ISSN:2319-2607

IISUniv.J.Sc.Tech. Vol.2(1),38-42 (2013)

RESEARCH ARTICLE

Cadmium Toxicity in Cereals –*In Vitro* Study

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Abstract

Today Abiotic stress is a major global problem limiting crop productivity. Stress factors like heavy metals, salinity and high temperature are a serious problem limiting the yield potential of modern cultivars, specially the cereals. Stress causes nutritional imbalances in the plant causing reduction in water uptake and toxicity, decreasing the production. This is posing a serious problem and needs to be evaluated. Such studies if done in field conditions would be time taking and cumbersome and therefore the present study was conducted under *in vitro* conditions taking *Eleusine coracana* (L.) Gaertn variety PR202 as the model plant. To limit the study, effects of only cadmium was evaluated. Seeds taken as explant were inoculated on callus induction medium with varied $Cd(NO_3)_2$ levels (100µM, 300 µM and 500 µM). For callus induction, cadmium was found to be tolerable only upto 100 µM concentration. Induced Callus were sub cultured on maintenance media and then on regeneration medium (MS + 1mg/l NAA) supplemented with same level of the heavy metal . Cd above 100 µM concentration was found to be completely inhibitory for callus induction as well as for plantlet regeneration.

Keywords

$Cd (NO_3)_2$, Heavy metal, *in vitro,* MS media

Introduction

Stresses are increasing drastically because of pollution, declining availability of good quality water and land degradation. Abiotic stresses like salinity, heavy metals and pesticides are the primary cause of crop failures in India. It has been estimated that about 8.6 million ha of land is affected by salinity in India (Pathak, 2000). Heavy metals make a significant contribution to environmental pollution as a result of human activities such as mining, smelting, electroplating, energy and fuel production, power transmission, intensive agriculture, sludge dumping and military operations (Nedelkoska and Doran, 2000). Cadmium, an industrial pollutant is constantly rising in the environment due to activities such as mining , smelting and refinement of zinc, manufacturing and use of fungicide and phosphorous fertilizers, metallurgy etc. Although Cd is not an essential mineral nutrient for plants , it is easily absorbed by the root system , causing a decrease in transpiration and photosynthesis (Bazzaz *et al*, 1974) and an increase in the respiratory rates (Lee *et al*,1976; Lamoreaux and Chaney, 1978). Cadmium being a highly toxic metal pollutant of soil , affects nutrient uptake and homeostasis, inhibiting root and shoot growth and thus yield. Zn supplementation to the medium has been reported to reduce lipid peroxidation, electrical conductivity and lipoxygenase activity induced by Cd (Aravind *et al*, 2003).

Cereals constitute a major source of food for the human population of the world. Important cereals in day to day use are rice, wheat and millets. From all the millets *Eleusine* (Fig.1, 2) contains more percentage of different nutrients as compared to rice and Wheat. It is a rich source of calcium, magnesium and potassium. *Eleusine* is nutritionally very rich and is a staple food for poor and invalids (Panda, 1999). Due its nutritional superiority and requirement by poor people production needs to be improved. *Eleusine coracana* mainly growing in dry condition faces heat, salinity and heavy metal stress (specially cadmium and nickel) which may cause negative effect on its yield and there feels *an* urgent need to develop stress tolerant plants to improve the yield. Stress tolerant plants can be developed by breeding and transgenesis but these are complex processes. Tissue culture techniques offer an easy and important tool in developing stress tolerant variants (Nabors and Dykes, 1985) and results if tested in field conditions may give an easy solution to the problem.

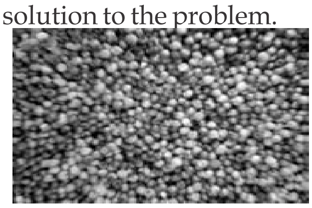

Fig.1. Seeds of *Eleusine coracana*

Fig.2. Mature Plant of *Eleusine coracana* in field

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Looking to the scope and realizing its importance, the present study was undertaken to investigate the effects of different concentrations of Cd on callus induction and plant regeneration. *E. coracana* being highly responsive in tissue culture studies was chosen as a model plant to study the effect of heavy metal stress on cereals.

