantity while growing food production (Ahldberg *et al*., 2006). In addition, the proper combination of soil type and plant species is essential to growing healthy crops with promising characteristics. This factor must be taken into account before choosing the optimum amount of sewage sludge as a fertilizer in agriculture (Singh & Agarawal, 2008). When plants are grown in soil that has a sewage sludge amendment, they have an increase in antioxidant potential (Nagajyoti *et al*., 2010).

The focus of the present study is on *V. unguiculata* (Cowpea). This plant was selected for cultivation in several sewage sludge soil amendments ranging from 0-100% proportion of sewage sludge. *V. unguiculata* is used as food and a prominent constituent of Asian diet. It is an important source of protein. It grows in semi-arid areas and is also known locally as “poor man’s meat.”

The objective of this study was to grow *V. unguiculata* in several amendments of soil and to find the optimum mixing ratio of sewage sludge to soil that could provide the crop with promising nutritional, antioxidant and functional attributes. This work is unique as it is discussing the effects of soil amendment using sewage sludge on *V. unguiculata*.

2. Methodology

2.1 Sample collection

The urban areas of Sargodha (a densely populated city of Pakistan) were selected for sewage sludge collection. For this purpose, the sampling was performed at a central drainage outlet, which is the disposal and accumulation site for all waste from industrial and residential areas around Sargodha. A team from the local municipality helped with sample collection. Non-reacting bags made of plastic were used to store the samples. All samples were safely transported to the University College of Agriculture at the University of Sargodha in Pakistan.

2.2 Soil amendments with sewage sludge

Several proportions of sewage sludge were mixed with soil. For this purpose, sandy soil from agricultural land of the University College of Agriculture was selected. Several combinations of sewage sludge with soil were prepared in varying proportions. The ratio of sewage sludge were 0%, 20%, 40%, 60%, 80% and 100% in the soil. The mass of each soil amendment prepared was 10 kg. Three pots for plant growth were designated for each sewage sludge soil amendment. Designating three pots to each sewage sludge and soil amendment was necessary to carry out experiments in triplicate. There were six soil amendments, including pure soil and pure sewage sludge. Therefore, the total number of pots was 18, and each pot consisted of 4 to 5 plants. All plants were grown for three months in an outdoor environment, under sun, and with the same biotic and abiotic factors.

2.3 Determination of antioxidant capacity

Antioxidant capacity of the plants grown in amendments of soil with sewage sludge (ranging from 0-100% proportion of sewage sludge) was determined by using multiple methods. These included the determination of total phenolic and flavonoid contents, ascorbic acid content, radical and ABTS+ scavenging potentials along with lipid peroxidation.

2.4 Determination of total phenolic content (TPC)

The Folin–Ciocalteu reagent was used for the determination of the total phenolic content. This determination was carried out using methanol extracts obtained from roots, stems, leaves and fruits of the plants which were grown in soil amended with sewage sludge. For this purpose, 200 µL of methanol extract were used separately for the roots, stems, leaves and fruits of the plants. In each extract sample, 142 mL of 7.5% sodium carbonate were added along with 800 µL of Folin–Ciocalteu reagent. In each reaction mixture, 7.0 mL of distilled water were added to obtain a final volume of 150 mL for each reaction mixture. All reaction mixtures were kept in the dark at room temperature for 2 hours. All absorbance was measured at 765 nm on a Cecil-7200 CE double beam spectrophotometer. The gallic acid standard was used, and all total phenolic content results for *V. unguiculata* were presented as gallic acid equivalents (mg/g). All measurements were performed in triplicate (Polshettiwar *et al*., 2007).

2.5 Extraction and evaluation of total flavonoid content (TFC)

To extract total the flavonoid content from different parts of *V. unguiculata*, a total weight of 10 g from each plant sample was subjected to extraction with 200 mL of 80% methanol. The process was completed over five hours by using a Soxhlet extractor. Next, the samples were filtered and centrifuged at 3000 rpm for 20 min. The supernatant layer was used to estimate the total flavonoid content.

