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Efficient Somatic Embryogenesis and Plantlet Regeneration Induced from Mature Embryo Culture under Heavy Metals Stress Conditions in a Millet Crop Sorghum [*Sorghum bicolor* (L.) Moench]

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The present study aims to establish the somatic embryogenesis and plantlet regeneration in *Sorghum bicolor* (*cv.* PAC501) under heavy metals (lead and cadmium) stress conditions. Mature embryos were inoculated in MS-medium containing (10mg/L, 25mg/L, 50mg/L, 75mg/L, and 100mg/L) of $PbSO_4$ and $CdCl_2$ (10mg/L, 25mg/L, 50mg/L, 75mg/L, 100mg/L, 150mg/L, and 200mg/L) each with IAA (1.0mg/L), BAP (0.5mg/L), zeatin (0.1mg/L), and proline (0.7mg/L). Significantly, high frequency (87.2±0.21%) of somatic embryogenesis was initially obtained with IAA (1.0mg/L), BAP (0.5mg/L), zeatin (0.1mg/L), and proline (0.7mg/L) while in comparison, 2,4-D (2.5mg/L) along with same concentrations of BAP, zeatin, and proline was proved as relatively less efficient (67.4±0.64%) for somatic embryogenesis. Furthermore, results reveal that sub-culture of embryogenic callus on cytokinins; BAP, Kn, and Zn (0.1mg/L, 0.5mg/L, and 1.0mg/L) of each added regeneration medium, kinetin (0.5mg/L) was recorded as the most effective cytokinin (77.8±0.93%) for plantlets regeneration. Moreover, during heavy metals treatments, the least frequency (2.9±3.32%) of lead tolerant somatic embryos was obtained with $PbSO_4$ (75mg/L) in presence of the same concentrations of IAA, BAP, zeatin, and proline. Significantly, the very low frequency (3.7±0.67%) of lead tolerant plantlets regeneration was recorded with $PbSO_4$ (75mg/L) and Kn (0.5mg/L). In contrast, during $CdCl_2$ stress treatment, the cadmium tolerant somatic embryos (3.1±3.24%) were obtained with $CdCl_2$ (150mg/L) and IAA, BAP, zeatin, and proline. Further, cadmium tolerant plantlets (4.23±2.33%) were also achieved on medium containing kinetin (0.5mg/L) with $CdCl_2$ (100mg/L) indicating that lead proves to be more toxic for somatic embryogenesis and plantlets regeneration than cadmium. Later, the regenerated tolerant plantlets were transferred to pots and gradually acclimatized in greenhouse.

Key words: Heavy metal, Millets, Mature embryo, Regeneration, Somatic Embryogenesis, Sorghum

Sorghum or *Sorghum bicolor* (L.) Moench is the fifth most cultivated crop all over the world. It is commonly used as food, fodder, fiber sources, and biofuel (Gnansounou *et al.,* 2005; Rooney *et al.,* 2007). Interestingly, sorghum has natural adaptability towards harsh environments and exhibits its tolerance against drought, salinity and water logging (Hadebe *et al.,* 2017; Huang, 2018; Varoquaux *et al.,* 2019).

Heavy metal stress is one of the major abiotic stresses which affect badly the plant's growth and development. The main source of heavy metal contamination is identified as industrial effluent, battery usages, usage of organic and inorganic fertilizers, mining etc. Moreover, heavy metals gradually accumulate in plant and animals through food chain that cause the adverse impacts on the physiology and biochemical process in organisms (Muratova *et al.,* 2015).

Interestingly, sorghum is widely used as crop against phytoremediation of heavy metal contamination in soil (Marchiol *et al.,* 2007; Soudek *et al.,* 2014; Zhuang *et al.,* 2009). Furthermore, lead and cadmium have been found as the most toxic heavy metals that contaminate the soil in India (reported by Ministry of Jal Shakti, India-2019). Also, as per WHO opinion, cadmium is one of the most toxic heavy metals which are highly dangerous to human health. The minimal concentration of these metalloids can cause severe toxicity in metabolism of living organism (Drouhot *et al.,* 2014; Camizuli *et al.,* 2018).

The mechanism of plant tolerance to heavy metals can be divided into avoidance strategies, leading to limitations of cadmium uptake, and tolerance strategies, including accumulation and storage of cadmium by binding it to amino acids, proteins and peptides (Tran and Popova, 2013). The main symptoms of cadmium phytotoxicity are visually manifested through inhibition of plant growth, leaf chlorosis, and necrosis of above head and underground organs (Hernadez and Cooke, 1997).

In vitro regeneration technique is one of the most efficient methods to achieve crop improvement for high yield, tolerance to abiotic stresses through genetic biotechnology (Tiecoura *et al.,* 2003) and somaclonal variation (Matheka *et al.,* 2008; Radchuk *et al.,* 2012). In order to overcome the existing challenges, *in vitro* plant tissue culture is known as an alternative approach to enhance the tolerance of the plant against abiotic factors like salinity, drought, heavy metal stresses etc., that are treated as the major factors of reducing the productivity of the crops (Vikrant, 2015).

Moreover, it is obligate necessary to build the well-structured establishment of somatic embryogenesis and plantlet regeneration under abiotic stress or mutagenic conditions for the recovery of tolerant millet crops (Ceasar and Ignacimuthu, 2009; Vikrant, 2015). Hence, it is imperative now to develop sorghum plant as stress tolerance that can sustain and show productivity even during adverse climatic conditions.

MATERIALS AND METHODS

Collection and Sterilization of Plant Material

Sorghum bicolor (cv.PAC501) seeds were collected from IIMR, Hyderabad (India). The seeds were initially surface sterilized using 'tween-20' followed by 70% of ethanol (v/v) for 30 seconds. Further, explants were then treated with $HgCl_2$ (0.1%) for 5 minutes followed by the washing with sterilized distilled water (4-5 times) in laminar air flow chamber. Sterilized sorghum seeds were further subjected to 3-4 hours of soaking and later mature embryos were excised easily from the seeds and used as explants. The sterilized mature embryo explants were subjected to nutrient medium further.

Nutrient Medium for Callus induction and Somatic Embryogenesis

Excised mature embryos were inoculated in MS-basal medium containing auxins (2,4-D and IAA) along with cytokinins (BAP and zeatin), amino acid (proline), sucrose (3%w/v) and agar (0.8% w/v) **(Table 1).** The cultures were incubated at 25±2°C in the dark for callus induction and subsequently sub-cultured in respective fresh nutrient medium at every two weeks.

Nutrient Formulation for Plantlet Regeneration

After six weeks, the embryogenic callus was sub-cultured to MS-regeneration medium supplemented with various concentrations of cytokinins (BAP, kinetin and zeatin) and proline **(Table 2).** These cultures were

incubated at 25±2°C with 16/8h (light/dark) photoperiod for 14 days. The germination of somatic embryos and regeneration of plantlets were observed after 10 days of sub-culture.

