Indian Journal of Plant Sciences ISSN: 2319–3824(Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/jps.htm
2016 Vol.5 (1) January-March, pp.119-125/Kumari et al.
Research Article

IMPACTS OF CADMIUM AND MANGANESE ON *IN VITRO* SEED GERMINATION AND SEEDLING GROWTH OF HORSEGRAM

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ABSTRACT
Heavy metal stress is one of the key factors for crop yield along with other abiotic stresses such as drought, salinity, extreme temperature and light. Due to natural and anthropogenic activities heavy metal pollution is increasing daily. Hence, it is must to know the effects of heavy metals in depth on crop growth and development ultimately yield levels. In the present work we assessed the impacts of cadmium (Cd) and manganese (Mn) on seed growth of important food crop horsegram (*Macrotyloma uniflorum*) under *in vitro* conditions. We observed the toxic effect of cadmium on horse gram seed germination and in contrast manganese promoted the germination. To know further details into the differences in seedling growth pattern, we estimated the shoot and root lengths of both heavy metal treated seedlings along with controls. Both the shoot and root lengths were reduced in high concentrations of cadmium. At the same time high concentration of manganese also exhibited decreased root lengths and we got mixed results with shoot lengths in manganese treatments. Moreover, we compared the effects of both the elements and possible reason for modifications in growth has been discussed. This work may be helpful to know the insights of susceptibility/resistance of crop growth under these heavy metal stresses.

Keywords: *Horsegram, Germination, Cadmium, Manganese, Toxicity*

INTRODUCTION
Soil pollution has become one of the major threat to overall yield in almost all the major field grown crops because of both natural and anthropogenic causes. In addition, release of industrial waste discharges which contains toxic heavy metals also leads to soil pollution (Chand *et al.*, 2015). The anthropogenic activities such as urbanization, mining, usage of chemical fertilizers and pesticides are the main sources of heavy metal pollution. Heavy metal toxicity has been known for several years and it is interesting that the advanced biotechnological research on this area is focused in recent times (Adrees *et al.*, 2015; WHO, 2015). In general irrigation with polluted water causes the heavy metal accumulation not only in the soil but also in seedlings and plant tissues. In addition, agricultural land is being contaminated by heavy metals such as chromium (Cr), cadmium (Cd), cobalt (Co), lead (Pb), nickel (Ni), manganese (Mn) etc. Biologically both essential and non-essential heavy metals exist and depending on plant species, the accumulation varies. Though some plants exhibited tolerance to soils with high heavy metal concentrations, most of them exhibited deleterious effects growing in such areas (Guala *et al.*, 2010; Bae *et al.*, 2016). In the present investigation we choose cadmium as non-essential and manganese as essential heavy metal to know their impacts on horse gram seed and seedling growth under *in vitro* conditions. The composition of the culture medium is an important factor for the successful establishment of an *in vitro* system for seed and seedling growth and development. In contrast studies in *in vivo* pot based system contains soil which includes other nutrients.
Accumulation of excess heavy metals (including essential) in living organisms also causes the excessive production of reactive oxygen species (ROS). It is a well-known fact that the accumulation of ROS leads to variations in morphological, biochemical and physiological aspects of plants including seed germination, seedling growth, chlorosis, protein degradation, lipid per oxidation etc. (Lux *et al.*, 2015). Several studies have been conducted in order to identify the impacts of various heavy metals such as Cd and Mn in different crop species (Weiquiang *et al.*, 2005; Pedas *et al.*, 2008). Cadmium acts as one of the most toxic and highly portable element from soil to plants in the environment. Initially the excess amount of cadmium was observed in roots and a very low amount will be transferred to shoots and so it leads to

damage of root tissue first (Houshmandfar and Moraghebi, 2011). Excess Cd caused a number of toxic symptoms in plant growth and inhibition of photosynthesis. Other activities such as nitrogen metabolism, water and nutrient uptake will be reduced by the heavy metal cadmium (Tao *et al.*, 2015). In *Pisumsativum* oxidative stress was observed in leaves and root tissues by different concentrations of Cd (Dixit *et al.*, 2001). In contrast manganese is an essential trance element for plant growth specifically for enzyme regulation and activation of certain metabolic activities. It is also essential in water-splitting system of photosystem II, which supplies electrons for photosynthesis (Singh *et al.*, 2011). Growth promoting nature of Mn on mung bean seed germination was studied by Roy and Bera (2000). However, the excess amount of essential manganese also becomes unfavorable to growth as noticed by Anjum *et al.*, (2015) recently.

