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Nickel, drought stress, anatomy, morphology, *Cucurbita pepo* L.

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Assessment of Morphoanatomical Modifications in *Cucurbita pepo* L. in Response to Combined Drought and Nickel Stress

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Abstract

Background: Nickel (Ni) is a toxic heavy metal and causes human health risks as well as plant abnormalities. The present study aimed to determine morpho-anatomical features of *Cucurbita pepo* L. var. fastigata under combined drought and Ni stress in greenhouse experiment.

Methods: Seedlings were treated with four irrigation levels to induce drought stress 14 ml, 11.2 ml, 8.4 ml and 5.6 ml (abbreviated as S0, S1, S2 and S3) along with 25 ppm of Nickel.

Results: The results were analyzed after 5th and 10th days and data revealed that seed germination rate and stem diameter was significantly ($P \leqslant 0.05$) increased while stem length, root length, root diameter, the number of secondary roots, leaf area, fresh weight, and dry weight were decreased. Among anatomical characteristics epidermis, xylem, phloem tissues, cortex, trichomes, medullary rays, endodermis, pericycle, palisade, mesophyll cells, and stem mid rib were adversely affected under water stress (S1 and S2) while plants under combined Ni stress showed significant ($P \leqslant 0.05$) reduction in cortex diameter, and increase in epidermis thickness, vascular bundles size except for S3 treatment level.

Conclusion: It was concluded that combined drought and Ni stress positively affected the morphological features (roots, stem length, and leaf area) of the *Cucurbita pepo* plant as compared to separate water and Ni stress, preventing plant wilting.

Introduction

In agriculture both water and heavy metals stress has both positive and negative effects on the anatomy of plants including vegetables like marrow, *Cucurbita pepo* var. fastigata. Marrow belongs to the Cucurbitaceae family. It has a light-greenish appearance. Water stress can reduce the growth of plants causing the distribution of natural flora and affecting their performance more than any others environmental factor. It is considered the major factor affecting respiration, stomatal movement, and photosynthesis and thus restricts plant growth which results in less agricultural production. It may lead to food scarcity [1]. Drought stress may be the result of various factors such as sufficient water uptake due to shallow soil, low and mercurial rain, high rate of transpiration or less supply of water towards roots [2,3]. Plants show various changes at molecular, physiological, cellular, morphological, and anatomical levels in response to abiotic stresses, especially drought stress [4, 5]. Guerfel et al. revealed that tissues that were exposed to conditions of low water availability had small sized cells along with thickened vascular bundles and cell walls [10]. It was also observed that plants in response to water stress show various anatomical adaptations like an increase in epidermis, cortex and xylem vessel diameter along with the increase in sclerenchyma fibers in the phloem of the stem under drought stress [2, 6, 7].

Over the last few decades, heavy metal stress has received greater attention among all the abiotic stresses [8, 9] which cause environmental contamination mainly due to industrialization, and also due to agricultural practices, traffic activities as well as mining and ore processing [10-12]. Heavy metals such as arsenic (As), cadmium (Cd), lead (Pb), and nickel (Ni) are toxic even at low concentrations as are non-biodegradable and make related to the group of metalloids and metals having a relatively density greater than 4 gcm-3 [13-15]. There are 53 naturally occurring heavy metals some of which are essential and others non-essential to plants [16]. Ni is an essential element that contributes in metabolic activities and plays the role as an enzyme (urease). While a catalyst in enzymes it helps the legumes fix nitrogen. Change in concentration of Ni in plants from the required range which is between 0.05-5 ppm (WHO) can cause an effect on morphology as well as the anatomy of plants. Previous study revealed that both metal (Ni, Cu, Co, and Cr) and water stresses in red maple (*Acer rubrum* L.) reduced the vessel density in stems and vessel size in roots while only metal stress reduced the stomatal density and chlorophyll content [17].

Several studies have focused on the response of plants to single stress in the last decades but in the field experiments at different regions globally, plants are subjected to two or more abiotic stresses simultaneously [18, 19]. In-addition, *Cucurbita pepo* is widely used as salad globally [20]. Industrial and mining activities release toxic metals, and contaminate the arable fields nearby, and cause human health risks [21]. Therefore, the *Cucurbita pepo* was selected as the research model plant to check the growth and anatomical features of the plant in response to combined drought and Ni stress.