Heavy Metal Stress Treatments

In order to evaluate the effects of lead (Pb) metalloid stress on callus induction, somatic embryogenesis and plantlet regeneration, concentrations (10mg/L, 25mg/L, 50mg/L, 75mg/L, and 100 mg/L) of $PbSO_4$ (w/v) were used along with the combination of IAA (1.0mg/L), BAP (0.5mg/L), zeatin (0.1mg/L), and proline (0.7mg/L) **(Table 3)**.

Similarly, to determine the effects of cadmium metalloid stress on sorghum regeneration, the mature embryo explants were inoculated in MS-medium containing various concentrations (10mg/L, 25mg/L, 50mg/L, 75mg/L, 100mg/L, 150mg/L, and 200mg/L) of $CdCl_2$ (w/v) in combination with IAA (1.0mg/L), BAP (0.5mg/L), zeatin (0.1mg/L), and proline (0.7mg/L) **(Table 5)**.

Plantlets Regeneration under Heavy Metal/s Stress

The embryogenic calluses were sub-cultured to the regeneration medium containing kinetin (0.5mg/L) along with respective concentrations of $PbSO_4$ and $CdCl_2$ **(Table 4 & 6)** respectively for somatic embryo germination followed by complete plantlet regeneration.

Acclimatization

In vitro regenerated plantlets were further transferred to the plastic pot containing the vermicompost, sand, and soil in 1:1:1 proportion. These potted plantlets were maintained under the greenhouse conditions for the hardening.

Statistical Analysis

Each experiment was performed three times and the data for mean percentage of somatic embryogenesis was calculated using the formula (No. of callus forming somatic embryos/Total no. of callus x 100). Furthermore, the Duncan's Multiple Range Test (DMRT) was undertaken to calculate its significance value.

RESULTS

In present study, the mature embryos were used as explants to induce the embryogenic callus formation followed by differentiation of somatic embryos in sorghum crop using MS-medium fortified with various concentrations and combinations of auxins and cytokinins along with or without proline **(Table 1).**

Callus induction and Somatic Embryogenesis

In basal medium, mature embryos were found to show germination symptoms and developed into young plants after 3-5 days **(Fig. 1A)** of culture initiation while the explants inoculated in nutrient medium supplemented with 2,4-D (1.0mg/L, 2.0mg/L, 2.5mg/L, and 3.0mg/L) alone was proved to be less efficient for the induction of callus followed by somatic embryogenesis. Moreover, the maximum embryogenic frequency (15.3±3.51%) was observed with 2,4-D (2.0mg/L) while the minimum (9.7±3.58%) was recorded with higher concentration of (3.0mg/L) 2,4-D **(Table 1)**.

Also, the explants that were treated with various concentrations (1.0mg/L, 2.0mg/L, 2.5mg/L, and 3.0mg/L) of 2,4-D along with Zn (0.1mg/L), were found to be little effective for callus induction and differentiation of somatic embryos while higher concentrations (2.0mg/L and 2.5mg/L) of 2,4-D could be reasonably good for compact and nodular callus induction (**Fig. 1B)** which could show later somatic embryogenesis (49.6±2.14% and 50.9±1.63%) respectively **(Table 1).**

Moreover, maximum number of somatic embryos per callus (15.1±0.89) was recorded in explants that were treated with 2,4-D (2.5mg/L) in combination with Zn (0.1mg/L) after 14-days of culture initiation. Significantly, further high concentration (3.0mg/L) of 2,4-D with Zn (0.1mg/L) was turned out to be considerably inhibitory for callus induction and somatic embryogenesis (22.3±0.33%) while even the low concentration (1.0mg/L) of 2,4-D was proved to be inefficient for callus induction and somatic embryogenesis (11.1±1.42%).

Furthermore, MS-medium fortified with 2,4-D and Zn was proved to be more efficient for induction of callus followed by differentiation of somatic embryos when BAP (0.5mg/L) and proline (0.7mg/L) were added. Significantly, after 14-days of culture initiation, mature embryo was found to show compact and nodular callus formation from the slightly germinated mature embryos particularly growing with (2.0mg/L or 2.5mg/L), zeatin (0.1mg/L), BAP (0.5mg/L), and proline (0.7mg/L) and later these nodules were observed to be converted into somatic embryos **(Fig. 1B & C)** respectively.

However, the other combinations and concentrations of PGRs exhibit relatively less embryogenic callus formation. The combination of 2,4-D (2.0mg/L or 2.5mg/L), zeatin (0.1mg/L), BAP (0.5mg/L), and proline (0.7mg/L) exhibits efficient and high embryogenesis (63.2±1.67% and 67.4±0.64%) respectively. Moreover, maximum number of somatic embryos differentiated per callus (39.2±0.15) was obtained with the explants that were growing with 2,4-D (2.5mg/L) along with Zn (0.1mg/l), BAP (0.5mg/L) and proline (0.7mg/L). Results indicate that BAP (0.5mg/L) and proline (0.7mg/L) were proved to be effective to enhance the frequency of callusing and somatic embryogenesis when these were used in presence of 2,4-D and Zn.

Meanwhile, the other auxin IAA (1.0mg/L, 2.0mg/L, 2.5mg/L, and 3.0mg/L) was used for callus induction which is significantly inefficient to induce the callus and somatic embryo formation. The embryogenic frequency (21.3±1.53%) was recorded with high concentration of 2,4-D (2.5mg/L). The higher (3.0mg/L) concentration and lower concentration (1.0mg/L) does not promote the embryogenic callus induction (18.7±1.53% and 19.0±1.0%) respectively.

Significantly, IAA was proved to be more effective for callus induction and somatic embryogenesis in combination with Zn, BAP and proline than same combination of Zn, BAP and proline with 2,4-D. Initially, mature embryo were treated with various concentrations (1.0mg/L, 2.0mg/L, 2.5mg/L and 3.0mg/L) of IAA along with Zn (0.1mg/L) and IAA (2.5mg/L) in combination with Zn (0.1mg/L) was proved to be the most effective combination in terms of compact and embryogenic callus formation **(Fig. 1D)** and moreover, this medium resulted the maximum embryogenic frequency (32.4±1.38%) while the same combination 2,4-D (2.5mg/L) with Zn (0.1mg/L) was observed to be more efficient and resulted (50.9±1.63%) indicating that 2,4-D auxin is more effective than IAA with zeatin (0.1mg/L) to trigger embryogenic potentials of the mature embryo explant tissues.

During further studies, various concentrations of IAA (1.0mg/L, 2.0mg/L, 2.5mg/L, and 3.0mg/L) with Zn (0.1mg/L), BAP (0.5mg/L), and proline (0.7mg/L) were tested to evaluate the morphogenic responses of PGRs during mature embryo culture. After 2-3 weeks of culture initiation, both concentrations (1.0mg/L and 1.5mg/L) of IAA with Zn (0.1mg/L), BAP (0.5mg/L), and proline (0.7mg/L) were turned out to be equally efficient for showing the induction of nodular and compact callus followed by differentiation of somatic embryos (**Fig. 1E & F**) respectively. However, IAA (1.0mg/L) with Zn (0.1mg/L), BAP (0.5mg/L), and proline (0.7mg/L) was proved to be the most efficient combination of nutrient medium for induction of callus and differentiation of typical and well-defined somatic embryos (**Fig. 1E).**

Furthermore, this combination of IAA and other additives could show the maximum frequency (87.2±0.21%) of embryogenic callus formation and this medium was also recorded as the best combination in terms of maximum number (55.3±0.33) of somatic embryos per embryogenic callus (**Table 1**). Interestingly, same concentration (1.0mg/L) of 2,4-D in combination with the same concentrations of Zn (0.1mg/L), BAP (0.5mg/L) and proline (0.7mg/L) was emerged as the less potent (61.8±1.15%) for callus induction and somatic embryogenesis during embryo culture.

