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Research Article

Role of Manganese Forms on Black Gram (*Vigna mungo*) Seedling Growth

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Abstract: The impact of concentration of manganese (Mn^{2+}) forms on early seedling growth and some physiological attributes of black gram (*Vigna mungo* L.) have been reported in the current study. An adequate amount of 22.32 µg/mL (as in control solution) of Mn^{2+} was found to be crucial for proper growth and it also greatly impacts the process of photosynthesis and the amount of chlorophyll in the growing seedling. Reduced growth was observed as the concentration of Mn^{2+} was increased from 50 ppm up to 250 ppm. Reduced growth is due to various non-enzymatic coping mechanisms invoked by the plant to ease the metal stress which has several side effects on key plant growth attributes. One such defense strategy to reduce metal overload is the production of proline that can dilute the excess metal content. Chlorophyll content with respect to the age of the seedling is also studied and it brought interesting results.

Keywords: Mn Toxicity, Growth Analysis, Black Gram

1. INTRODUCTION

Manganese (Mn^{2+}) is a micronutrient metal that is essential for many metabolic, physiological, and growth processes of plants including respiration, synthesis of enzymes and photosynthesis, etc. [1]. Essential enzymes necessary for proper plant growth such as nitrate reductase, and isocitrate dehydrogenase require Mn^{2+} for their biosynthesis [2]. Manganese is also utilized as a cofactor of the Mn^{2+} -dependent-superoxide-dismutase enzyme. It also regulates the synthesis of fatty acids, carotenoids, acyl lipids, and nitrogen metabolism [1, 3, 4]. Manganese also contributes to the functionality of photosystem II (PSII) when water molecules split into oxygen, and also in the protection of photosystem II from photodamage [5].

In comparison with aluminum, copper, and other metals, Mn^{2+} has a higher tendency of mobility for translocation from roots to the upper part of the plant, therefore, the toxicity induced by manganese shows symptoms clearly in the upper parts of plants. The effect of toxicity caused by manganese is greatly attributed to the type of species, age of the plant, environmental temperature and intensity and quality of light, etc. Several studies describe these visual features such as reduced pigmentation, reduced leaves size, and reduced height of the plant caused by manganese toxicity [6-9].

Mn^{2+} is the most stable and water-soluble form of Mn^{2+} present in soil and is responsible for the majority of Mn^{2+} -induced contamination and toxicities. Certain environmental conditions like lower pH of the soil, low quantities of organic matter, and decreased redox potential can greatly increase the risk of Mn^{2+} toxicity. Darkening of leaves, brown spots on leaves, black specks on the stem, and crinkled leaves are some of the most commonly induced Mn^{2+} toxicity. Literature information revealed high quantities of Mn^{2+} in the soil causes impaired carbon dioxide absorption in *Citrus grandis* (Pomelo) seedlings [9], reduced content of chlorophyll A in *Pisum sativum* L. (Common Pea) [10, 11], and *Glycine max* L. (Soybean) [12].

Vigna mungo is mainly grown in the Indian

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subcontinent, comprises a major part of Punjabi cuisine, where it is locally called "urad ki daal" in the local Punjabi language. It is largely being used to make daal from both wholes and split seeds. It is an erect annual herb with dense hairs and sub-erect and trailing branches. These beans were originally placed in the *Phaseolus* genus of the *Fabaceae* family but have recently been transferred to the *Vigna* genus of the legume family because of the difference of the two genera in biochemistry, the structure of pollen, style, and stipules [13].

Literature study on the subject suggests that the effect of manganese on growth parameters in early seedlings and growing plants is important in the understanding pattern of manganese distribution though out the plant body under metal overload, the underlying physiological mechanisms at molecular levels of manganese toxicity, and its tolerance. In this study, we have reported for the first time the role of manganese in two of its common forms found in biological systems (Mn^{+2} and Mn^{+7}), on the growth of early seedlings of *Vigna mungo* as a step forward towards understanding the role of micronutrient-manganese in the development of early seedlings.

2. MATERIAL AND METHODS

Vigna mungo species were used throughout the experiments. The seeds were obtained in a single lot from local seed suppliers in Karachi. The seedlings were grown in Hoagland hydroponic nutrient solution in custom-made hydroponic-styled pots facilitated with lids with some ¼ inch holes approximately 1 inch apart, as shown in Figure 1. All pots were placed in a partial shade environment and as soon as the primary leaves had unfolded, the test solutions of various oxidation states of manganese were introduced in all test pots in concentrations ranging from 50 to 250 parts per million (ppm). Seedlings were grown in a soil-less water-culture half-strength Hoagland solution [14]. For this purpose, analytical grade chemicals were dissolved in double-distilled water with a balanced composition of macro and micro-nutrients to prepare the Hoagland hydroponic culture (Table S1), while Table S2 lists the recipe to prepare the half-strength Hoagland solution.