Methods

Seed collection and experimental trial

The seeds of the experimental plant *Cucurbita pepo* L. were collected from local the market of Okara, Punjab Province, Pakistan. The whole experiment was confined to 15 days and a mixture of sand and clay was used as the soil in a ratio of 1:1. Seeds were surface sterilized with H_2O_2 (10%) for 15 minutes and sown in the soil below 1 cm of the soil surface by exposing their tips to sunlight after soaking them in water for 24 hours. 5 seeds were sown at 4 cm distance in each pot in randomized complete block design (RCBD) in four replicates. After the sprouting of seeds 48 pots were divided into two experiments, one as water stress (as control, T) and another as an experiment treated with 25 ppm of Ni metal (Tm). Each experiment consisted of 4 levels of water as S0 S1, S2, and S3 which represented 14 ml, 11.2 ml, 8.4 ml, and 5.6 ml correspondingly.

Analysis of experiment

After two selected intervals i.e. 5th (A1) and 10th (A2), day of treatment experimental sets was analyzed for different morpho-anatomical analyses of *Cucurbita pepo* L. Germination of seeds was observed regularly till most of the seeds germinated i.e., the 5th day of the experiment.

Morphological study

The parameters like length, the diameter of stem and root, number of secondary roots, and leaf area were measured and analyzed after 1st and 2nd intervals of stress while fresh weight and dry weight were analyzed at the end of the experiment.

Anatomical study

For the anatomical study, cross sections of roots, stem, and leaf were taken from three different points (apex, center and base) of roots, stem and leaf, and were stained with Safranine and Fast green. The prepared were examined under Compound Light Microscope and photographed.

Statistical Analysis

After the collection of data for different morphological traits, Statistix 8.1 was used with one-way ANOVA.

Microsoft Excel 16 was used to develop figures and graphs.

Seed germination	T					Tm				
	D1	D2	D3	D4	D5	D1	D2	D3	D4	D5
	3.00 a	3.25a	3.25 a	4.00 a	4.00 a	2.50 a	4.25 b	4.25 b	4.50 b	4.50b
	1.50 a	3.25 b	3.50 a	4.00 b	4.00 b	2.00 b	3.50 a	3.50 a	4.25 a	4.25 a
	2.25 a	4.25 b	4.25 a	4.50 a	4.50 a	2.00 b	3.50 a	3.50 b	3.75 b	3.75 b
	2.25 b	3.00 b	3.25 b	3.25 b	3.50 b	2.75 a	4.25 a	4.25 a	4.00 a	4.00 a
	2.25 b	3.25 b	3.50 b	3.75b	4.00 b	2.25 a	4.00 a	4.25 a	5.00a	5.00 a
	3.00 a	4.00 a	4.25 a	4.25 a	4.25 a	2.00 b	3.75 b	3.75 b	4.00 b	4.00 b

Table 1: Average rate of seed germination (%) of *Cucurbita pepo* L. (n=4). Different lowercase letters denote a significant difference ($P \leq 0.05$) while similar letters indicate the non-significant difference among treatments. An abbreviation represents, T = Control, Tm= Experimental set with Ni metal stress, D1= Seed germination after 1st day D2= Seed germination after 2nd day, D3= Seed germination after 3rd day, D4= Seed germination after 4rth day, D5= Seed germination after 5th day

Results

On the base of transverse sections (Figure 1) internal features of leaf, stem, and root were studied under combined water and Ni metal treatments.