Materials and Method

Callus Induction, Embryogenic Callus Formation and Plant Regeneration

Eleusine coracana (L.) Gaertn of family Poaceae was taken as the model plant for the study (Common Name: Finger millet, Vernacular Name: Ragi). Seeds of agronomically superior and released variety (PR-202) of ragi (Fig. 2) were used as explant which were procured from Agricultural University, Bangalore. Basal medium used in the study was MS medium (Murashige and Skoog, 1962) with sucrose 3% (w/v), agar 0.8-1% (w/v) and pH 5.8. Seeds were surface sterilized in 0.1 percent mercuric chloride solution for 3 minutes , rinsed several times in distilled water and then inoculated on callus induction medium. (MS medium + 2 mg/l 2, 4-D and 0.5 mg/l Kn). Aseptic culture was carried out in laminar air flow chamber. Cultures were incubated in a growth chamber equipped with two air conditioners and temperature controlled at 26±1°C. A photoperiod of 16 hours alternating with 8 hours of darkness was maintained. After four weeks of incubation, the compact green, nodulated sectors of callus were separated from non embryogenic watery callus and subcultured on MS medium with 0.2mg/l 2,4D. The callus developed after 5-6 passages were cultured on regeneration medium (MS medium + 1.0 mg/l NAA) and number of shoots regenerated after 4-6 weeks were counted (Control)

Effects of Heavy Metal (Cd) on Callus Induction and Plant Regeneration

To study the effects of cadmium, seeds of *Eleusine coracana* were inoculated on callus induction medium with varied Cd levels (100µM, 300 µM and 500 µM). The amount of callus formed in each case was recorded and after 3-4 weeks all the calli were transferred to MS medium with 0.2mg/l 2,4-D (callus maintenance media) with the same concentration of cadmium as was in callus induction medium . The callus so formed was transferred on regeneration media i.e MS+1mg/l NAA + same concentration of cadmium as was in callus induction medium . In this case, cadmium was present in both callus induction and regeneration medium.

Effects of Cd on Plant Regeneration when Induction Medium had no Cadmium.

Seeds were inoculated on MS Medium supplemented with 2mg/l 2,4D + 0.5mg/l Kinetin (control). The amount of callus formed in each case was recorded and after 3-4 weeks all the calli were transferred to MS medium with lower concentration of 2,4-D(0.2mg/l) for callus maintenance without heavy metal and then on regeneration media (MS+1mg/l NAA) with varied Cd levels (100µM, 300 µM and 500 µM). In this experiment heavy metal were added only in regeneration media . Callus induction medium was free of cadmium. Approximately 4-5 seeds were cultured in each flask and approximately 5 replica of each concentration were made. Approximately 250mg of callus was transferred to each flask for regeneration.

Statistical Analysis- The observations recorded for various experiments were subjected to statistical analysis. Standard Deviation was calculated in each case.

Results and Discussion

Callus Induction, Embryogenic Callus Formation and Plant Regeneration

Mature seeds of *Eleusine coracana* were inoculated on normal callus induction medium. After 2-3 weeks of inoculation of seeds , two types of calli were observed : healthy, compact, nodulated, dark green embryogenic callus along with white, friable, watery, translucent and non embryogenic callus (Fig.3a and 4a). Amount of callus formed was recorded . The embryogenic sectors were transferred to maintenance medium, excluding the non embryogenic portion of the callus after every 3-4 weeks . With each subculture, the number of embryoids increased profusely. When the embryogenic calli were transferred on regeneration media , plantlet formation was observed. The plantlets were healthy and green with well developed root and shoot axis (Fig.3b & 4e) (Control).