Horse gram is a leguminous crop and grown in dry land areas of tropics and subtropics. It is a nutritious food crop and also used as cattle feed in various regions in India. It is also used as source of anti-oxidants, useful for digestion process and seeds have capacity to reduce hyperglycemia (Bolbhat Sadhashiv and Dhumal Kondiram, 2012). There were few works conducted on horse gram using different heavy metals such as lead, chromium and nickel (Reddy *et al.*, 2005; Pati *et al.*, 2014; Arzoo *et al.*, 2014). Modifications of antioxidant activity were observed with lead treatments in horse gram (Reddy *et al.*, 2005). Arzoo *et al.*, (2014) proved that nickel also exhibited the phytotoxic nature in this crop. Similarly, high level of chromium exhibited toxicity in this crop was observed by Pati *et al.*, (2014). We also demonstrated the impacts of various heavy metals on seed growth of both pearl millet and green gram crops (Gangaiah *et al.*, 2013; Neelesh Babu *et al.*, 2014). In the present investigation we conducted the experiments under *in vitro* conditions in order to know the possible mechanism of effects of Cd and Mn on horse gram which is most reliable method when compared to pot based *in vivo* method.

MATERIALS AND METHODS

We used the local variety of horsegram as source of plant material for the present investigation. Further we used cadmium chloride monohydrate ($CdCl_2.H_2O$) and manganese sulphate monohydrate ($MnSO_4.H_2O$) as heavy metal sources for *in vitro* growth assays. The glassware was washed thoroughly with commercial detergents and later cleaned using running tap water and further rinsed with distilled water. All the washed glassware must be oven dried before going to be use.

Media and its Combinations

The media used in the present study were very simple i.e. with heavy metal and without nutrients. The media contains distilled water with 1, 10, 20, 50 and 100 ppm concentrations of respective heavy metals i.e. Cd and Mn. Both tap water and distilled water media were used as controls without adding any heavy metal (Table-1). pH of the medium was maintained 5.8 and 0.8 percent agar was added and was melted. Later 15 ml of melted medium was dispensed into each test tube. Culture test tubes containing media were autoclaved for 15 min at 121^{o}C in an autoclave. Immediately after the completion of sterilization, they were placed in a slanting position to get more surface area. All the requirements for inoculation were transferred inside the laminar air flow chamber for *in vitro* seed inoculation.

Surface Sterilization of the Horsegram Seed Materials

Without surface sterilization process it is highly impossible to maintain *in vitro* cultures with seed or any explant material. All the horsegram seeds were initially washed in running tap water for 3-5 min to get them free from dust particles. Further decontamination of the seeds was performed in laminar air flow chamber by treatment with 70% ethanol for 1 min and later with a solution of 0.1% mercuric chloride [$HgCl_2$ (w/v)] for 10 min in the process of surface sterilization. Surface sterilization was followed by 3 rinses with sterile distilled water. Then the seeds were blotted on sterile filter paper sheets before inoculating them on respective medium in test tubes.

Growth Conditions

All the surface sterilized seeds were inoculated in laminar flow chamber and tubes were incubated in a culture room at 25 ± 2°C with a relative humidity of 50-60%. All the cultures were maintained with 16 h photo period at a photo flux density of 40-50 μEm^2s^{-1} with white fluorescent tubes in culture stands.

Observations of the cultures were done every day and was estimated the percentage of seed germination and also lengths of both shoots and roots of the seedlings per culture. All the data were collected on 15th day (after two weeks) and a minimum of three replicates were involved in each experiment and conducted thrice. The mean and standard error was calculated using excel programming techniques.

RESULTS AND DISCUSSION

Both physical and chemical properties of heavy metals are likely to be influence the seed growth and development. The results including percentage of seed germination and lengths of shoot and roots of heavy metal treated horsegram seedlings were documented.

Analysis of Cadmium Treated Seeds

Treatment of horse gram seeds and seedlings with cadmium metal proved adverse impacts (Figure I). Seed germination and seedlings were initiated from the 1, 10, 20, 50 and 100 ppm media along with both tap water (control 1) and distilled water (control 2) media. We noticed around ninety percentage of seed germination in both the controls. Growth inhibition was observed at above 20 ppm concentration of cadmium. Particularly we observed 45 % and 30 % of seed germination in 50 and 100 ppm of cadmium (Figure 1 and 2).