The anatomy of the leaf showed the presence of trichomes on both upper and lower epidermis, healthy cells of both spongy and palisade parenchyma, and scattered xylem and phloem tissues in the unstressed plant (T), while in plants treated with Ni metal number of trichomes decreased, midrib region became flattened and width of all cells of mesophyll, xylem, phloem, cortex, and epidermis was reduced, and length increased (S0). Lower epidermis and xylem and phloem cells also got damaged. Plants under treatment S1 revealed damaged and rough lower epidermis containing ridges and furrows, squeezed midrib area, damaged xylem tissue and under treatment S2 trichomes number reduced significantly showing presence on mid rib region, upper and lower epidermis similar to leaf as under S1 treatment and damaged and small size xylem and phloem tissues. In the case of S3 treatment trichomes number increased, cells of lower epidermis got damaged but midrib region, cells of palisade and spongy mesophyll and xylem and phloem tissues increased significantly as compared to all S0, S1 and S2 treatments.

In case of stem without any treatment (T) cells of epidermis, cortex, xylem, and phloem tissues were healthy. Oval-shaped air passage, the large number of trichomes, and medullary ray area were also observed. Under treatment of Ni metal, (S0) stem anatomy showed thickened epidermis, broad area and damaged cells of the cortex, increase size of xylem and phloem tissues, decreased width of medullary ray while trichomes were thick and large in number. Under S1 treatment thickness of the epidermis increased further, cells of the cortex were squeezed, the size of damaged vascular bundles (xylem and phloem) became small and trichomes were thin and prominent while the breadth of the medullary ray increased.

Similar results were observed under the S2 treatment except for damaged epidermis and xylem and phloem tissues further decreased in size due to which medullary ray width increased. The number of trichomes decreased substantially and some cortical cells formed the air passage due to death. Under S3 treatment stem anatomy revealed a thin epidermis, the broad area of the cortex and air passage, enlarged sized xylem and phloem and the emergence of new bundles, and a large number of trichomes while some cortical cells became damaged.

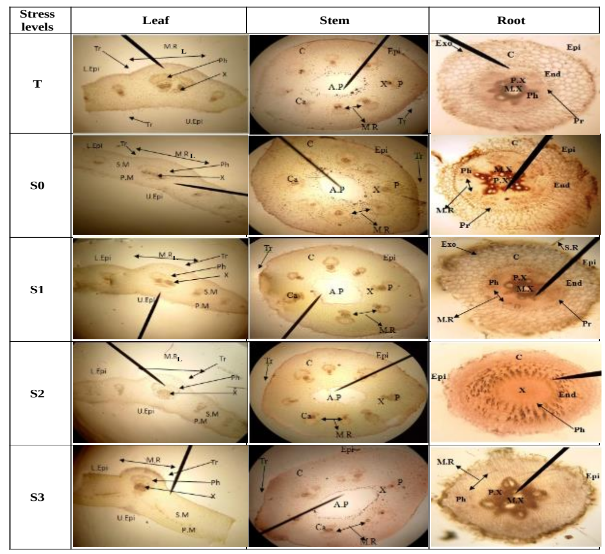

Figure 1: Anatomical view of cross sections of different parts of Cucurbita pepo after 10 days of combined Ni metal and drought stress, Abbreviations represents U. Epi (Upper Epidermis), P.M (Palisade Mesophyll), S.M (Spongy Mesophyll), X (Xylem), P.X (Protoxylem), M.X (Metaxylem), Ph (Phloem), L. Epi (Lower epidermis), M.RL (Mid Rib of leaf), Epi (Epidermis), Exo (Exodermis), End (Endodermis), Pr (Pericycle), C (Cortex), Ca (Cambium), M.R (Medullary Ray), A.P (Air Passage), Tr (Trichomes).

The root anatomy revealed thickened walled cells of the epidermis, diffused exodermis, large and oval-shaped parenchyma cells of the cortex, prominent endodermis and pericycle centered metaxylem, radiated protoxylem surrounded by phloem in the plant supplied with 14 ml water (T). In case of plant under S0 some cells of epidermis, cortex and exodermis became damaged, protoxylem had taken central potion and metaxylem was radiated. Medullary rays were formed due to the separation of phloem tissues. Under S1 treatment root anatomy indicated thick-walled but damaged cells of the epidermis and centered metaxylem, radiated protoxylem, cortical, and exodermis cells were also damaged. The size of xylem and phloem tissues became small and the area of medullary rays increased in breadth while under S2 treatment all cells of the epidermis, exodermis, cortex, endodermis, pericycle, and vascular bundles (xylem and phloem) got extremely damaged and the distinction between protoxylem and metaxylem lost due to their diffusion.

