MJFAS
Malaysian Journal of Fundamental and Applied Sciences

Ungadau *et al.* | Malaysian Journal of Fundamental and Applied Sciences, Vol. 19 (2023) 616-622

RESEARCH ARTICLE

Morphological and Biochemical Responses of *Vigna unguiculata* ssp. *sesquipedalis* in Different Zinc Concentrations

Fenny Ungadau, Raihana Ridzuan, Fazilah Abd Manan*

Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia, 81300 UTM Johor Bahru, Johor, Malaysia

Abstract Zinc is an essential trace element required by plants. However, high zinc concentrations can lead to environmental pollution and plant toxicity. This research aimed to investigate how plants respond to different concentrations of zinc (0, 100, 200 and 300 ppm) in soil, using *Vigna unguiculata* ssp. *sesquipedalis* (Yard long bean) as a model plant. A total of 12 parameters were collected including plant morphological characteristics such as plant height, leaf number, yield, root length, pod length and fresh weight. Furthermore, key biochemical properties including chlorophyll content, total protein content, total phenolic and flavonoid content in plants were analyzed, in addition to soil pH and electrical conductivity. These parameters were used to determine the morphological and biochemical responses of plants under zinc-stress conditions. The results indicated that different concentrations of zinc significantly decreased the leaf number and pod length *V. unguiculata*. Soil electrical conductivity was significantly high at 200 ppm zinc. Significant changes in total protein were observed in stems and pods. Moreover, the total phenolic content in leaves showed a significant increase with higher zinc concentrations, while the opposite trend was observed for total phenolic content in *V. unguiculata* pods. In summary, varying concentrations of zinc had a significant impact on various morphological and biochemical properties of *V. unguiculata,* exhibiting a distinct pattern specific to each organ. This suggests that *V. unguiculata* is responsive, adaptive and capable of tolerating abiotic stress induced by a broad range of zinc concentrations.

Keywords: *Vigna unguiculata,* Zinc, Physico-chemical properties, Soil properties.

Introduction

There are many biotic and abiotic factors that affect plant growth and development. Among the abiotic factors are drought, salinity, temperature and heavy metals. Metals such as copper (Cu), zinc (Zn) and iron (Fe) are the essential components for metabolic processes linked to various biochemical and physiological purposes in living organisms including plants [1]. Unlike animals, plants are sessile and need specific defense mechanisms to avoid or exclude stress factors. Nevertheless, plants are both sensitive and resistant to the aforementioned factors depending on their level of exposure as well as their unique defensive systems [2]. Plant survival under rapidly changing environments has sparked interest in studying the stress responses of plants. These responses are produced as a result of chemical alteration that occurred at the molecular and cellular level, as well as the physiological level. In addition, other factors such as the physico-chemical parameters of soil such as moisture, water content and pH value regulate and aid plants' ability to absorb nutrients available in the soil. It also affects plant stress response considering excessive essential and non-essential nutrients can result in plant toxicity [3,4].

Zn is one of the micronutrients that play various roles in plants. It serves as an enzyme cofactor for proteins and is involved in membrane integrity and stabilization [5]. Zn assists the plant system to adopt the symplastic or apoplastic pathway to allow nutrient movements to the xylem [6]. Zn also involved in nitrogen metabolism, phytohormone regulation, act as a defense system against oxidative stress, and plays a crucial in photosynthesis [7]. Zn concentrations in the soil are often found in the range of 150 and 300 ppm which are ten-fold concentrations from 15 to 30 ppm are assumed as a permissible limit in plant tissues [5]. However, hyperaccumulator plants have been found to take up to 10,000 ppm Zn in their aerial parts [5]. Abnormalities can occur in plants exposed to excessive or insufficient levels of Zn. Visual signs of high Zn levels in leafy plants are typically observed in young leaves, where a yellow or purplish-

***For correspondence:**
m-fazilah@utm.my

Received: 8 Nov. 2022
Accepted: 27 July 2023

red color become visible [8]. In the mature leaves, necrosis may occur, leading to a reduction in the number of leaves. Other than that, when Zn reaches their threshold level than their sufficient level in plants, the symptoms in morphology changes as shown through the plant height, root length and fresh weight are common during the treatment period and after plant harvest. Excess Zn ions can inhibit the uptake of other nutrients, resulting a decrease in efficiency of cellular functions and other regulations. Production of Reactive Oxygen Species (ROS) as the consequence of the high concentrations of heavy metals accumulating in plant tissue could lead to oxidative stress formation which eventually causes damage to cellular tissue, thus affecting plant growth [9]. Activating defense mechanisms is important for the plant when facing this phytotoxic condition.