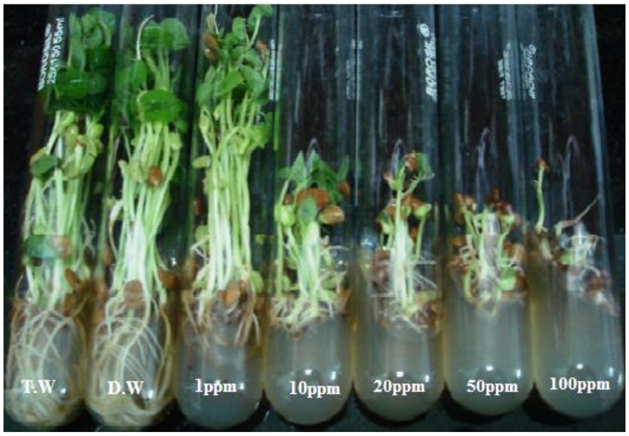

Figure 1: Effect of Cadmium on Seed Germination and Seedling Growth of Horse Gram

T.W- Tap water sample, D.W-Distilled water sample, 1, 10, 20, 50 and 100 ppm sample

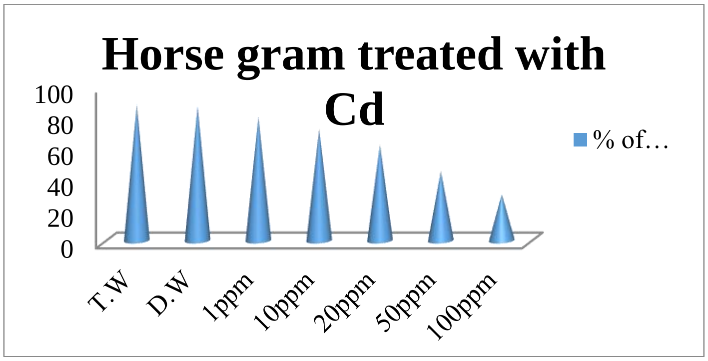

Figure 2: Effect of Cadmium on Seed Germination of Horse Gram

The above data is collected after 2 weeks. Values above represented are mean of 3 replicates

Heidari and Sarani (2011) also emphasized that the mustard seed germination was severely damaged by cadmium. In extent eighty percentage of germination was observed in 1 ppm Cd medium when compared to germination at high concentrations. Shoot and root lengths were also affected with high concentrations of cadmium. Increasing cadmium exhibited decreased lengths of both shoots and roots respectively (Figure 1 and 3). Specifically we observed negligible root length values in seeds treated with high concentration (Figure 1 and 3). Similar toxic nature of cadmium was also proved in milk thistle (Kharamipour *et al.*, 2011). Overall, high concentration of cadmium will lead to damage of the growth of horse gram but does not show any impacts at low concentrations. These results also indicate that the phytotoxicity of non essential cadmium did not affect the seedling growth until it crosses its critical level.

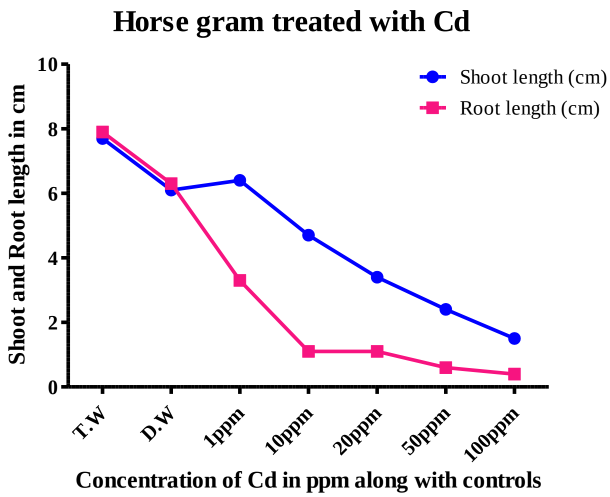

Figure 3: Effect of Cadmium on Early Seedling Growth of Horse Gram
The above data is collected after 2 weeks. Values above represented are mean of 3 replicates

Analysis of Manganese Treated Seeds
Parallel experiments were conducted to screen with manganese on horse gram seeds under similar conditions as mentioned above. We observed unaffected growth promotion in seed germination with increasing Mn concentrations (Figure 4 and 5). Hundred percentage of germination was observed in all the treatments with all the healthy seeds (Figure 4). Similarly Roy and Bera (2000) observed 100ppm manganese favors 100% seed germination in mung bean, because it is an essential heavy metal for plant growth. Moreover increasing Mn exhibited minor reduction of length of shoots was observed in our investigation except at 20ppm where the shoot lengths was suddenly increased (Figure 4 and 5).