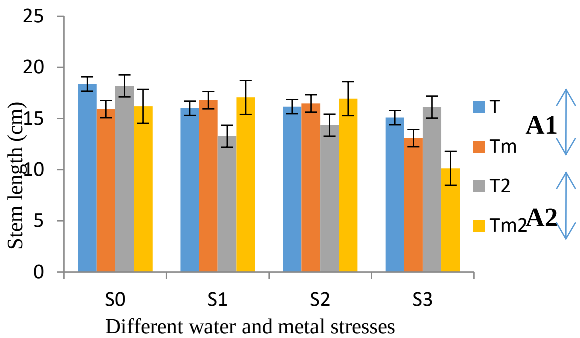

Figure 2: Average length of stem

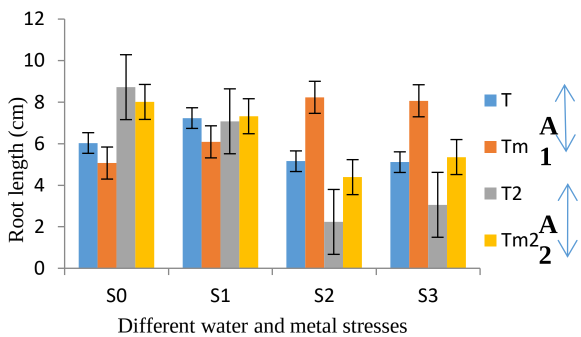

Figure 3: Average root length

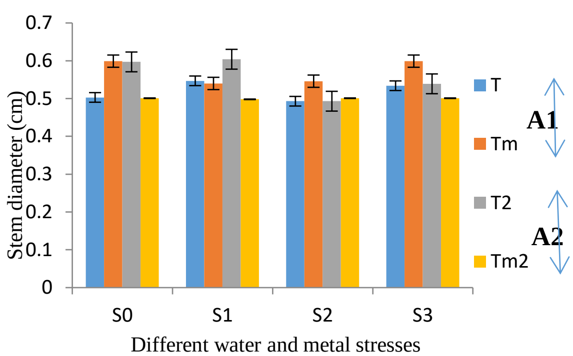

Figure 4: Average diameter of stem

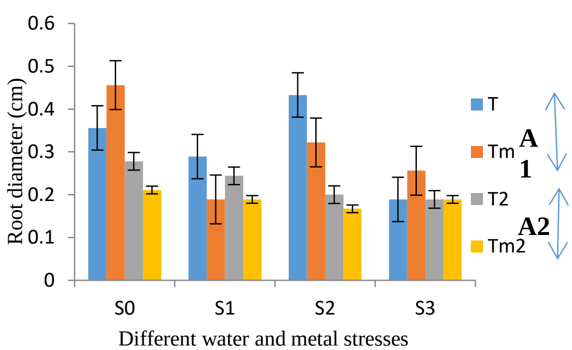

Figure 5: Average root diameter

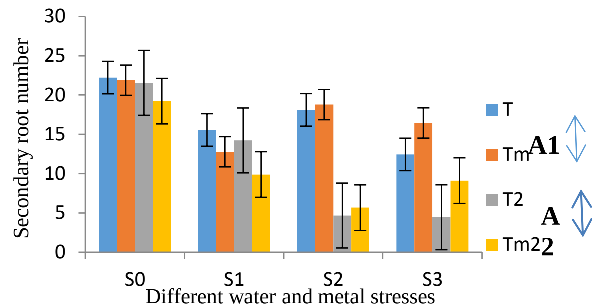

Figure 6: Average number of secondary roots

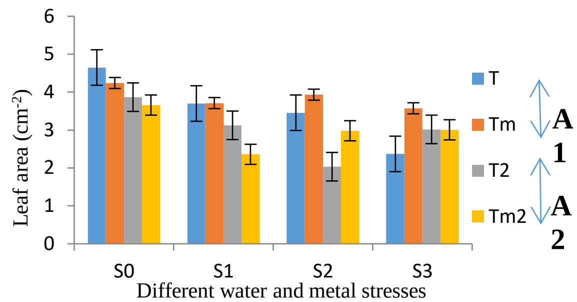

Figure 7: Average leaf area

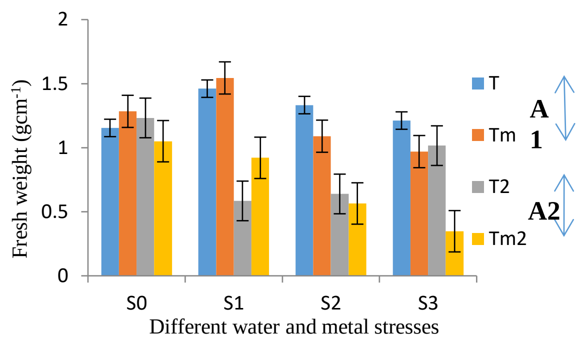

Figure 8: Average fresh weight

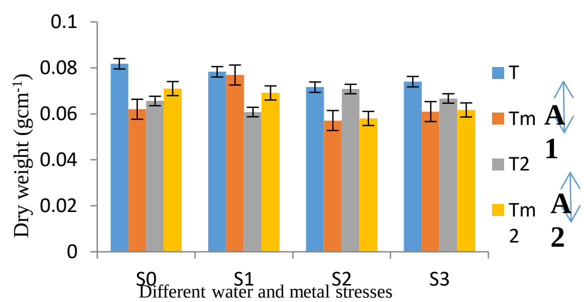

Figure 9: Average dry weight of *Cucurbita pepo* L.

Figure 2-9: Graphical representation of morphological characters of root, stem and leaves of *Cucurbita pepo* L. under different water and metal stress. An abbreviation represents. A1= Analysis of Experimental set after 5 days of treatment, A2= Analysis of Experimental set after 10 days of treatment, T = Experimental set without any metal stress, Tm= Experimental set with Ni metal stress S0 = Experimental sets under 0% water stress, S1= Experimental sets under 40% water stress, S2= Experimental sets under 60% water stress, S3= Experimental sets under 80% water stress

However, root anatomy under S3 treatment showed an absence of exodermis, endodermis and pericycle, damaged, and thick-walled epidermal cells, damaged cortex, expanded medullary rays and enlarged, and healthy xylem (protoxylem and metaxylem) and phloem tissues.

In the present research work, the anatomical features of leaf, stem and root were studied under combined water, and metal treatment. This is the first study on the anatomy of *Cucurbit pepo* L.