Vigna unguiculata ssp. *sesquipedalis* (*V. unguiculata*), also known as asparagus bean, Chinese long bean or common bean belongs to the family of Fabaceae but is often preferred as Leguminosae ranked as subspecies of *sesquipedalis* [10]. *V. unguiculata* is a good source of essential amino acids, low carbohydrates, and fibre-rich plant food [10]. *V. unguiculata* is an annual climbing flowering vegetable with indeterminate growth habits, requiring support for its pods which tend to grow in pairs [11]. In both medical and pharmaceutical industries, *V. unguiculata* extracts is utilized in the formulation of therapeutic drugs as it contains phytonutrients and antioxidants which are useful in preventing damage at the cellular level. *V. unguiculata* can survive without fertilizer or supplement feeding since it is able to fix nitrogen from the air on its own [12].

Unfortunately, nowadays, environmental contamination due to anthropogenic activities has largely affected our water, air and land, including agricultural soil. Zn contamination may occur due to excessive use of phosphate fertilizer, fungicides and burning of fossil fuels. Since plants are able to accumulate Zn in their roots, the phytotoxic effects might harm other living organisms including animals and humans. Synergistic impact of *V. unguiculata* with mercury-reducing bacterium strain MELD1 has also been suggested as one of the phytoremediators for mercury removal [13]. Therefore, ensuring food safety is necessitates serious attention. This study aims to determine the response of *V. unguiculata* grown in soil treated with different concentrations of Zn, examining both its morphological traits and biochemical properties.

Materials and Methods

Planting Materials

In this experiment, *V. unguiculata* ssp. *sesquipedalis* plant was used (Figure 1). The seeds were sown in a seedling tray and allowed to grow for one week before being transplanted into pots. Approximately 3 kg of organic soil were weighed and filled in each pot with the size of (12 cm width 12 cm height, 12 cm, 12 cm length). On day-30, *V. unguiculata* plants were treated with different concentrations of Zn solution at 0, 100, 200 and 300 ppm and the plants were harvested after a month.

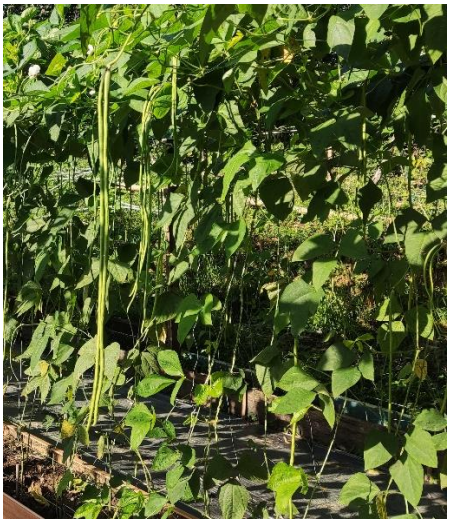

Figure 1. *Vigna unguiculata* ssp. *sesquipedalis* plant

Morphological Traits and Chlorophyll Content

Plant height, leaves number, root length, pod length, pod number, and fresh weight were determined. Plant heights were measured using a rope, followed by height estimation using a measuring tape. The root and pod length were directly measured using a centimeter ruler. The leave number and pod number were manually counted including the young leaves and pods. An analytical balance was used to weigh the fresh weight of *V. unguiculata*. The chlorophyll content in *V. unguiculata* leaves was measured using the soil plant analysis development (SPAD) meter provided in the laboratory.