The growth of the beans was constantly monitored and it was noted that for both test and control pots, the seedling started to grow from the 5^{th} to 7^{th} day of germination. The data was recorded from 10 to 20 days of germination that used primary leaves of the plant, root, and hypocotyl. These were measured, weighed, and dried.

The area of the leaves was simply determined by drawing the outlines of the leaves on paper with the help of a graphite pencil.

The leaves were dried afterward and stored in small glass vials with plastic lids until their chlorophyll concentration per unit area was determined spectrophotometrically. About a half gram of fresh leaves was cut into small pieces and grounded in acetone and was centrifuged at 3000 RPM (rcf = 1107 x g) for about 20 min. Then the supernatant was separated with about 20 ml acetone. The absorbance of samples was measured at wavelengths of 661.6, and 644.8, nanometers for chlorophyll A, and chlorophyll B respectively. The following equations were employed for calculating the quantities of pigments per unit area on the surface of the leaves [15].

Chlorophyll "A" = (11.24 X E661.6) – (2.04 X E 644.8)

Chlorophyll "B" = (20.13 X E644.8) – (4.19 X E 661.2)

E = absorbance wavelength

It is a common observation that the chlorophyll concentration per square area would be greater in the full-sized leaves as compared to younger leaves. When it comes to studying the concentration of chlorophyll in the presence of higher doses of heavy metals, the necessity to study the effect of age naturally arises.

It is very important to know whether there had been a natural increase or decrease in the chlorophyll concentration during the study regardless of the concentration of heavy metal. This study was also important for the fact that etiolated leaves may not regain the chlorophyll level unless there is a soluble carbohydrate supply [1]. Another very important relationship that must be counted during the study of the chlorophyll concentration is that the presence of cotyledons, the chlorophyll concentration

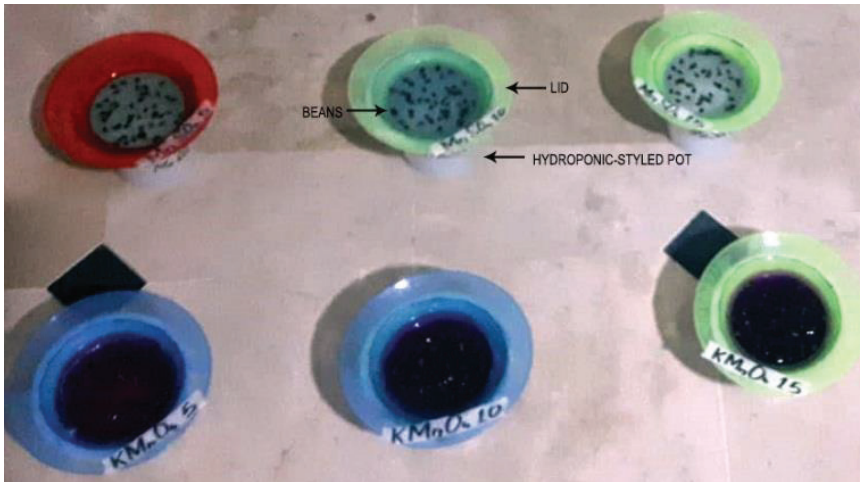

Fig. 1. Hydroponic-styled pots for germinating seedlings

before and after the shedding of the cotyledon was therefore also studied. To count for the effect of age, the leaves were reaped at four different stages of development: - (i) "Age-A" primary leaves reaped after they unfolded, (ii) "Age-B" as the cotyledons being shed, (iii) "Age-C" as the primary leaves were of considerable size and (iv) "Age-D" at the 20th day of germination.

3. RESULTS AND DISCUSSION

Results of our studies showed the overwhelming effect of manganese toxicity on seedling's morphology, germination, chlorophyll concentration, and pigmentation, especially of the manganese in its 2+ oxidation state. As the doses were increased, the effect worsened which is thought to be the side effects of plant defense mechanisms. Whenever plants employ one of these defense strategies to tolerate or detoxify the heavy metal stress, it has a direct effect on various growth parameters such as physiological malfunctioning of the vascular bundle that results in poor growth rate, limited germination, reduced length and diameters of epicotyl and roots to name a few [6]. Figure 2 shows the impact of Mn^{2+} toxicity on seedling germination of *Vigna mungo.*

Similar results were observed in the first 10 days of germination when various doses of Mn^{2+} were introduced to the pots before the emergence of the primary leaves. A direct relationship of reduced growth was observed as the doses of Mn^{2+} were increased and the test pots. Table 1 lists the morphological parameters observed in seedlings of age 10-12 days after germination after they were exposed to the Mn^{2+}.