The hypodermis and xylem and phloem tissues are deformed and damaged due to metal and water treatment (S1 and S2). The pericycle was observed thicker under S2 treatments as compared to the control. Under metal treatment (S0) mesophyll cell size, and cortical cell area increased while lower epidermis thickness, the thickness of midrib, and diameter of xylem vessels decreased in *Cucurbita pepo*. Under combined metal and water treatment (S3) cortical cell area, xylem and phloem tissue size and trichome number and thickness increased and were not damaged. As the water level decreased combined with Ni metal (S3) trichome density, the number of mesophyll cell and size of vascular bundles (xylem and phloem tissues) increased. These tissues were not damaged.

Discussion

Climate change and water scarcity, as well as heavy metal pollution, have become global issues especially in terms of the yield of food crops [22]. The effect of Ni heavy metal and water stress on morphology and anatomy of Cucurbita pepo has been studied in this study. The seed germination increased under both metal and drought stress and these results are consistent with the previous studies [23]. T experiment showed the best results of seed germination because in the T experiment *Cucurbita pepo* received no metal (Ni) and drought stress. Plants remain healthy and therefore best germination.

The average stem length was increased under combined stress but reduced in response to water stress. These outcomes were confirmed by various researchers previously [24,25] in soybean, young maize and Capsicum annum plants respectively. An increased root growth due to water stress was reported in sunflower [26], *Catharanthus roseus* [27] maize and wheat [28].