In order to determine the total flavonoid content in roots, stems, leaves and fruits of *V. unguiculata*, a colorimeter assay was employed. For this analysis, double distilled water (4.0 mL) along with 1.0 mL of diluted plant extract was poured into a flask. Directly after, 0.6 mL of NaNO2 (5%) was added in the flask. After 5 min., a total volume of 0.5 mL of aluminium chloride (10%) and 2.0 mL of 1.0 M sodium hydroxide were added. The contents of each flask were diluted to 10 mL by adding distilled water. Absorbance of the resulting pink colored solution was noted at 510 nm. The results were determined as epicatechin equivalents (mg/g). The determination was conducted in triplicate as adopted by Marinova *et al*. (2005).

2.6 Quantitative determination of ascorbic acid

The indophenol titration method was employed in order to evaluate the ascorbic acid content of plant samples. The homogenization and extraction of samples was carried out in a metaphosphoric acid solution. Vitamin C was titrated at pH 0.6 against 2, 6-dichlorophenol-indophenol solution in the presence of formaldehyde. This was a reported titration method. The end point of this titration method was a pink color as described by Favell (1998).

2.7 Radical scavenging ability

Free radical scavenging potential was evaluated by DPPH assay using extracts from roots, shoot, leaves and fruits (Bukhari *et al*., 2014). A total volume of 5.0 mL freshly prepared solution of DPPH was added to 2.0 mL volume taken from the plant root extract. The procedure was repeated with extracts from the shoots, leaves and fruits. The absorbance (λmax) for all sample extracts was recorded at 515 nm at intervals of 0, 0.5, 1.0, 2.0, 5.0, and 10.0 minutes. The calibration curve was plotted for each sample extract in order to determine the radical scavenging potential.

2.8 Scavenging assay (ABTS+)

The ABTS+ radical cation scavenging assay was used in the present study. The ABTS+ radical cation was prepared by passing a 2, 2'- azino-bis (3- ethylbenzthiazoline-6-sulphonic acid) aqueous solution (5.0 mM) through MnO2. A 0.2 mm fisher brand membrane was used to eliminate extra MnO2 from the filtrate. Extracted samples were diluted with a 5.0 mM phosphate buffer saline (PBS, pH 7.4). The dilutions were adjusted to show 0.700 (±0.020) absorbance at 734 nm in a standard cell. A mixture of 1.0 mL from each extract along with 5.0 mL of ABTS+ radical cation solution was prepared, and absorbance was recorded after 10 minutes and at ambient temperature for each sample. The phosphate buffer solution (PBS) was taken as the control. Trolox was used as the standard for the calibration curve. The antioxidant potential for all samples under study was reported as the Trolox equivalent g-1 of plant tissue (Iqbal *et al*., 2007).

2.9 Lipid peroxidation

The malondialdehyde (MDA) content was determined in order to measure lipid peroxidation. It is the product of lipid peroxides reacting with thiobarbituric acid (TBA). Malondialdehyde cannot stand pH less than 7 (Zornoza *et al*., 2002). A total volume of 2.0 mL of trichloroacetic acid (TCA–TBA–HCl reagent: 15.00% (w/v) TCA; 0.37% (w/v) TBA and 0.25 mM HCl) was used in the extraction of 0.1 g of the plant sample. All extracts from plants were placed in a water bath at 90 0C for 30 minutes. The removal of the flocculent precipitate was carried out by centrifugation at 11,000 rpm for 10 minutes at 4 0C. Then the samples were cooled. The absorbance of all extracts was corrected for non-specific turbidity (Iqbal *et al*., 2005).

3. Results and discussion

3.1 Total phenolic content

Antioxidant potential determines the ability of a plant to resist and control oxidative stress which can be caused by free radicals (Sinha *et al*., 2007). Total phenolic content determination is a part of phytochemical evaluation for a certain plant under study. Therefore, the total phenolic content of *V. unguiculata* was also determined spectrophotometrically by using a Folin-Ciocalteu reagent. The results were expressed as gallic acid equivalents. Phenolic compounds are believed to play a direct role in anti-oxidative action. Previous studies have focused on the biological activities of plant derived phenolic compounds (Jayaprakasha *et al*., 2007); These phenolic compounds can be potential antioxidants, free radical scavengers, and can reduce the negative impact of active metal ions by chelating those metal ions with the specific structure of phenolic compounds (Jayaprakasha *et al*., 2007). The total phenolic content of *V. unguiculata* is shown in Figure. 1.