Soil Physico-chemical Properties

Soil pH was determined using a digital pH meter to assess the hydrogen ions concentration as it is one of the factors influencing plant growth and Zn ion uptake. After harvesting, the soils were collected in a separate plastic bag and used for electrical conductivity estimation. The electrical conductivity (EC) value was estimated using the digital EC meter. Approximately 150 g of soil was weighed into a glass jar for each treatment, mixed with 100 ml of distilled water and left to stand for 10 mins. The EC meter electrode was immersed in the solution until the value stabilized and then recorded. The unit of EC is µS/cm.

Protein Extraction and Protein Assay

Protein extraction was performed according to Wang *et al*. (2006) with some modifications [14]. The leaves, stems, roots and pods were grounded separately. After 0.2 g of powder was obtained, it was then transferred into a 2 ml microcentrifuge tube. One ml of 10% TCA/acetone (10% v/v) was added and mixed well by vortexing. It was then centrifuged at 15,000 g for 4 min at 4°C. The supernatant was removed by careful pipetting. One ml of 80% methanol and 0.1 M ammonium acetate was added. After being vortexed, it was centrifuged again at 15,000 g for 4 min at 4°C. The supernatant was then removed. The next step was the acetone wash by filling the tube with 1 ml of 80% acetone. It was vortexed until the pellet was fully dispersed, centrifuged and the supernatant was removed as above. The samples were air-dried at room temperature for 10 min to remove residual acetone. In the protein extraction and precipitation step, 0.6 ml of phenol reagent and 0.6 ml of SDS buffer as starting material were added into the samples, thoroughly mixed and incubated at room temperature for 5 min. Then, the mixture was centrifuged again at 15,000 g for 4 min under 4°C. The upper phenol phase was transferred into a new microcentrifuge tube of about 0.4 ml and filled with 0.8 ml of 80% methanol containing 0.1 M ammonium acetate and was stored at -20°C overnight. It was then centrifuged again on the second day at 15,000 g for 6 min under 4°C. The supernatant was removed, and a white pellet was visible. The obtained pellet was then washed once with 1 ml of 100% methanol and once with 1 ml of 80% acetone. For each washing step, it was followed by vortexing and centrifugation as above. The pellet was air-dried briefly and then dissolved in an SDS sample buffer. Protein quantification was performed using the Bicinchoninic acid (BCA) method using Bovine Serum Albumin (BSA) for the standard calibration curve. The total protein in the leaves, stem, roots and pod was determined using spectrophotometer at 562 nm.

Methanol Extraction, Total Phenolic and Total Flavonoid Assay

The leaves, stems, roots and pods were dried in the oven for 6 days until it reached their constant weight. The extracts were obtained following Mohankumar *et al*. (2018) method with slight modifications [15]. About 0.3 g of leaves, stem, roots and pod were extracted by mixing with 1 ml of 80% methanol. The mixture was then placed in an incubator shaker at 50°C for 2 hours at 200 rpm. Next, it was centrifuged at 1000 g for 15 min under 4°C. The supernatant was then transferred into new microcentrifuge tubes and wrapped with aluminum foil before keeping it under -4°C.

The phenolic compound in the leaves, stems, roots, and pods of *V. unguiculata* was determined by using Folin-Ciocalteu's reagent and gallic acid was used for the standard preparation [16]. The phenolic content in these parts was calculated using the linear equation from the prepared gallic acid standard curve. Extract solution, 50 µl was pipetted into the 15 ml falcon tube, mixed with 2.5 ml of Folin Ciocalteu's reagent and let stand for 5 minutes. Then, 2 ml of 7% sodium carbonate was added and shaken thoroughly. The mixture was then incubated for 30 minutes at room temperature. Absorption was determined using spectrophotometer at 765 nm.

Flavonoid content in the leaves, stems, roots and pods of *V. unguiculata* was determined by the colorimetric method using aluminium chloride and quercetin as the standard [17]. The total flavonoid content in the mentioned *V. unguiculata*'s parts was calculated using the equation obtained from the prepared standard quercetin. Extract, 5 µl was taken and pipetted into the falcon tubes. Next, 150 µl of 5% sodium nitrate was added then let stand for 5 minutes. After that, 150 µl of 10% of liquid aluminium chloride and let stand for another 5 minutes. The mixture was then added with 1 ml of 1 M sodium

hydroxide and the mixture was diluted to a volume of 5 ml with double distilled water. Absorption was determined using spectrophotometer at 510 nm.