Fig.3-a. Callus induced from seeds of *Eleusine coracana* on MS + 2, 4-D (2 mg/l) +Kn (0.5 mg/l)

Fig.3-b. Regeneration from embryogenic callus on MS+ NAA(1mg/l)

Effects of Heavy Metal (Cd) on Callus Induction and Plant Regeneration

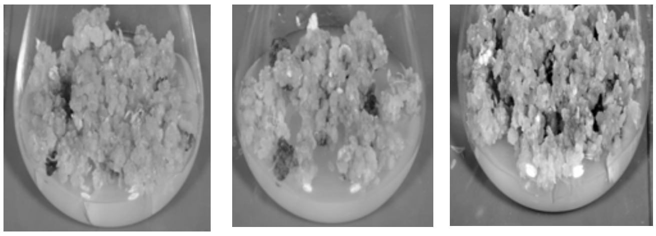
Seeds of *Eleusine coracana* were inoculated on callus induction medium with varied Cd levels (100µM, 300µM and 500 µM). Proper callus formation was observed on medium with 100 µM concentration of Cd (Fig.4b) Callus was nodulated, compact , embryogenic along with soft , watery and non embryogenic callus. Callus formation on media with 300 and 500 µM of Cadmium (Fig.4c-d) were poor (Table 2). Callus on control induction media was more compact as compared to callus induced on media with 100 µM Cadmium. This shows that Cd have an inhibitory effect , although tolerable upto 100 µM concentration . The embryogenic sectors of calli from all the concentrations were then transferred to maintenance medium with the same concentration Cd as in callus induction media . The non embryogenic portion of the callus was excluded during subculture. The callus developed after 3-4 subcultures (maintenance media were same everytime as their respective initial media) was transferred on regeneration media with varied Cd levels (same concentration of cadmium as was in their callus induction medium). After 2-3 weeks shoots appeared and their number was counted. From all the Cadmium levels, regeneration was observed only on medium with 100 µM Cadmium but even this was worse than control (Fig.4f). There was no regeneration on media with higher levels of Cd .

Table 1. Callus induction medium in *E. coracana* with various concentrations of Cadmium

Medium	Concentration of Cd(µM)
MS + 2,4-D + Kn (Control)	Nil
MS + 2,4-D + Kn+Cd	100
MS + 2,4-D + Kn +Cd	300
MS + 2,4-D + Kn +Cd	500

Table 2. Callus induction and plant regeneration on media with varied concentration of Cd in induction and regeneration media

Concentrations of Cd in Callus Induction Medium(µM) and subsequent plant regeneration media	Amount of callus formed / explant (mg)	Number of shoots/ seed callus on plant regeneration medium ± S.D.
0(C)	250 (C)	12.4 ± 6.4(C)
100	150	3.3±0.4
300	72	0
500	39	0

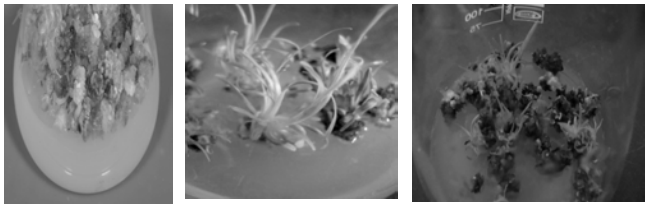


(a) (b) (c)

Fig.4a. Callus induction on MS+2, 4-D and kn (control)

Fig.4b. Callus induction on MS+2, 4-D and kn +Cd (100µM)

Fig.4c. Callus induction on MS+2, 4-D and kn+Cd (300µM)

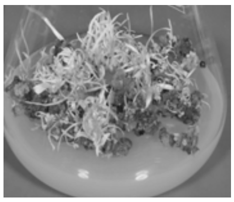


(d) (e) (f)

Fig.4d. Callus induction on MS + 2, 4-D and kn +Cd (500µM)

Fig.4e. Regeneration on MS+ NAA from callus induced on normal induction medium

Fig.4f. Regeneration from callus (developed on medium with 100µM Cd) on medium with Cd (100µM)



(g)

Fig.4g. Regeneration on MS+NAA + 100µM Cd from callus induced on normal induction medium