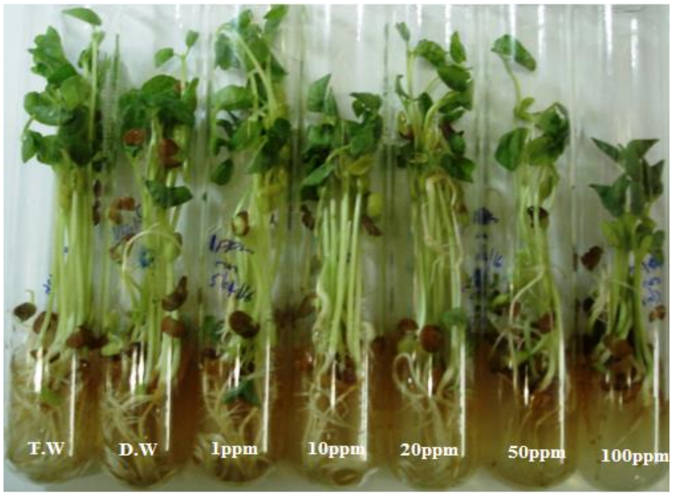

Figure 4: Effect of Manganese on Seed Germination and Seedling Growth of Horse Gram
T.W- Tap water sample, D.W-Distilled water sample, 1, 10, 20, 50 and 100 ppm samples

Root lengths were decreased with increased Mn concentrations. Similarly, Bhupendra *et al.*, (2014) proved that the higher concentration of manganese showed reduced germination and root lengths in green and black gram. The entire seedlings colour was same, indicating that the chlorophyll synthesis was similar in all the manganese treatments. Further we are working to reveal the concept of 'critical level' of Mn on horse gram growth and development which may give some clues to know the role of this element at early and later stages. Similarly Shaik *et al.*, (2013) also proved that the severe toxic nature of cadmium when compared to manganese in wheat. This work may be useful to deals with the various morphological, physiological and functional insights of heavy metal stress on crop plants and outlines the scope for future research and the possibilities for remediation.

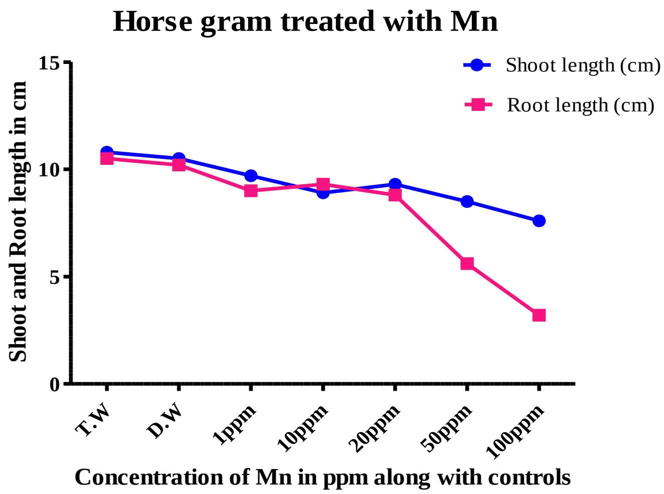

Figure 5: Effect of Manganese on Early Seedling Growth of Horse Gram
The above data is collected after 2weeks. Values above represented are mean of 3 replicates

Table 1: Media Combinations Used in *in Vitro* Conditions

S.No.	Name of the Heavy Metal	Concentrations used in PPM					Control 1	Control 2
1	Cadmium or Manganese Treatment	1	10	20	50	100	Tap water Only	Distilled Water only

Conclusions
Over all we observed the adverse effects of cadmium when compared to other heavy metal manganese in this investigation. Surprisingly essential heavy metal manganese also showed minor effects on seedling growth and development. Further research may unveil some answers specifically for unexpected results with manganese.

ACKNOWLEDGEMENTS
The authors are thankful to the Agri-Science Project, Ministry of Industries and Commerce, Government of Andhra Pradesh, India and partial support from CSIR grant to Thummala Chandrasekhar.

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