Germination of Somatic Embryo and Plantlets Regeneration

The embryogenic calli were sub-cultured into the regeneration medium supplemented with various equivalent concentrations (0.1mg/L, 0.5mg/L, and 1.0mg/L) of BAP, Kn and Zn**.** In case of embryogenic callus that was transferred to MS-basal medium without addition of PGRs, somatic embryos were observed to show quick germination and develop into plantlets **(Fig. 1G)** and the frequency of plantlet regeneration exhibited with basal medium was found to be (63.2±0.27%) and number of regenerated plantlets per embryogenic callus (12.8±0.79%) was recorded (**Table 2**).

Moreover, in order to evaluate the effects of various cytokinins on somatic embryo germination and plantlets regeneration in sorghum millet, various cytokinins (BAP, kinetin and zeatin) at equivalent concentrations (0.1mg/L, 0.5mg/L, and 1.0mg/L) were tested. Results indicate that Kn (0.5mg/L) was proved to be the most potent cytokinin for promoting somatic embryo germination followed by plantlets regeneration **(Fig. 1H)** and moreover, the maximum frequency (77.8±0.93%) of

embryogenic calli showing plantlets regeneration and also the maximum number of regenerated plantlets per embryogenic callus (55.3±0.80) were recorded.

However, in case of BAP cytokinin, the maximum frequency (37.8±0.54%) of plantlet regeneration was observed with embryogenic calli growing on (0.1mg/L) of BAP supplemented medium while zeatin (0.5mg/L) could show maximum plantlets regeneration frequency (32.6±0.43%).

Significantly, during this study, results indicate that among the tested cytokinins (BAP, Kn, and Zn) at equivalent concentrations (0.1mg/L, 0.5mg/L, and 1.0mg/L), Kn was proved to be the most effective cytokinin in terms of promoting germination of somatic embryos and plantlets regeneration while BAP and Zn were proved to be little ineffective (**Table 2**). The regenerated plantlets were later transferred to the pot containing soil with vermicompost for acclimatization under greenhouse conditions **(FIG. 1I)**.

Effects of Lead Stress on Somatic Embryogenesis

After establishment of the best nutrient medium containing IAA (1.0mg/L), zeatin (0.1mg/L), BAP (0.5mg/L), and proline (0.7mg/L) for callus induction and somatic embryogenesis during mature embryo culture in sorghum millet, heavy metals stress treatments were undertaken for induction of heavy metals tolerant somatic embryos and plantlets regeneration. Hence, mature embryo was cultured into the nutrient medium supplemented with IAA (1.0mg/L), zeatin (0.1mg/L), BAP (0.5mg/L), and proline (0.7mg/L) along with various concentrations (10mg/L, 25mg/L, 50mg/L, 75mg/L, and 100mg/L) of $PbSO_4$.

In control MS-medium contained with IAA (1.0mg/L), Zn (0.1mg/L), BAP (0.5mg/L), and proline (0.7mg/L), mature embryo explants were observed to produce the compact and nodular callus **(Fig. 2A)** while during lead stress treatments**,** lower concentrations of $PbSO_4$ (10mg/L and 25mg/L) were proved to be equally effective to show compact and nodular callus formation **(Fig. 2B & C)** respectively. Furthermore, compact calli induced on control and $PbSO_4$- treated nutrient media could show somatic embryos differentiation after 20 days of culture initiation **(Fig. 2D, E & F**) respectively. The control medium exhibits the high potential of somatic embryogenesis **(Fig. 2D)** while the callus grown with the lower concentration of $PbSO_4$ (10mg/L) was found to be little less efficient for somatic embryogenesis **(Fig. 2E)**.

Moreover, further higher concentration (25mg/L) of $PbSO_4$ was observed to cause significantly inhibitory for the induction of somatic embryos **(Fig. 2F)**, Later it begins to turn gradually browning and begins to show gradual toxicity. During the further higher concentration (50mg/L) of $PbSO_4$ treatment, induced calli could show compact callusing but was proved to be inhibitory to differentiate somatic embryos significantly rather produce rhizogenic callus which started to produce the root and eventually dried up **(Fig. 2G).** Moreover, the high concentration of $PbSO_4$ (75mg/L) was observed to be considerably toxic in which the calluses were necrosed after few days **(Fig. 2H)** while during very high concentration of $PbSO_4$ (100mg/L) treatment, mature embryos were found to be completely non-responsive for callus induction indicating that 100mg/L of $PbSO_4$ proves to be highly toxic for *in vitro* morphogenesis in sorghum mature embryo culture.

In sorghum, the lower concentration (10mg/L) of $PbSO_4$ shows inhibitory effects towards the embryogenesis. The somatic embryogenesis up in high frequencies (71.9±1.56% and 64.4±0.61%) were observed in explants that were treated with the lower concentrations (10mg/L and 25mg/L) of $PbSO_4$, which are significantly lower than the frequency (87.2±0.21%) of somatic embryogenesis that was obtained during control experiment without $PbSO_4$ treatment. Moreover, further increase in $PbSO_4$ concentration (50mg/L) was proved to be significantly inhibitory (26.9±2.24%) for the induction of callus and differentiation of somatic embryo **(Table 3)**.

However, the least mean frequency (2.9±3.32%) for somatic embryogenesis was observed in explants that were treated with high concentration (75mg/L) of $PbSO_4$ solutions **(Table 3)**. However, very high concentration (100mM) of $PbSO_4$ shows callus formation poorly and such calli were failed to induce somatic embryogenesis indicating the toxicity level of $PbSO_4$ for morphogenesis.

Plantlets Regeneration under Lead - Heavy Metals Stress

During the regeneration of plantlets, MS-regeneration medium supplemented with kinetin (0.5mg/L) without addition of $PbSO_4$, was considered as control experiment that leads to achieve the high frequency of plantlet regeneration **(Fig. 2I)**. Moreover, the lower concentrations (10mg/L and 25mg/L) of $PbSO_4$ were failed to show the inhibitions in plantlet germination from somatic embryos **(Fig. 2J & K)** respectively. However, the higher concentration (50mg/L) of $PbSO_4$ was turned out to be strongly inhibitory for the plantlet regeneration. Later, 12-week-old lead tolerant regenerated plantlet grown with 25mg/L of $PbSO_4$ was gradually acclimatized in plastic cup containing vermicompost, sand, and soil in 1:1:1 proportion under greenhouse conditions (**Fig. 2L**).