The diameter of roots was enhanced in the experiment. Contrarily, the root diameter of maize, and rice decreased in response to water stress[29,30]. A decrease in the number of roots was observed in young maize plants under drought stress [29]. The leaf area in Sorghum was significantly reduced due to water stress [31]. At an extreme level of drought stress with heavy metal the fresh weight was increased sharply and an increase in biomass under Cu and Pb heavy metal stress was reported in *Zea mays* L. [32]. Sridhar et al also reported similar results in *Brassica juncea* [33]. The negative effect of combined stress on the average dry weight of plant was reported and that result was dissimilar to the experimental results of Gomes et al and Ahmad et al. [34, 35].

The observed seed germination and positive effects in Cucurbita pepo are attributed to high levels of antioxidative enzymes and translocation of nutrients from root to shoot. The vegetable accumulates high amount of Ni suggesting the name hyperaccumulator to plant. The high yield of hyperaccumulator, *Cucurbita pepo*, can help in reducing, and curing diabetes, high cholesterol levels and the obesity like diseases which are spreading day by day in the world.

The changes in the anatomy of roots, stems, and leaves reveal the water status and physiological functions of plants in response to abiotic stress. The size of the xylem and phloem tissues, cortex area, and epidermis thickness decreased and were damaged in various studies also [36, 37]. A decrease in diameter and cross-sectional area of the root, central cylinder, the thickness of cortex, midrib, upper epidermis, and vascular bundles was observed by various researchers [38, 39].

The hypodermis and xylem and phloem tissues are deformed and damaged due to metal and water treatment (S1 and S2). The same features were observed in root stem and leaf due to the accumulation of Cd, Pb, and Hg in stellar and hypodermal regions in *Bruguiera sexangula* [40]. The pericycle was observed thicker under S2 treatments as compared to the control which was similar to the results of Benkova et al. [41].

The effect of metal treatment on the anatomy of plant was found similar to the results of several researchers [42-44]. Makbul and his fellow reported the increase in epidermis thickness and vascular bundles in root, stem, and leaf under water stress [22] but this study indicated the reverse results under the extreme water deficit level (S3) [45]. Under Cd stress cortex, vascular bundles and trichomes were positively affected in *Cenchrus ciliaris* were revealed by Mukhtar and his colleagues [43]. In experimental sets under 80% water stress (S3) trichome density, number of mesophyll cells, and size of vascular bundles (xylem and phloem tissues) increased. This increase may be due to the beneficial effects of water on *Cucurbita pepo*.

The anatomical features related to trichome density, number of mesophyll cells and size of vascular bundles were also revealed by various researchers in *Lotus creticus*, *Solanum melongena* and *Cynodon dactylon* under water stress only but in the damaged form [46, 47, 48]. The increase in these features and their healthy condition may be adaptation of *Cucurbita pepo* under increased water deficit level and metal stress together.

It was concluded that the combined effect of water and metal stresses significantly ($P \leqslant 0.05$) increased the rate of seed germination and average length of stem while fresh weight and dry weight seems to be decreased. The stem diameter initially decreased but then increased in S3 treatment (combined water and metal stresses). In case of anatomy vascular bundles size, cortical cell area, trichome density and epidermis thickness in all plant parts decreased and damaged under separate stresses but increased and found healthy under S3 treatment (combined Ni and Drought stresses). An inverted behavior was observed in all parameters except average length of stem in S3 treatment of combined stresses. These changes might be the adaptations for *Cucurbita pepo* plant to the changing environment. Therefore, it could be perceived that combined drought and Ni stress positively ameliorates the morphological and anatomical features of *Cucurbita pepo* as compared to separate water and metal stress, preventing wilting and other losses. The results suggested that the Cucurbita pepo plant can be used for Ni soil remediation and can decrease the agricultural soil pollution.

Competing Interest

The authors declare that they have no conflict of interests.

Author Contributions

Saira Bano conducted experiment, Syeda Anjum Tahira supervision and refinement of experiment, Muhammad Ibrahim data analysis and evaluation, Shaheena Umbreen technical support, Saba Younas assist in drafting experiment, Sana Tahir editing and technical support, Shafiq ur Rehman reviewing and editing, Sidra Abdul Ghani reviewing.

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