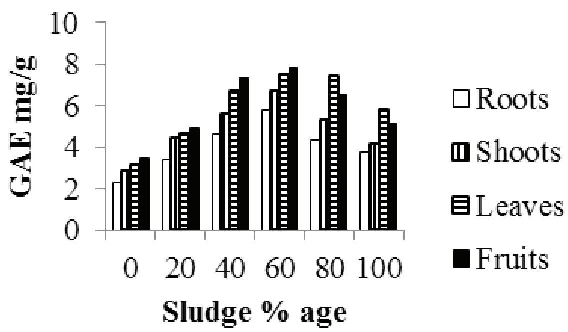

Fig 1. Variation in total phenolic content, gallic acid equivalent (GAE mg/g), of various parts of Vigna unguiculata grown in different amendments of sewage sludge and soil.

The results reveal that in roots, shoots and fruits, the maximum phenolic content was found with the soil possessing 60% of the sewage sludge proportion, whereas the phenolic content decreased in the soil possessing 80% and 100% sewage sludge proportion. In the case of leaves, the phenolic content was directly proportional to the increasing sewage sludge content in the soil up to 80% proportion. However, the phenolic content was less in the pure sewage sludge. In fact, metal ions (Cr= 29-52 ppm, Pb= 16-80 ppm, Fe= 15-76 ppm, Cu= 14-205 ppm, Zn= 54-655 ppm, Ni= 19-27 ppm determined by atomic absorption spectroscopy) were found to be present in the sewage sludge which is known to generate free radicals, peroxides and superoxides. These species can cause cellular toxicity along with unwanted morphological changes in the plant. In order to overcome such circumstances, a built-in antioxidant defense system is present within the plants. At lower proportions, the metal ions present in the sewage sludge do not show any negative impact on the phenolic content of the plant; it is due to the fact that the metal ions get scavenged by the chelating action of the built-in antioxidant defense system of the plant (Hassinen *et al*., 2011). On the other hand, an increase in the concentration of metal ions within the sewage sludge (80% and 100%) results in the induction of severe stress on the plant. As a consequence, a defensive antioxidant system of the plant turns out to be insufficient and fails to counter these generated species within the plants grown at higher proportions of the sewage sludge. It results in the decrease of total phenolic content in all parts of the plant. The plants have a threshold level to inhibit the oxidative stress induced by the metal ions. When the concentration of the metal ions surpasses this level, it may cause deteriorating effects on the plant nutrients as well as antioxidant attributes (Nagajyoti *et al*., 2010). Therefore, it reveals that under higher proportions of sewage sludge, the total phenolic content decreased in all parts of *V. unguiculata*. This decrease can be attributed to the over production of free radicals as well as peroxides by the metal ions.

3.2 Total flavonoid content

Flavonoids (polyphenolic compounds) are found in vegetables, fruits, and beverages. They possess beneficial biochemical and antioxidant properties. The accumulation of heavy metals in the plants results in the production of reactive oxygen species (ROS). These ROS possess carcinogenic and nephrotoxic activities. The flavonoids are considered natural antioxidants on the basis of their metal binding property. Flavonoids can reduce the production of free radicals and ROS (Bukhari *et al*., 2013). Therefore, determining the flavonoid content in the plants under study was necessary in order to find out the ability of the plant to act as a potential source of free radicals and ROS scavengers. Total flavonoid content in *V. unguiculata* was expressed as epicatechin equivalents (Figure. 2). The results clearly show that when *V. unguiculata* was grown in soil possessing 60% sewage sludge, it showed a maximum flavonoid content in all parts of the plant compared to the control and other soil types possessing lower percentages of sewage sludge.

However, the flavonoid content decreased significantly in all parts of the plant grown under higher proportions of sewage sludge (80% and 100%). In fact, the oxidative stress induced by metal ions in the plant grown in amended soil was enhanced due to the production of ROS.