The lower concentrations (10mg/L and 25mg/L) of $PbSO_4$ were found to show efficient plantlet regeneration (51.9±1.23% and 45.9±0.37%) respectively, however, frequency of plantlet regeneration was obtained lesser than the regeneration frequency recorded in control treatments. The lowest plantlets regeneration frequency (23.8±0.29%) was recorded with concentration (50mg/L) of $PbSO_4$. The toxicity of Pb highly affects the frequency of plantlet regeneration from somatic embryos (3.7±0.67%) that was observed with 75mg/L of $PbSO_4$ **(Table 4)**.

Effects of Cadmium - Stress on Somatic Embryogenesis

Mature embryo explants that were treated with IAA (1.0mg/L), Zn (0.1mg/L), BAP (0.5mg/L), and proline (0.7mg/L) without $CdCl_2$ could produce the compact and nodular callus while during cadmium stress treatments**,** similar to $PbSO_4$-treatment, the lower concentrations of $CdCl_2$ (10mg/L and 25mg/L) were failed to show inhibitory response in terms of callus induction **(Fig. 3A & B)** respectively. However, the higher concentration (75mg/L) of $CdCl_2$ could show significant inhibitions caused by the toxicity of $CdCl_2$ but even though compact and nodular callus formation **(Fig. 3C)** was observed**.**

Furthermore, compact calli induced on $CdCl_2$ - treated nutrient media (10mg/L, 25mg/L, 50mg/L, 75mg/L, 100mg/L, and 150mg/L) could show somatic embryos differentiation after 3-4 weeks of culture initiation **(Fig. 3D-H**) respectively. The lower concentrations (10mg/L and 25mg/L) of $CdCl_2$ medium exhibited the high potential of somatic embryogenesis **(Fig. 3D & E)** respectively while the callus grown with the further higher concentrations of $CdCl_2$ (50mg/L and 75mg/L) were found to be little less efficient for somatic embryogenesis **(Fig. 3F)**. Moreover, in comparison to $PbSO_4$, cadmium chloride even at very high concentrations (100mg/L and 150mg/L) could be effective to induce callusing and somatic embryogenesis **(Fig. 3G & H**) respectively. However, there were symptoms of gradual brownish or necrosis of non-embryogenic calli grown on higher concentrations (75mg/L, 100mg/L, and 150mg/L) of $CdCl_2$- treatments (**Fig. 3F, G & H**) respectively. Moreover, brownish appearance of non-embryogenic tissues could be due to toxicity caused by high concentrations of $CdCl_2$.

In comparison to control experiment (87.2±0.21%), during $CdCl_2$ treatments, the maximum frequency (69.2±0.7%) of somatic embryogenesis was obtained with the lower concentration (10mg/L) of $CdCl_2$. indicating the inhibitory response of $CdCl_2$. Moreover, with the further increase in $CdCl_2$ concentrations, gradual reductions in frequency of somatic embryogenesis were recorded and therefore, the minimum frequency (03.1±3.24%) was obtained with very high concentration (150mg/L) of $CdCl_2$-treatments. However, there was no significant difference in terms of embryogenesis frequency obtained with 10mg/L of $CdCl_2$ (69.2±0.7%) and (66.9±1.63%) that was recorded with 25mg/L of $CdCl_2$ concentration in nutrient medium. Significantly, even the high concentration (100mg/L) of $CdCl_2$ proves to be inefficient to cause heavy metal stress inhibition response on embryogenesis (39.2±2.26%). Moreover, very high concentration (200mg/L) was turned out to be lethal **(Table 5)**.

Plantlets Regeneration under Cadmium - Heavy Metals Stress

The embryogenic callus was transferred into the nutrient medium consisted of kinetin (0.5mg/L) along with respective $CdCl_2$ concentrations **(Table 6)**. The plantlet germination from somatic embryos was recorded as the maximum frequency (77.8±0.93%) with control and eventually reduced (61.9±0.43% and 57.5±0.38%) in case of embryogenic calli that were transferred to the nutrient media consisted of 10mg/L

and 25mg/L of $CdCl_2$ respectively along with Kn (0.5mg/L). The higher concentration (50mg/L) of $CdCl_2$ is efficient enough to support the tolerant plantlet regeneration **(Fig. 3I)**.

While the least frequency of plantlet regeneration (13.3±3.21%) was obtained in embryogenic calli treated with 75mg/L of $CdCl_2$ solutions **(Table 6)** whereas, the high concentration (100mg/L) is exhibiting high toxicity and affects the germination frequency negatively (4.23±2.33%). Moreover, very high concentration (150mg/L) is proved to be lethal. Finally, *in vitro* regenerated plantlet (**Fig. 3J)** grown under $CdCl_2$ (50mg/L) stress conditions was further transferred to plastic cup for gradual acclimatization **(Fig. 3K)** in greenhouse.

Table 1: Sorghum (*Sorghum bicolor* L.), effects of auxins (2,4-D and IAA) in combination with cytokinins (Zn and BAP) and proline on percentage of explants showing embryogenic callus induction and number of somatic embryos per embryogenic callus during mature embryo culture in MS- nutrient medium.

Concentration of Auxins (mg/L)		Concentration of Cytokinins (mg/L)		Concentration of Proline (mg/L)	Percentage of Embryogenic Callus (Mean ± SD)	No.of Somatic Embryos/ Embryogenic Callus (Mean ± SD)
2, 4- D	1.0	-		0	12.3±2.09	10.1±0.64
	2.0				15.3±3.51	12.5±2.21
	2.5				13.3±0.57	11.4±3.41
	3.0				9.7±3.58	02.7±4.72
	1.0	**Zn**	0.1	0	11.1±1.42	03.1±3.41
	2.0				49.6±2.14	14.7±2.38
	2.5				50.9±1.63	15.1±0.89
	3.0				22..3±0.33	11.1±0.63
	1.0	**Zn + BAP**	0.1 + 0.5	0.7	61.8±1.15	34.5±1.27
	2.0				63.2±1.67	33.5±0.86
	2.5				67.4±0.64	39.2±0.15
	3.0				54.5±1.71	16.2±0.27
IAA	1.0	-		0	19.0±1.0	8.2±2.22
	2.0				19.3±0.57	8.5±3.41
	2.5				21.3±1.53	9.1±3.57
	3.0				18.7±1.53	7.8±2.51
	1.0	**Zn**	0.1	0	23.2±0.25	11.3±0.71
	2.0				27.7±1.53	12.1±0.37
	2.5				32.4±1.38	13.2±0.32
	3.0				12.4±2.12	6.91±0.33
	1.0	**Zn + BAP**	0.1 + 0.5	0.7	87.2±0.21	55.3±0.33
	1.5				81.1±0.52	49.4±0.82
	2.0				74.2±1.44	34.8±1.16
	2.5				61.7±1.93	29.2±2.14
	3.0				48.3±0.33	14.2±1.71

Table 2: Sorghum (*Sorghum bicolor* L.), effects of various concentrations of cytokinins on percentage of embryogenic callus showing plantlets regeneration and number of regenerated plantlets per embryogenic callus during mature embryo culture in MS-nutrient medium supplemented with BAP, Kn, and Zn.