3.1 Effect on Plant Morphology (Growth)

Generally, heavy metals can interrupt cell processes and interact with vital bio-molecules such as proteins and DNA, this is because they have a high affinity for thiol groups in these molecules. These interactions can lead to the production of reactive oxygen species (ROS) [16-18]. Severe morphological, metabolic, and physiological damages in plants can result from these toxic interactions. These damages may include chlorosis, protein degradation, reduced growth, and germination inhibition [8, 19]. Several studies reveal these damages mainly depend upon the concentration of heavy metals the plants were exposed to or the effect of the dose of heavy metals in combination with environmental conditions [8].

Nature has provided plants with some defense strategies to reduce the toxicity of heavy metals. One important defense mechanism is to remove the heavy metals by phytochelation and production of metallothioneins complexes at intracellular levels occasionally and intercellular levels more frequently [8]. These defensive detoxification processes can come into action at sites where toxicity occurs, as a result of defensive action, the produced phyto-complexes are removed from the affected sites by a process of vacuolar requisitioning. A non-enzymatic defensive mechanism to reduce heavy metal toxicity is the production of a proteogenic amino acid called proline (Pro), which acts essentially as a solute and helps greatly in the detoxification of heavy metals

[20, 21].

A combined effect of deteriorated xylem and phloem cells in the vascular bundles as a result of one of these stressful processes adopted by the plant is thought to be responsible for the reduced growth of the seedlings in this study. This combined effect may involve the slumped ability of the xylem in moving water and minerals upwards and contracted phloem cells leading to the deficient distribution of nutrients in the plant body [8, 22, 23].

The effect of Mn^{7+} was observed to be similar in results but with a mild-to-low toxic effect on plants morphology that may be due to the fact that Mn^{7+} can easily get reduced into Mn^{2+}, this conversion may not be 100 % in the context of any plant defense mechanism hence there is a mixture of both species and mild-to-low toxic effect is observed. Table 2 lists the results obtained for Mn^{7+} tests, and growth reduction under the influence of Mn^{7+} is shown in Figure 3.

3.2 Effect on Photosynthesis

For all photoautotrophs in the Kingdom Plantae, it is well known that the photosystem-II is responsible for the production of oxygen by photosynthesis. This photosystem mainly consists of a water-oxidizing complex (WOC) that has a cluster of 4 manganese atoms that catalyzes the production of oxygen from water during the photosynthetic process. The oxidation states of manganese are now known to be "3 Mn atoms in 3+ oxidation states while one atom in 2+ oxidation state" [24].

The four-manganese-atom inorganic core of the photosystem-II extracts electrons from water by a series of four consecutive redox steps denoted as S steps or states. These states are frequently labeled as S_0, S_1, S_2, and S_3 [24]. The subscript number refers to the oxidized equivalent obtained by the successive electron removals. Although it is not known so far, the individual oxidation states of manganese atoms in all four S states in the redox process however, in the presence of excess Mn^{2+} the photosynthetic ability of the plant is observed to be reduced that can be associated with the dis-balancing effect on the inorganic core resulting in interfering the redox chain reaction of electron removal [24]. Our studies confirm this hypothesis, as we introduced higher doses of Mn^{2+} to the test seedlings, the reduced growth and pale coloration of the leaves confirms the reduced photosynthesis, a clear indication of malnutrition and is caused by the malfunctioning of the vascular bundles.

3.3 Effects on Pigmentation

As the doses of the manganese were increased especially of Mn^{2+}, when the primary leaves unfolded, they noticeably appeared pale-colored. For the structure of the chlorophyll molecule, the central magnesium atom is in a 2+ oxidation state, when the seedlings were exposed to the excessive quantities of Mn^{2+}, a competition reaction presumably started between Mn^{2+} and Mg^{2+}. Due to the difference in the redox potential of these two ions, Mg^{2+} can get replaced by Mn^{2+} which can lead to the disintegration of chlorophyll molecules. Figure 4 and figure 5 show the impact of high concentrations of manganese ions on leaves.