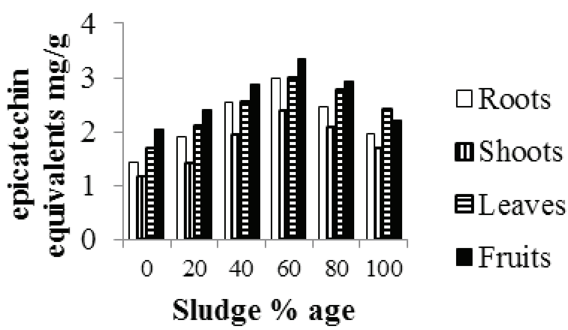

Fig. 2. Variation in total flavonoid.

content, epicatechin equivalent (mg/g), of various parts of Vigna unguiculata grown in different amendments of sewage sludge and soil

To counter this oxidative stress, plants exhibit different metabolic variations which include the higher production of flavonoids to overcome the induced stress. It means that the antioxidant attributes of the plant improve under stress conditions. However, this improvement lasts up to a certain limit. The plants grown under higher proportions of sewage sludge results in an excessive generation of ROS, and an increased production of free radicals occur due to the high content of metal ions. This disturbs the metabolism of the plant. It ultimately leads towards a lower flavonoid content in the plant (Michalak *et al*., 2006).

3.3 Total ascorbic acid content

Ascorbic acid has the ability to scavenge free peroxyl radicals. It can also inhibit cytotoxicity induced by oxidative stress. The deficiency of ascorbic acid in plants may lead to severe biological disorders. The ascorbic acid content is known to increase under stress conditions (Bukhari *et al*., 2013). Therefore, the total ascorbic acid content determination was carried out in order to establish the ability of *V. unguiculata* as a potential inhibitor of cytotoxicity induced by oxidative stress. The results indicated that the total ascorbic acid content increased with the increase in the proportion of sewage sludge (Figure. 3). This shows that a sewage sludge increase can be associated with an increase in scavenging ability.

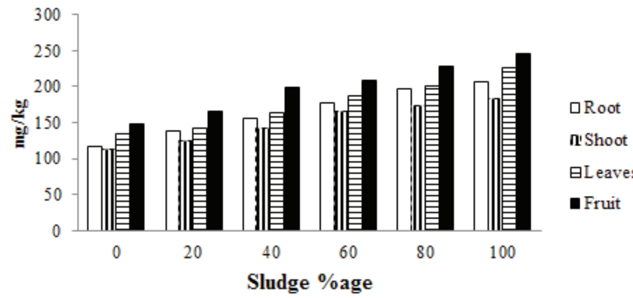

Fig. 3. Variation in ascorbic acid content in different parts of Vigna unguiculata grown in different amendments of sewage sludge (mg kg-1).

3.4 Radical scavenging assay

The DPPH is well known for its stability and is normally used for the determination of free radical scavenging potential and antioxidant potential of several botanical resources (Pietta, 2000). The presence of a nitrogen atom in the center of a DPPH radical is responsible for its ability to act as a free radical in a solution showing λmax at 517 nm. The components possessing an antioxidant ability within the samples donate hydrogen and the transform DPPH radical to 1-diphenyl-2-picrylhydrazine. This donation of the hydrogen atom forms a stable complex among DPPH and antioxidant components existing within the sample under consideration. As a result, the reduction in color intensity of the DPPH solution occurs, and this change in color intensity helps to evaluate the antioxidant ability of the sample.

Free radical scavenging activity of methanol extracts from the roots, shoots, leaves, and fruits of *V. unguiculata* was measured. All extracts from different parts of the plant revealed considerable a scavenging ability of up to 60% soil amendment with sewage sludge (Figure. 4). This means that the soil possessing a 60% sewage sludge is the most suitable one because at this proportion of sewage sludge, plant parts showed their maximum DPPH scavenging ability. On the other hand, at higher proportions of sewage sludge, this scavenging ability decreased in all extracts from different parts of *V. unguiculata*.

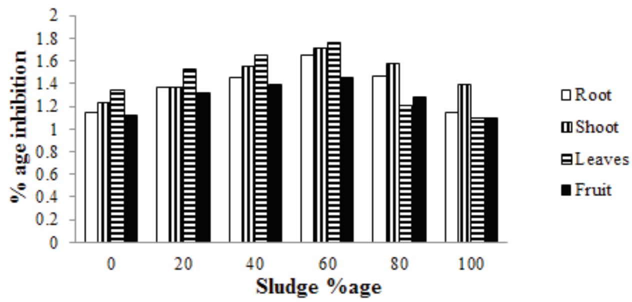

Fig. 4. Variation in DPPH radical scavenging activity in different parts of Vigna unguiculata grown in different amendments of sewage sludge and soil.