Concentration of Cytokinins (mg/L)		Percentage of Embryogenic Callus showing Plantlets Regeneration (Mean±SD)	No. of Regenerated Plantlets/ Embryogenic Callus (Mean±SD)
BAP	0	63.2±0.27	12.8±0.79
	0.1	37.8±0.54	17.5±1.53
	0.5	27.3±0.63	11.3±0.20
	1.0	21.4±0.27	10.8±0.71
Kn	0.1	45.8±0.67	28.3±1.63
	0.5	77.8±0.93	55.3±0.80
	1.0	33.3±0.77	12.9±0.81
Zn	0.1	26.2±0.37	11.5±1.41
	0.5	32.6±0.43	13.9±0.93
	1.0	28.4±0.25	11.9±0.71

Table 3: Sorghum (*Sorghum bicolor* L.), effects of lead (Pb) heavy metal stress on percentage of somatic embryogenesis and number of somatic embryos per embryogenic callus during mature embryo culture in MS-medium supplemented with (IAA, zeatin,BAP, and proline) and various concentrations of $PbSO_4$.

Concentration (mg/L)	Concentration of $PbSO_4$ (mg/L)	Percentage of Somatic Embryogenesis (Mean±SD)	No. of Somatic Embryos/ Embryogenic Callus (Mean±SD)
IAA (1.0) + Zeatin (0.1) + BAP (0.5) + Proline (0.7)	0	87.2±0.21[a]	55.3±0.33
	10	71.9±1.56[b]	44.1±1.14
	25	64.4±0.61[c]	39.2±0.95
	50	26.9±2.24[d]	12.9±0.56
	75	02.9±3.32[e]	0.4±3.81
	100	0	0

Table 4: Sorghum (*Sorghum bicolor* L.), effects of lead (Pb) heavy metal stress on percentage of plantlet regeneration and number of regenerated plantlets per embryogenic callus during mature embryo culture in MS-medium supplemented with Kn and various concentrations of $PbSO_4$.

Concentration of Kn (mg/L)	Concentration of $PbSO_4$ (mg/L)	Percentage of Plantlet Regeneration (Mean±SD)	No. of Regenerated Plantlets / Embryogenic callus (Mean±SD)
0.5	0	77.8±0.93[a]	55.3±0.80
	10	51.9±1.23[b]	39.2±1.32
	25	45.9±0.37[c]	33.5±2.19
	50	23.8±0.29[d]	17.8±1.78
	75	03.7±0.67[e]	1.4±3.91
	100	0	0

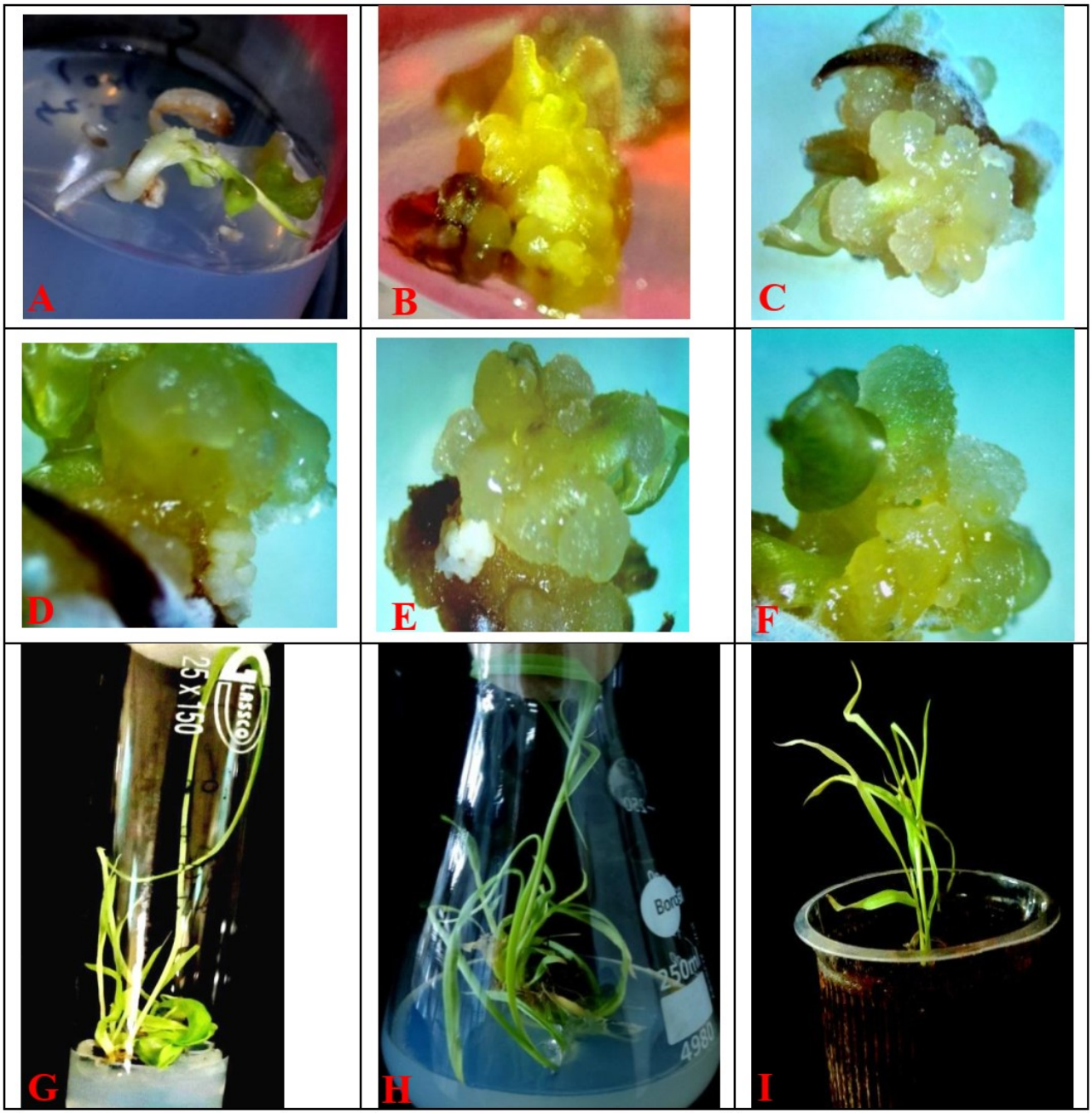

Figure 1: *Sorghum bicolor* L. Mature embryo culture on MS- medium supplemented with various concentrations of auxins (2.4-D and IAA) and cytokinins (Zn and BAP) either alone or in combinations, also in presence or absence of proline;