Magnesium ion in chlorophyll molecule is bonded to four nitrogen atoms by coordinate covalent bonds. These four nitrogen atoms have a greater tendency to become covalently bonded to Mn^{2+} ions. Hence this reaction is favored and is the main reason for the seedlings to have pale pigmentation [1].

Results confirmed the hypothesis, that quantities of chlorophyll per unit area in leaves of all ages of seedlings decreased and this reduced pigmentation is proportional to the doses of Mn^{2+} the seedlings were treated with. Table 3 to table 6 list the chlorophyll concentration for all ages with respect to averaged leaf area and fresh weight of leaves per unit area for all ages sequentially. The numbers of plants for which the average data have been collected are as follows: for age-A: 16 plants, for age-B: 24 plants, for age-C and age-D: 32 plants. A closer look at the results can easily reveal a direct relationship of reduced chlorophyll concentration as the doses of the Mn^{2+} increases with one exception, a comparatively better chlorophyll concentration was detected at age-B, this is because the cotyledons were just being shed, the higher concentration of chlorophyll A and B can be attributed to the dissolved carbohydrate supplied by the cotyledons even in the presence of higher doses of Mn^{2+}.

Fig. 2. Reduced germination and growth under Mn^{2+} exposure

Table 1. Growth Analysis and Morphology on *Vigna mungo* exposed to Mn^{2+}

Mn^{2+} Dose (ppm)	**Germination** (%)	**Root Length** (cm)	**Epicotyl**		**Leaf Area** (cm^2)
			Length (cm)	**Morphology**	
Control	90	6.9 + 0.2	13.5 + 0.1	ERECT	4.4 ± 0.1
50	80	5.8 ± 0.1	11.0 ± 0.1	ERECT	3.8 ± 0.2
100	65	4.3 ± 0.2	10.4 ± 0.2	CURVED	3.0 ± 0.3
150	50	3.0 ± 0.2	9.2 ± 0.4	CURVED	2.4 ± 0.2
200	25	2.2 ± 0.1	5.5 ± 0.1	DOWN	1.8 ± 0.2
250	10	1.1 ± 0.1	3.8 ± 0.3	DOWN	1.2 ± 0.1

Table 2. Growth Analysis & Morphology on *Vigna mungo* exposed to Mn^{7+}

Mn^{7+} Dose (ppm)	**Germination** (%)	**Root Length** (cm)	**Epicotyl**		**Leaf Area** (cm^2)
			Length (cm)	**Morphology**	
CONTROL	95	7.0 + 0.1	13.0 + 0.1	ERECT	4.2 ± 0.1
50	82	6.1 ± 0.1	11.2 ± 0.1	ERECT	3.5 ± 0.1
100	70	5.4 ± 0.1	10.8 ± 0.1	CURVED	3.6 ± 0.2
150	65	3.7 ± 0.1	8.1 ± 0.1	CURVED	2.9 ± 0.1
200	35	2.9 ± 0.1	6.4 ± 0.1	CURVED	2.1 ± 0.1
250	25	2.0 ± 0.1	2.1 ± 0.2	DOWN	2.0 ± 0.1

Fig. 3. Mildly reduced germination and growth under Mn^{7+}

Fig. 4. Mn^{2+} exposure, reduced chlorophyll

Fig. 5. Mn^{7+} exposure, similar but mild impact

Table 3. Chlorophyll content for "age-A" of *Vigna mungo* exposed to Mn^{2+}

Mn^{2+} Dose (ppm)	**Avg. Leaf Area** (sq.cm)	**Avg. Fresh Weight** (per sq.cm (g))	**Chlorophyll A** (per sq.cm (mg))	**Chlorophyll B** (per sq.cm (mg))
CONTROL	3.72 ± 0.1	0.0097	0.0097	0.0058
50	3.30 ± 0.1	0.0088	0.0080	0.0048
100	2.86 ± 0.1	0.0060	0.0070	0.0042
150	02.5 ± 0.1	0.0053	0.0042	0.0025
200	01.9 ± 0.1	0.0038	0.0028	0.0017
250	01.3 ± 0.1	0.0029	0.0016	0.0010

Table 4. Chlorophyll content for "age-B" of *Vigna mungo* exposed to Mn^{2+}

Mn^{2+} Dose (ppm)	**Avg. Leaf Area** (sq.cm)	**Avg. Fresh Weight** (per sq.cm (g))	**Chlorophyll A** (per sq.cm (mg))	**Chlorophyll B** (per sq.cm (mg))
CONTROL	09.8 ± 0.1	0.0257	0.0354	0.0212
50	08.7 ± 0.1	0.0233	0.0293	0.0176
100	07.5 ± 0.1	0.0160	0.0255	0.0153
150	06.8 ± 0.1	0.0140	0.0153	0.0092
200	05.0 ± 0.1	0.0102	0.0103	0.0061
250	03.6 ± 0.1	0.0077	0.0060	0.0036