3.5 Scavenging activity (ABTS+)

The scavenging of the stable ABTS+ radical cation (2, 2'-azino-bis (3-ethylbenzo thiazoline-6-sulfonic acid) is one popular method used to measure the antioxidant ability of an unknown sample. The ABTS+ behaves as a peroxidase substrate possessing the ability to become oxidized by hydrogen peroxide. As a result, it produces a metastable radical cation. The comparative capability of hydrogen donating moieties in order to scavenge ABTS+ radical cation is determined at 734 nm (Ozgen *et al*., 2006). The radical scavenging ability revealed that the plant contains components presenting similar activity towards active radical species present in biological systems (Ozgen *et al*., 2006). The scavenging potential of the methanol extracts from *V. unguiculata* is presented in Figure. 5. It has been observed that ABTS+ scavenging ability in all extracts from different parts of the plant increased up to the specimen with 60% sewage sludge proportion in the soil. On the other hand, at higher proportions of sewage sludge with soil, it decreased significantly. Therefore, the maximum antioxidant ability was recorded when plants were grown in soil with 60% sewage sludge.

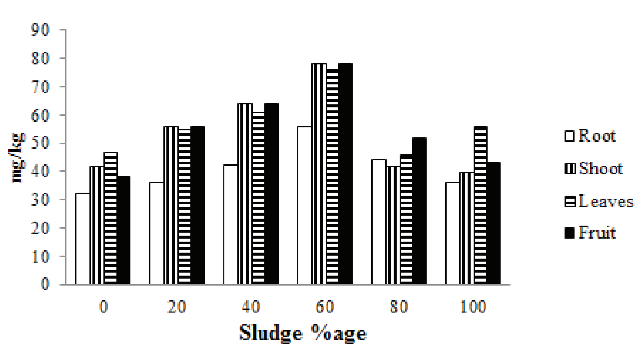

Fig.5. Variation in ABTS+ scavenging activity of extracts from different parts of Vigna unguiculata grown in different amendments of sewage sludge and soil (mg kg-1).

3.6 Lipid peroxidation

The lipid peroxidation causes death of biological cells by the oxidative damage of lipids in cell membranes. Lipid peroxidation increases with an increase in the uptake of heavy metals by plants and results in the accumulation of malondialdehyde (MDA). The measurement of lipid peroxidation was carried out in *V. unguiculata* in terms of malondialdehyde (MDA) for determining the extent of variation in lipid peroxidation in plant extracts grown in different proportions of sewage sludge in soil. Figure. 6 clearly shows that the MDA content in all parts of *V. unguiculata* increased with the increasing proportion of sewage sludge in the soil, which is due to the increase in the free radical production under metal stress. A similar trend is reported in the literature for leaves and shoots of different plants (Sinha *et al*., 2009; Bharagava *et al*., 2008).

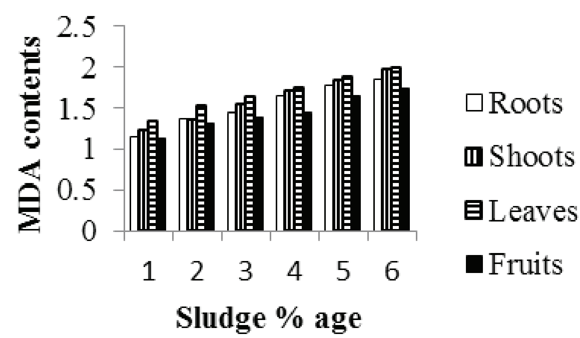

Fig. 6. Lipid peroxidation (MDA content) of various parts of Vigna unguiculata grown in soil, sewage sludge as well as in amended soil with different proportions of sewage sludge.