(A) In control or MS-basal medium, mature embryo germinates and develops into young plant– after 3-5 days of culture initiation; **(B)** Mature embryo shows inhibited germination and induces callus induction followed by differentiation of somatic embryos on medium supplemented with 2,4-D (2.5mg/L) and Zn (0.1mg/L) **(C)** Mature embryo with slight germination induces compact and nodular callus formation followed by somatic embryogenesis on medium supplemented with 2,4-D (2.5mg/L), Zn (0.1mg/L), BAP (0.5mg/L), and proline (0.7mg/L) **(D)** Explant induces callus formation with some nodular structures leading to somatic embryogenesis on medium added with IAA (2.5mg/L) and Zn (0.1mg/L) after 2-3 weeks of culture; **(E)** Compact callus differentiates somatic embryos on medium supplemented with IAA (1.0mg/L), Zn (0.1mg/L), BAP (0.5mg/L), and proline (0.7mg/L) **(F)** Somatic embryogenesis on medium supplemented with IAA (1.5mg/L), Zn (0.1mg/L), BAP (0.5mg/L), and proline (0.7mg/L) – after 2 weeks of culture initiation; **(G)** Somatic embryo germination and plantlet regeneration from embryogenic callus on transfer to MS-basal medium (**H)** Plantlet regeneration on MS-medium supplemented with Kn (0.5mg/L) – after 10-12 weeks of culture initiation; **(I)** Potted plantlet – After 7 days of Transplantation.

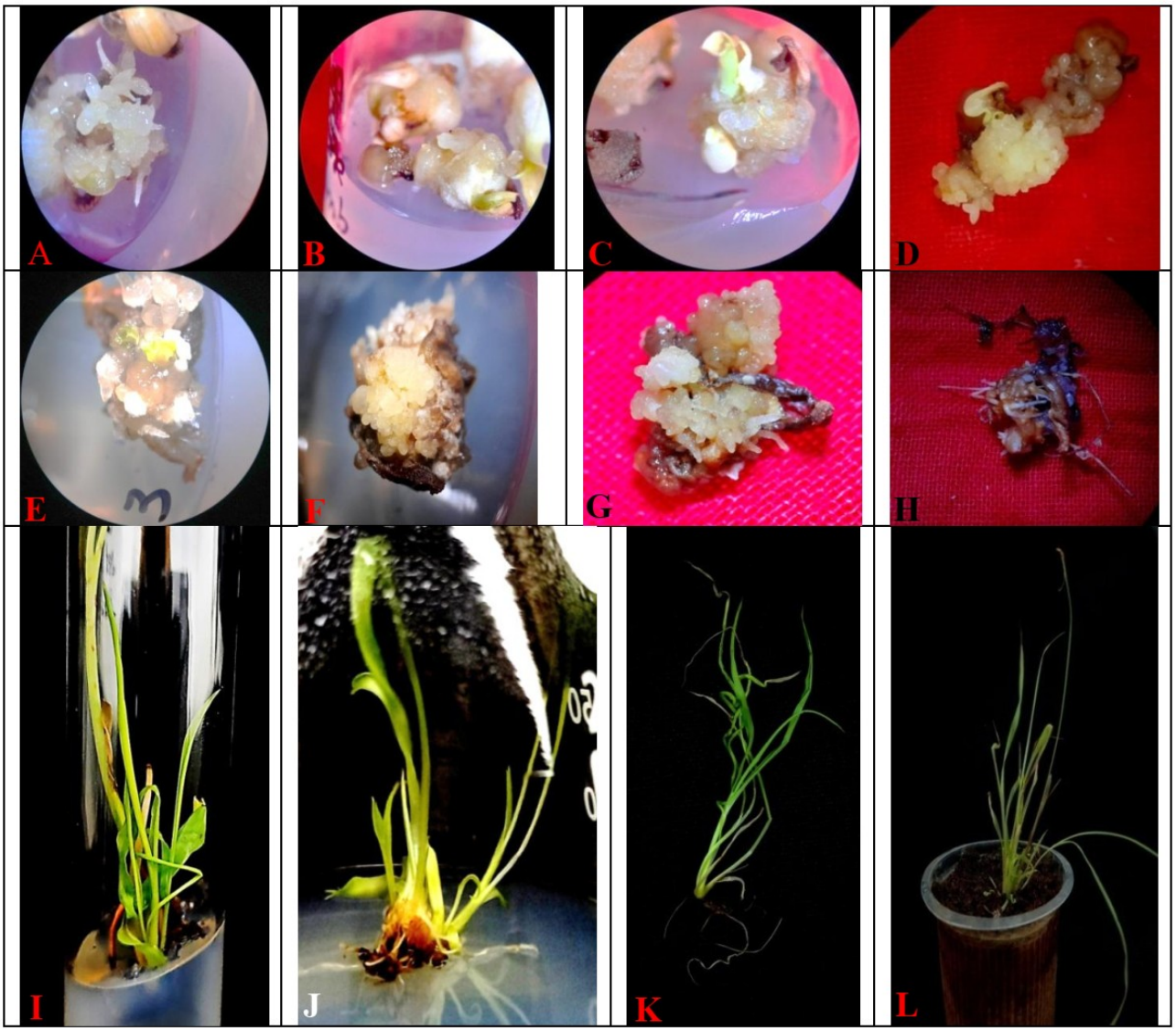

Figure 2. *Sorghum bicolor* L. Mature embryo culture on MS-medium supplemented with IAA (1.0mg/L), Zn (0.1mg/L), BAP (0.5mg/L), and proline (0.7mg/L) as control and also supplemented with various concentrations of $PbSO_4$ showing induction of callus and somatic embryogenesis;

(A) Induction of callus from mature embryo in control MS-medium without $PbSO_4$ **(B)** Compact callus formation from mature embryo treated with 10mg/L of $PbSO_4$ **(C)** Callus formation under 25mg/L of $PbSO_4$ **(D)** Somatic embryogenesis in control MS-medium without $PbSO_4$ **(E)** Differentiation of somatic embryos on induced compact callus growing with 10mg/L of $PbSO_4$ **(F)** Formation of embryogenic compact callus growing under MS-medium supplemented with 25mg/L of $PbSO_4$ **(G)** Somatic embryogenesis in mature embryo derived callus growing with 50mg/L of $PbSO_4$ **(H)** Mature embryo derived callus treated with 75mg/L of $PbSO_4$ gradually shows necrosis without differentiation of somatic embryos– after 21-days of culture initiation **(I)** Regeneration of plantlets form embryogenic callus on transfer to MS-medium supplemented with 0.5mg/L of Kn without $PbSO_4$ (**J)** Plantlet regeneration on transfer of embryogenic callus to MS-medium added with 0.5mg/L of Kn and 25mg/L of $PbSO_4$– after 12- weeks of culture initiation; **(K)** Regenerated plantlet grown on 0.5mg/L of Kn along with 25mg/L of $PbSO_4$ under *ex vitro* condition **(L)** $PbSO_4$ (25mg/L) - treated tolerant plantlet under acclimatization conditions after 11-days of transfer.