Table 5. Chlorophyll content for "age-C" of *Vigna mungo* exposed to Mn^{2+}

Mn^{2+} Dose (ppm)	**Avg. Leaf Area** (sq.cm)	**Avg. Fresh Weight** (per sq.cm (g))	**Chlorophyll A** (per sq.cm (mg))	**Chlorophyll B** (per sq.cm (mg))
CONTROL	13.5 ± 0.1	0.0353	0.0257	0.0154
50	12.0 ± 0.1	0.0321	0.0213	0.0128
100	10.4 ± 0.3	0.0219	0.0185	0.0111
150	09.4 ± 0.1	0.0193	0.0111	0.0067
200	06.9 ± 0.2	0.0140	0.0075	0.0045
250	04.9 ± 0.1	0.0106	0.0043	0.0026

Table 6. Chlorophyll content for "age-D" of *Vigna mungo* exposed to Mn^{2+}

Mn^{2+} Dose (ppm)	**Avg. Leaf Area** (sq.cm)	**Avg. Fresh Weight** (per sq.cm (g))	**Chlorophyll A** (per sq.cm (mg))	**Chlorophyll B** (per sq.cm (mg))
CONTROL	18.6 ± 0.1	0.0484	0.0485	0.0291
50	16.5 ± 0.2	0.0440	0.0402	0.0241
100	14.3 ± 0.3	0.0301	0.0349	0.0209
150	12.9 ± 0.2	0.0265	0.0210	0.0126
200	09.5 ± 0.3	0.0192	0.0141	0.0084
250	06.8 ± 0.2	0.0146	0.0082	0.0049

4. CONCLUSION

Our results showed that the quantity of Mn^{+2} in the control pot (22.32 ug/ml) was necessary for proper seedling development, no visible sign of growth malfunction was observed, even at a moderately higher level of Mn^{+2} (50 ug/ml), but as the concentration of Mn^{+2} was further increased, deteriorations were observed and found to be concentration-dependent i.e., as the concentration was increased, the more prominent effect was observed. Beyond 50 ug/ml of Mn^{+2}, the epicotyl started curving down, chlorophyll concentration and percent germination were also reduced. For Mn^{+7}, similar but mild effects were observed. The impacts of manganese toxicity were seen as the result of the plant's non-enzymatic defensive measures to reduce the metal overload. The high concentration of Mn^{+2} in the system can cause the disintegration of the chlorophyll molecule, primarily affecting the pigmentation and ultimately contributing to the malfunctioning of chloroplast, and causing the reduced chlorophyll concentrations. The mild impact on the overall growth observed in the case of Mn^{+7} was attributed to its ability to get reduced into Mn^{+2} in the biological system. The experiments perfumed to observe chlorophyll in the aging seedling showed that at the age where the cotyledons were just being shed (age-B), the chlorophyll concentration was found comparatively better than expected and it was attributed to the carbohydrates supplied by the cotyledons.

5. CONFLICT OF INTEREST

There is no conflict of interest.

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Supplementary Data

Table S1. Composition of macro and micro-nutrient in Hoagland culture

S. No.	**Macronutrients**	**Quantity** (mg/100ml)
1	$Ca(NO_3)_2$	22.48
2	KCl	7.22
3	KH_2PO_4	13.22
4	$MgSO_4$	23.98
S. No.	**Micronutrients**	**Quantity** (g/L)
1	H_3BO_4	2.4
2	$MnCl_2/MnSO_4.H_2O$	1.48/1.31
3	$ZnSO_4.4H_2O$	0.11
4	$H_2MoO_3/NaMoO_4.H_2O$	0.5/0.083
5	CuSO4	0.78
6	$Fe(EDTA)^{-2}$	24.8 g $FeSO_4$.$7H_2O$+25 mg EDTA in 500 ml distilled water with few drops of KOH

Table S2. Half-Strength Hoagland Recipe

S. No.	**Chemicals**	**Volume of Stock Solution** (mL)
1	$Ca(NO_3)_2$	1.5
2	KCl	1.0
3	KH_2PO_4	1.2
4	$MgSO_4$	0.8
5	Micronutrients	0.5
6	$Fe(EDTA)^{-2}$	0.6