Effects of Cd on Plant Regeneration when induction medium had no cadmium

Seeds were inoculated on MS Medium supplemented with 2mg/l 2,4-D + 0.5mg/l Kinetin. After 3-4 weeks the calli were transferred to normal MS medium with lower concentration of 2,4-D(0.2mg/l) for callus maintenance . Embryogenic callus from maintenance medium without heavy metal (MS + 0. 2 mg/l 2,4-D) were further transferred on regeneration medium (MS + 1mg/l NAA) supplemented with varied concentrations of Cd (100 µM, 300 µM and 500 µM) to see the effects of Cd concentrations on regeneration of *Eleusine coracana* , when callus induction and maintenance media are free of cadmium. After 2-3 weeks, differentiation of shoot buds took place in medium containing 100μM concentration of Cd (Fig.4g). Number of shoots per callus piece was counted. Regeneration was worse than control. Shoots were healthier in this experiment than the previous one, since cadmium was added only in regeneration medium (Fig.4g). Callus induction and maintenance of embryogenic callus was conducted on, media without cadmium.

Table 3. Plant regeneration on media with Cadmium only in regeneration media

Concentrations of Cd in Plant Regeneration Medium[a] (μM)	Mean number of shoot / Callus Piece ± S.D.
0	13.4 ± 1.45
100	6.8 ± 2.2
300	0
500	0

The results of the present study show that cadmium has an inhibitory effect on callus induction as well as plant regeneration. Regeneration was better when cadmium was present only in the regeneration medium i.e. callus induction medium was free of heavy metal (Table 3). In other words we can conclude that the toxic effect of heavy metals can be reduced if seeds are made to germinate in a soil free of heavy metal. Whatever the case may be, it is proved that cadmium has a toxic effect on plant regeneration and growth.

Eleusine coracana mainly grows in dry condition and faces the toxicity of cadmium severely which have a negative effect on its yield. In *Holarrhena antidysenterica* L. similar toxicity had been reported when nodal parts were treated with $CuSO_4$, $Pb(NO_3)_2$ and $CdCl_2$. (Agrawal and Sharma, 2006). Cu and Zn significantly reduced seed germination and root growth in *Carum carvi* L. (caraway) and *Foeniculum vulgare* L. (Jeliazkova and Craker, 2002). In cabbage, Zn, Cu, Cd, Pb and Hg when present in excess, reduced glutathione (GSH) greatly and increased the soluble and immobilized peroxidase activity (Brzyska et al, 2001). These effects seem to be related to Cd 116 induction of premature senescence in plants (Van Assche *et al*, 1988). Cadmium, being a highly toxic metal pollutant of soil, affects nutrient uptake and homeostasis, inhibits root and shoot growth and yield. It also gets accumulated in crops and then enters the food chain with a significant potential to impair animal and human health The application of sewage sludge, city waste, and Cd containing fertilizers cause the increase of Cd content in soils.

All the above result and discussion points toward the toxicity of cadmium, not only in *Eleusine* but in other plants as well. The stress caused by these abiotic factors is decreasing the yield posing a problem towards a country 's economy and needs to be rectified urgently. It has been shown that the nutrient level in the medium has a profound effect on callus induction and subsequent plant regeneration (Preece *et a*l, 1995; Poddar *et al*, 1997; Ramage and Williams, 2002). There also occurs interplay between nutrients and plant growth regulators (PGRs) during the course of *in vitro* growth and differentiation (DeFossard, 1974). The effect of various micronutrients on barley culture have also been investigated by Dahleen and Bregitzer (2002) and several fold increase in regeneration of green plants was observed by increasing some of the nutrients in the culture medium. Optimized levels of copper have also been reported to improve plant regeneration from callus cultures of barley (Bregitzer *et al*, 1998; Castillo *et al*, 1998; Chauhan and Kothari, 2004), rice (Sahrawat and Chand, 1999), finger millet (Kothari *et al*, 2004), sorghum (Nirwan and Kothari, 2003) and wheat (Tahiliani and Kothari, 2004). Sahasrabudhe *et al*, 1999 found an increase in the embryogenic response of indica rice on higher concentrations of boric acid. Looking to these studies it seems that same approach if applied in field conditions could be useful in overcoming toxicity of heavy metals in cereals. Keeping this view in mind the present study would be pursued further.

Acknowledgment

This work was supported by International College for Girls, The IIS University, Jaipur-302020.

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