Figure 3: *Sorghum bicolor* L. Mature embryo culture on MS-medium supplemented with IAA (1.0mg/L), Zn (0.1mg/L), BAP (0.5mg/L), and proline (0.7mg/L) as control and also supplemented with various concentrations of $CdCl_2$ showing induction of callus and somatic embryogenesis;

(A) Induction of callus from mature embryo treated with 10mg/L of $CdCl_2$ **(B)** Callus formation from mature embryo treated with 25mg/L of $CdCl_2$ treatment **(C)** Callus formation under 75mg/L of $CdCl_2$ **(D)** Somatic embryogenesis in callus treated with 10mg/L of $CdCl_2$ **(E)** Differentiation of somatic embryos induced on callus growing with 25mg/L of $CdCl_2$ **(F)** Development of embryogenic callus growing under MS-medium supplemented with 75mg/L of $CdCl_2$ **(G)** Somatic embryogenesis in callus treated with 100mg/L of $CdCl_2$ **(H)** Growth of embryogenic callus derived from mature embryo treated with 150mg/L of $CdCl_2$ – after 3-4 weeks of culture initiation; **(I)** Plantlet regeneration in embryogenic callus on transfer to MS- medium supplemented with 0.5mg/L of Kn and 50mg/L of $CdCl_2$ - after 13-weeks of culture initiation (**J)** Cadmium chloride (50mg/L) tolerant regenerated plantlet under *ex vitro* condition **(K)** Potted plantlet growing under $CdCl_2$ (50mg/L) stress condition after 8-days of transplantation.

Table 5: Sorghum (*Sorghum bicolor* L.), effects of cadmium (Cd) heavy metal stress on percentage of somatic embryogenesis and number of somatic embryos per embryogenic callus during mature embryo culture in MS-medium supplemented with various concentrations of $CdCl_2$ along with IAA, BAP, zeatin, and proline.

Concentration (mg/L)	Concentration of $CdCl_2$ (mg/L)	Percentage of Somatic Embryogenesis (Mean±SD)	No. of Somatic Embryos/ Embryogenic Callus (Mean±SD)
IAA (1.0) + BAP (0.5) + Zeatin (0.1) + Proline (0.7)	0	87.2±0.21[a]	55.3±0.33
	10	69.2±0.77[b]	44.0±0.54
	25	66.9±1.63[b]	43.7±2.16
	50	58.3±0.86[c]	39.2±1.77
	75	51.9±1.62[d]	37.4±0.82
	100	39.2±2.26[e]	11.2±3.15
	150	03.1±3.24[f]	1.3±4.38
	200	0	0

Table 6: Sorghum (*Sorghum bicolor* L.), effects of cadmium (Cd) heavy metal stress on percentage of plantlet regeneration and number of plantlets per embryogenic callus during mature embryo culture in MS-medium supplemented with kinetin and various concentrations of $CdCl_2$.

Concentration of Kn (mg/L)	Concentration of $CdCl_2$ (mg/L)	Percentage of Plantlet Regeneration (Mean±SD)	No. of Regenerated Plantlets/ Embryogenic Callus (Mean±SD)
0.5	0	77.8±0.93[a]	55.3±0.80
	10	61.9±0.43[b]	46.8±1.74
	25	57.5±0.38[b]	40.2±1.39
	50	39.2±0.49[c]	27.6±1.71
	75	13.3±3.21[d]	08.4±0.93
	100	4.23±2.33[e]	02.8±4.73

DISCUSSION

Generally, the biotic and abiotic stresses show negative impacts over plants and retard the growth and development by inhibiting the uptake of water and nutrients and compromise membrane permeability (Arif *et al.*, 2020). The contaminations of ground water, exploitation of land used for agriculture, crop plants, etc., are highly affected by the heavy metal/s in high concentration (Gismera *et al*., 2005; Fawzy, 2008).

The alteration of biochemical, physiological and metabolic pathway of crop plants significantly affect grain yield by heavy metal/s contamination (Hossain *et al*., 2010; Rascio and Navari-Izzo, 2011). *In vitro* regeneration via somatic embryogenesis is playing major significant role in crop improvement and also has proved a meaningful tool for gene transformation techniques in food crops (Tiecoura *et al*., 2003; Vikrant, 2015).

Induction of Callus and Somatic Embryogenesis

The suitable and competent explants have been suggested as primary requirements for the achievement of successful embryogenesis, plantlet regeneration and genetic transformation (Grootboom *et al*., 2008). Literature reveals that callus induction and plantlet regeneration have been established in sorghum crop by employing the various explants such as, immature inflorescence (Gupta *et al*., 2006; Jogeswar *et al*., 2007), shoot segment (Brar *et al.*, 1997), shoot tips (Bhaskaran and Smith, 2006) and mature embryo (McKinnon *et al.,* 1986).

As per the available reports, among various explant tissues identified, mature embryos are considered as potential explants for *in vitro* study deals with somatic

embryogenesis and plantlet regeneration because of being easily available abundantly throughout the year (Sudhakar *et al.,* 2004; Jha *et al.,* 2008; Vikrant, 2015). The present study aims with the impacts of heavy metals stresses on induction of embryogenic callus followed by tolerant plantlets regeneration during mature embryo culture.

Significantly, during previous studies on sorghum mature embryo culture, auxins have been found to be the main source to induce the somatic embryogenesis (Elkonin *et al*., 1995; Nguyen *et al.,* 2007; Gurel *et al.,* 2012; Chen *et al.,* 2015; Do *et al.,* 2016; Omer *et al*., 2019) along with or without the cytokinins like BAP and Kn (Belide *et al.,* 2017; Espinoza-Sánchez *et al.,* 2018). Additionally, the amino acids like proline are known to enhance the induction of embryogenic callus and plantlet regeneration in maize and sorghum (Rao *et al.,* 1995; Omer *et al.,* 2019).

The report states that the combinations of auxins and cytokinins induce the differentiation of embryogenic callus (Thomas and Maseena, 2006). However, the present study reveals that the combinations of auxin (1.0mg/L of IAA) and cytokinins (0.5mg/L of BAP and 0.1mg/L of zeatin) along with the amino acid (0.7mg/L of L-Proline) induce the highest frequency (87.2±0.21%) of embryogenic callus and somatic embryogenesis**.** Significantly, in contrast, other studies indicate the combination of other auxin 2,4-D along with BA, kinetin and zeatin increases the percentage of embryogenic callus induction in *Eleusine coracana* (Ceasar and Ignacimuthu, 2008), *Sorghum bicolor* (Belide *et al.,* 2017; Espinoza-Sánchez *et al.,* 2018).

However, during present study, 2,4-D alone was proved to be less efficient for callus induction and somatic embryogenesis while 2,4-D along with Zn resulted better combination and therefore, 2,4-D (2.0mg/L and 2.5mg/L) along with Zn (0.1mg/L) could show the almost equal frequency of embryogenic callus formation (49.6±2.14% and 50.9±1.63%) respectively. Moreover, in general 2,4-D auxin proves to be less potent than IAA in terms of callus induction and somatic embryogenesis. The other combination of auxins; 2,4-D and IAA, either alone or with cytokinins (BAP and zeatin) does not exhibit embryogenesis significantly **(Table 1)**.