Statistical Analysis

Experiments were carried out in triplicates and data are expressed as mean ± standard deviation of the mean. Data were statistically analyzed by one-way ANOVA using Statistical Package of Social Science (SPSS) software (IBM, version 27) with the least significant difference test, $p \leq 0.05$. The difference was then further analyzed by post-hoc test using the Duncan Multiple Range Test (DMRT) test to obtain the ranking group of each treatment by the independent variables to provide a presentable graph for each parameter studied.

Results and Discussion

Morpho-physiological Traits of *V.unguiculata* and Soil Physico-chemical Properties

Table 1 shows the morphological characteristics of *V. unguiculata* treated with different Zn concentrations after four weeks of treatment. The leave number of *V. unguiculata* treated with 300 ppm Zn was significantly lower than the control, 100 and 200 ppm Zn treatments. Similarly, the pod length of *V. unguiculata* at 300 ppm Zn was significantly lower than in the other treatments, although there were no significant differences observed in pod number. Increasing Zn concentrations, did not significantly change plant fresh weight, plant height, root length, pod number and chlorophyll content. However, increasing growth trend for plant height, leaf number, root length, pod length, pod number and fresh weight were observed for plants treated with 100 ppm Zn, before the value reduced again at higher Zn concentrations. Physico-chemical analyses were conducted on the soil for the control treatment and soil treated with different levels of Zn. For soil properties, pH at a neutral or almost neutral values were determined at the end of the experiment. No significant differences in pH value between Zn treatments was recorded. However, the electrical conductivity (EC) showed a significantly high value in soil treated with 200 ppm Zn.

Table 1. *V. unguiculata* ssp. *sesquipedalis* morphological characteristics, chlorophyll content and soil physico-chemical properties after four weeks of zinc treatment

Treatment (ppm)	Parameters				
	Plant height (cm)	**Leaf number**	**Root length (cm)**	**Pod length (cm)**	**Pod number**
0	308.67 ± 72.39^{a}	22.00 ± 01.73^{a}	9.00 ± 03.22^{a}	58.70 ± 04.98^{a}	2.00 ± 0.00^{a}
100	312.67 ± 64.69^{a}	24.00 ± 06.08^{a}	17.77 ± 03.96^{a}	60.40 ± 09.96^{a}	3.00 ± 1.73^{a}
200	252.33 ± 09.47^{a}	22.00 ± 02.89^{a}	11.77 ± 00.91^{a}	60.03 ± 06.95^{a}	2.00 ± 0.00^{a}
300	265.00 ± 12.49^{a}	9.00 ± 03.22^{b}	17.17 ± 06.26^{a}	39.37 ± 08.73^{b}	2.67 ± 0.58^{a}
Treatment (ppm)	**Parameters**				
	Fresh weight (g)	**Chlorophyll content**	**Soil pH**	**Electrical Conductivity (µs/cm)**	
0	42.13 ± 06.72^{a}	43.33 ± 1.20^{a}	6.67 ± 0.57^{a}	730.33 ± 129.56^{b}	
100	46.52 ± 27.19^{a}	43.47 ± 1.98^{a}	7.00 ± 0.00^{a}	821.33 ± 156.86^{b}	
200	39.18 ± 07.59^{a}	37.63 ± 1.55^{a}	6.67 ± 0.57^{a}	1164.33 ± 246.75^{a}	
300	20.26 ± 05.23^{a}	42.53 ± 4.38^{a}	6.00 ± 0.00^{a}	808.67 ± 77.08^{b}	

Note: Data expressed in mean ± standard deviation (n=3). Different letter indicates significant difference using Duncan Multiple Range Test (DMRT) at significant level $P \leq 0.05$