4. Conclusion

Results of the present study showed that *V. unguiculata* grown in different amendments of sewage sludge is well implemented as the concentration of antioxidants was enhanced. It has the ability to reduce the destruction triggered by unwanted oxygen species. However, such toxicity appears when the generation of these ROS surpasses the quenching ability of the normal antioxidative defensive mechanism. Analysis of data supported the probability that the immense rise of metallic concentration in higher amendments of the sewage sludge produced a large quantity of ROS which resulted in the oxidative stress. Hence, the oxidative stress reduced the level of antioxidants in all parts of *V. unguiculata* grown in higher soil amendments of sewage sludge proportion (80%) and also in pure sewage sludge. Therefore, the present study reveals the importance of sewage sludge in the agricultural industry for its potential use as a fertilizer. Amendment of sewage sludge with soil, up to a certain level, enhanced the antioxidant attributes in all parts of the plant. Soil amendment with 60% sewage sludge proved to be the most appropriate ratio because it showed the maximum increase in the antioxidant capacity in all parts of *V. unguiculata*.

The safe, economical and beneficial disposal of sewage sludge is recommended by the researchers. It should also be noted that the suitable ratio of soil amendment with sewage sludge determined in this study is specific to *V. unguiculata*. The suitable soil amendment proportion with sewage sludge may vary for other plants from other species.

This unique research has helped to determine suitable growth conditions for the important food crop, *V. unguiculata*. Adding these results to previous research will improve farming knowledge.

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اختلاف إمكانية استخدام مضاد الأكسدة لنبات اللوبياء الظفرية في التربة النقية والمعدلة

ذهيب سعيد، شاهد اقبال، عمر يونس، محمد برفايز، سيد م. بخاري، أسماء زايدي

قسم الكيمياء، جامعة Sargodha ،sargodha باكستان

قسم الكيمياء، الجامعة الحكومية، لاهور، باكستان

قسم الكيمياء، جامعة COMSATS، اسلام اباد، باكستان

ملخص

يناقش البحث فكرة اختلاف القدرة المضادة للأكسدة للوبياء الظفرية التي تم تحديدها باستخدام تجربة الأصيص في تربة نقية وتربة معدلة. ويشير البحث إلى أن تعديلات التربة تم إجراؤها باستخدام نسب مئوية مختلفة من حمأة مياه الصرف الصحي، وتم تقييم النسبة الملائمة لحمأة مياه الصرف الصحي في التربة من أجل غرض الزراعة. تم زراعة اللوبياء الظفرية في أصص منفصلة وبتعديلات مختلفة للتربة تشمل التحكم في التربة ونسبة حمأة مياه الصرف الصحي تصل إلى 20% و40% و 60%و80% في التربة، وكذلك حمأة مياه الصرف الصحي النقية. تم استخلاص محتويات النبات النامي عن طريق المذيبات المستخلصة وتم إخضاع كل العينات المستخرجة لعدد من التحليلات بغرض تحديد محددات النمو وكذلك متغيرات القدرة المضادة للأكسدة فيما بينها كعامل لتعديلات التربة. ومن خلال البحث، تم ملاحظة زيادة المحتويات المضادة للأكسدة بشكل خطي في كافة أجزاء النباتات التي نمت في تربة معدلة تصل نسب حمأة مياه الصرف الصحي فيها ما بين 20% و 40% و60%. بينما انخفضت بشكل كبير المحتويات المضادة للأكسدة في حالة النباتات التي نمت في تربة معدلة تصل نسبة حمأة مياه الصرف الصحي فيها إلى 80% وكذلك الحال في حمأة مياه الصرف الصحي النقية، ويرجع ذلك إلى الاجهاد المؤكسد المعزز والذي تسببه التركيزات الأعلي لمعدن الحديد الموجود في حمأة مياه الصرف الصحي. كما يشير البحث إلى أن النبات أظهر تحسنا كبيرا في إمكانية مضادات الأكسدة والتي يقدرها فحص الـ DPPH و +ABTs ودهون بيروكسيد وهي الطريقة التي تصل بتعديلات التربة لنسبة 60% من حمأة مياه الصرف الصحي. وعلي الجانب الآخر، أظهرت القدرة المضادة للأكسدة اتجاهًا تنازليًا بنسب مرتفعة لحمأة مياه الصرف الصحي في التربة.