Germination of Somatic Embryo and Plantlet Regeneration

Many studies reveal the regeneration of sorghum plantlets (Mishra and Khurana, 2003; Visarada *et al.,* 2003; Nirwan and Kothari, 2004; Pola and Mani, 2006; Kishore *et al.,* 2006; Baskaran *et al.,* 2006). The formation of multiple shoots from different explants such as mature embryo (Kuruvinashetti *et al*., 1998; George and Eapen, 1989), shoot apices (Harshavardhan *et al.,* 2002), leaf base (Mishra and Khurana, 2003), leaf segments (Pola and Mani, 2006), shoot tips (Seetharama *et al*., 2000; Kingley and Ignacimuthu, 2014) of sorghum has been reported in literature.

The supplementation of different concentrations (0.1mg/L, 0.5mg/L and 1.0mg/L) of cytokinins (BAP, kinetin and zeatin) were used during present study for regeneration of plantlets. The control medium without any hormones exhibits (63.2±0.27%) the average frequency of somatic embryogenesis while the supplementation of kinetin (0.5mg/L) supports to enhance the somatic embryo germination (77.8±0.93%) followed by plantlet regeneration. Similar results based on kinetin were also supported by many other studies, in which the cytokinins like kinetin or BAP produces the efficient shoot induction and plantlet regeneration in pearl millet (Mythili *et al*., 2001; Srivastav and Kothari, 2002), kodo millet (Nayak and Sen, 1989) and foxtail millet (Xu *et al.,* 1984).

In contrast, the regeneration medium containing BAP or zeatin does not efficiently support the plantlet regeneration. Among them, maximum frequency (37.8±0.54%) of plantlet regeneration was observed with 0.1mg/L of BAP while 0.5mg/L of Zn could show the maximum frequency (32.6±0.43%) of plantlets regeneration.

Effects of Heavy Metals Stress on Somatic Embryogenesis

Some heavy metals such as Cu (Prunhauser and Gyulai, 1993), Co (Chraibi *et al.,* 1991; Roustan *et al.,* 1989), Ni (Roustan *et al.,* 1989) and Cd (Patnaik *et al.,* 2005) were widely used as micronutrients in tissue culture, it plays significant role in morphogenesis in callus initiation (Roustan *et al.,* 1989; Rout *et al.,* 1998). Many cereal crops such as maize (*Zea mays*), sorghum

(*Sorghum bicolor*) and alfalfa (*Medicago sativa*) (Vijayarengan, 2005; Zhuang *et al.,* 2009) have the ability to accumulate the high amount of heavy metals.

During additional studies, the resistance and accumulation of cadmium by sorghum plants demonstrated that sorghum is tolerant to cadmium at a concentration of 0.5mM (Marchiol *et al.,* 2007; Kuraikose and Prasad, 2008; Epelde *et al*., 2009; Soudek *et al.,* 2014). Likewise, the lower concentration (10mg/L) of $PbSO_4$ reflects the maximum embryogenesis frequency (71.9±1.56%) which is comparatively less than control (87.2±0.21%). In contrast, the lower concentration (10mg/L) of cadmium found to exhibit more toxicity and proves to reduce the embryogenic frequency (69.2±0.77%).

Previous study suggests that millets show inhibitory effect like retard growth and less productivity is one of the adaptations of plant to overcome the heavy metal stress and it accumulates more Cd and Pb (Wuana *et al*., 2013). Moreover, the medium concentration (50mg/L and 75mg/L) of Cd tend to produce the embryogenic callus (58.3±0.86% and 51.9±1.62%) respectively. On the other hand, the Pb proves to be relatively inhibitory towards the callus induction and somatic embryogenesis. Significantly, the same concentrations (50mg/L and 75mg/L) of $PbSO_4$ exhibit the reduced frequencies of somatic embryogenesis (26.9±2.24% and 2.9±3.32%) respectively. Cadmium (Cd) alone is highly efficient to induce the somatic embryogenesis without 2,4-D in leaf-base culture of wheat (Patnaik *et al.,* 2005). Likewise, the higher concentrations (100mg/L and 150mg/L) of cadmium stress tend to produce the somatic embryogenesis (39.2±2.26% and 3.1±3.24%).

The inhibited growth in plants during heavy metal stresses causes the root browning, chlorophyll deformation result in chlorosis of leaves (Doganlar and Yurekli, 2009; Michel-López *et al.,* 2016; Okem *et al.,* 2016; Vijendra *et al.,* 2016). Likewise, the highest concentration 200mg/L of Cd is proved to be lethal. Pb is showing it's phytotoxicty which results in the complete inhibition of callus induction in higher concentration (100mg/L).

Plantlets Regeneration under Heavy Metals Stress Conditions

In present study, the germination frequency of somatic embryos was observed to be reduced to 51.9±1.23% in Pb and 61.9±0.43% in Cd compared with control (77.8±0.93%). This effect and accumulation of lead and cadmium pollution in sorghum crop (Liu *et al.,* 2014). The report states that increase in the concentration of Pb and Cd leads to the decrease in plant height and weight. The accumulation of cadmium was observed in the stem of shoot whereas the lead accumulated in the leaf of shoot crop (Liu *et al.,* 2014). Trans and Popova (2013), reported that the plants adapt a tolerance strategies in which plants can accumulate and store by binding the cadmium in protein monomers.

The regeneration of plantlets with higher concentration (75mg/L) of cadmium is inhibited upto 13.3±3.21%. This is due to the high accumulation of cadmium in plant metabolism. In contrast, in Pb stress the regeneration frequency (3.7±0.67%) in higher concentration (75mg/L) is found to be highly toxic that inhibits the plant growth.

The higher concentration (100mg/L) of Pb is highly toxic. In cadmium, the very high concentration (200mg/L) was proved to be lethal in present study which is mainly due to the necrosis caused by the cadmium (Hernadez and Cooke, 1997).

CONCLUSION

The present study successfully established the efficient protocols for somatic embryogenesis and regeneration of plantlets using mature embryo as explants under heavy metal stress conditions particularly lead and cadmium. The inhibition of embryogenesis and plantlet regeneration were observed under both the heavy metal stresses in comparison to control. The higher concentration (100mg/L) exerts the phytotoxicity in lead is highly lethal and affects the callus induction. However, the 100mg/L of cadmium tends to exert somatic embryogenesis (39.2±2.26%). Hence, the lead is proved to be highly toxic than the cadmium. Plantlet germination was also significantly retarded by lead and cadmium stresses. The production of crop plants with high tolerance and high productivity towards heavy metals is necessary through gene transformation technology for crop improvement in the pollution world.

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Authors Contribution Statement

The first author (RRM) of the manuscript has carried out the experimental part of the study, compilation of the statistical data and the manuscript preparation. Second author has designed the experimental set up and also edited the manuscript into presentable format.

Data Availability Statement

All statistical data supporting the findings of this study are available within the paper and its Supplementary Information provided in (**Tables 1-3)**. Images (**Fig. 1, 2 & 3**) are given in JPEG format. Authors are open to give the permission to use the data after publication of the research article in the concerned journal.

CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

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