Zn, when sufficiently taken up by the roots is distributed to plant organs to maintain homeostasis and stability of the cellular membranes, producing chlorophyll, regulates gene expression and photosynthesis process [18]. These factors are necessary to produce healthy plants with good morphological traits. The average Zn concentration considered sufficient for optimal plant growth ranged from 25 to 150 ppm, while the toxicity exerted when Zn is more than 400 ppm [19]. At high concentrations of heavy metals, plant fresh weight, dry weight, as well as yield number resulted in reduction as can be observed in spinach [20] and some other species. Plant fresh weight decreased due to plant size minimization, growth inhibition, reduction in the number of leaves and less seed production is also often associated with Zn toxicity [21]. Not just the aboveground tissues, plant roots are also affected by excessive heavy metals. In a previous study using *Arabidopsis thaliana*, primary root length was inhibited by excess Zn, but more lateral roots were found growing vertically [22]. The amount of Zn used in this study did not interfere with

most of *V. unguiculata* morphological traits except for leave number and pod length which significantly reduced at the highest Zn treatment (300 ppm). Some restrictions in molecular and cellular processes including biosynthesis of certain proteins or enzymes during growth might occur, but it was not clear enough to be seen up to the level where it may cause morphological deterioration in this range of Zn used. It shows that *V. unguiculata* has a high Zn tolerance capacity. Soil physico-chemical properties also influence plant growth and development. The appropriate soil pH ranged from 5.5 to 7 assist in the cation exchanges to increase the soil humidity and enhance Zn ion movement in the soil into the intracellular cell [4]. Besides pH, electrical conductivity is a good indicator of nutrient content in the soil. Otterson (2015) [23] and Reade *et al*. (2000) [24] stated that a high concentration of metal ions in the soil medium will proportionally increase its electrolyte conductivity. Thus, the EC value needs to be appropriate as an extremely high value will hamper plant growth.

Biochemical properties of *V.unguiculata*

The protein content in different parts of *V. unguiculata* showed a discreet pattern of Zn impact (Table 2). In the Zn-untreated plant, proteins are increasingly concentrated in the order of Pod < Stem < Root < Leaf. Meanwhile, when Zn have been supplied, the protein was concentrated in the following ascending order of treatment. For plants treated with 100 ppm Zn (Stem < Pod < Root < Leaf); 200 ppm (Pod < Root < Leaf < Stem), and 300 ppm (Stem < Pod < Root < Leaf). Protein content in the stems and pods of *V. unguiculata* were significantly altered by Zn treatment compared to proteins in leaves and roots.

Table 2. Effects of different zinc concentrations on the total protein concentration (µg/ml) in different parts of *Vigna unguiculata* ssp. *sesquipedalis* after four weeks of treatment

Treatment (ppm)	Total protein concentration (µg/ml)			
	Leaf	Stem	Root	Pod
0	1770.31 ± 68.36^{a}	1152.02 ± 33.21^{a}	1447.40 ± 95.10^{a}	967.83 ± 9.16^{c}
100	2120.13 ± 36.49^{a}	1032.18 ± 27.81^{b}	1212.36 ± 42.00^{a}	1162.87 ± 30.85^{a}
200	1881.58 ± 56.16^{a}	2099.97 ± 112.89^{a}	1448.94 ± 88.77^{a}	1106.12 ± 34.42^{ab}
300	1917.73 ± 385.22^{a}	1024.58 ± 12.55^{b}	1404.58 ± 102.41^{a}	1030.74 ± 47.10^{bc}

Note: Data expressed in mean ± standard error (n=3). Different letter indicates significant difference using Duncan Multiple Range Test (DMRT) at significant level P ≤ 0.05

As a cofactor for enzymes, Zn plays a very important role in protein biosynthesis. Proteins have crucial functions in driving the metabolisms networking and interacting with other cellular signalling. Therefore, upon receiving excessive heavy metals than plant needs, their system senses the changes and then activate a particular pathway to eliminate metal ions through the help of transporter protein. Excessive heavy metals also cause plant oxidative stress that affects plant central metabolism [25]. Protein content in different parts of plants including fruit/ pod, stem, root and leaf are affected and may also be due to the changes in protein structure and function. Alteration of protein content in plants indicates the occurrence of the physiological response of plants in heavy metal-stress conditions [26].

The total phenolic and flavonoid content in *V. unguiculata* plant parts is tabulated in Table 3 and Table 4. In comparison with the control, leaves of *V. unguiculata* treated with 100 ppm Zn had significantly higher total phenolics. In contrast, the amount of phenolics in pods was significantly reduced in plants with 100 ppm Zn. Different response pattern between leaf and pods towards zinc concentrations shows that this effect is organ-specific. On the other hand, the impact of Zn concentrations was not significant in the total flavonoid content in all parts of *V. unguiculata.*

Table 3. Total phenolic (µg GAE/g) content in different parts of *Vigna unguiculata* ssp*. sesquipedalis* after four weeks of treatment

Treatment (ppm)	Total phenolic content (µg GAE/g)			
	Leaf	Stem	Root	Pod
0	235.57 ± 30.39^{bc}	139.85 ± 5.29^{a}	454.20 ± 150.18^{a}	1025.74 ± 56.80^{a}
100	414.89 ± 41.61^{a}	266.42 ± 59.84^{a}	378.31 ± 34.86^{a}	298.82 ± 34.39^{b}
200	345.15 ± 52.74^{ab}	173.27 ± 13.32^{a}	442.07 ± 66.92^{a}	850.62 ± 108.25^{a}
300	204.80 ± 28.77^{c}	169.08 ± 24.44^{a}	240.45 ± 60.65^{a}	270.20 ± 43.52^{b}

Note: Data expressed in mean ± standard error (n=3). Different letter indicates significant differences using Duncan Multiple Range Test (DMRT) at significant level P ≤ 0.05

Table 4. Total flavonoid (µg QE/g) content in various parts of *Vigna unguiculata* ssp. *sesquipedalis* after four weeks of treatment

Treatment (ppm)	Total Flavonoid Content (µg QE/g)			
	Leaf	Stem	Root	Pod
0	158.34 ± 05.30[a]	26.65 ± 00.79[a]	27.66 ± 2.39[a]	30.13 ± 2.57[a]
100	177.84 ± 05.02[a]	26.76 ± 00.17[a]	25.69 ± 1.31[a]	32.45 ± 5.59[a]
200	149.35 ± 28.71[a]	26.65 ± 00.30[a]	33.06 ± 0.88[a]	37.26 ± 8.82[a]
300	122.49 ± 18.36[a]	26.19 ± 01.09[a]	29.83 ± 2.54[a]	27.81 ± 0.72[a]

Note: Data expressed in mean ± standard error (n=3). Different letter indicates significant differences using Duncan Multiple Range Test (DMRT) at significant level P ≤ 0.05

The phenolic compound is one of the secondary metabolites produced by plants in response to abiotic stress. Most plants have been shown to accumulate phenolic compounds when experiencing heavy metals toxicity as part of their defense mechanisms [27]. In previous study, potato plants treated with Zn nanoparticles showed an increased amount of non-enzymatic antioxidants including phenolic compounds [28]. The presence of phenolic compounds is important to help plants eliminate ROS, hence maintaining their cellular metabolisms and healthy physical growth, which in this case contributed mainly by the leaf part.

Conclusions

Zn concentration in soil influence plant morphological growth and biochemical properties of plants. *Vigna unguiculata* ssp. *sesquipedalis* is responsive towards different concentrations of Zn up to 300 ppm as shown in their morphological changes (leaf number and pod length) and biochemical properties such as total protein content and total phenolic content in plant organs. Significant changes in total protein content were determined in plant stems and pods. As for the total phenolic content, changes in TPC in plant leaves and pods were detected. For soil physico-chemical properties, the soil pH for all Zn treatments is almost at a neutral level. The soil electrical conductivity increased with increasing Zn concentrations. Since there were no severe effects to many other parameters tested, Zn up to 300 ppm is considered tolerable by *Vigna unguiculata* ssp. *sesquipedalis.* More studies need to be conducted in the future to understand the capacity of *Vigna unguiculata* to take up heavy metals especially Zn when supplied in excess levels to the soil.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

Acknowledgment

The authors expressed their gratitude to the Ministry of Higher Education Malaysia for awarding the Fundamental Research Grant Scheme under grant number FRGS/1/2018/STG05/UTM/02/4, as well as Universiti Teknologi Malaysia.

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