In vitro selection and characterization of cadmium-tolerant calli of *Tagetes erecta* and *Gomphrena globosa*

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Ornamental plants (OPs) are beneficial to remove, control and reduce heavy metals (HMs) in a process called 'phytoremediation'. This study evaluated the *in vitro* system-based phytoremediation properties of *Tagetes erecta* and *Gomphrena globosa* calli. Leaves from *in vitro* seed-grown *T. erecta* and *G. globosa* were used as an explant source for callus culture. Callus culture was found optimal in MS medium supplemented with 8 µM BAP + NAA for *T. erecta* and 2 µ M 2, 4-D for *G. globosa*. These plants were grown in their respective optimized (controlled) medium enriched with different amounts (50, 100, 150, 200, and 250 µM) of Cadmium (Cd) added in the form of Cadmium chloride ($CdCl_2$). The *in vitro* calli developed were evaluated for Cd stress tolerance based on callus diameter, growth tolerance index (GTI) and catalase (CAT) activity. Over four weeks, the callus diameters of *T. erecta* and *G. globosa* grown in different concentrations of Cd had lower growth than that of the controlled one. On the other hand, the GTI measured were greatest at 150 µM of Cd for both *T. erecta* (130.95%) and *G. globosa* (149.32%) suggesting a potential Cd tolerance. However, the CAT activity in *T. erecta* callus increased with the Cd concentration peaking at 150 µM then started declining while *G. globosa* callus showed the highest CAT activity at 50 µM of Cd. Thus, *T. erecta* callus showed greater Cd tolerance with the prospect of utilizing it for phytoremediation. The study also suggests growing *T. erecta* at 150 µM Cd for tolerant calli.

Keywords: Cadmium tolerance, callus, catalase activity, growth tolerance index, ornamental plants.

Exponential population growth has caused significant damage to the environment and ecosystems. One of the many factors affecting the environment is related to some heavy metals 'HMs' (Khan *et al.*, 2021). There are several toxic HMs, including Cadmium 'Cd' (Saini & Dhania, 2020) that poses a threat to biota even at a very low concentration. Consumption of food contaminated with Cd is hazardous to human health (Khan *et al.*, 2017). Accumulation of Cd can cause renal tubular dysfunction (Bernard, 2004) and affects the reproductive systems (Kumar & Sharma, 2019). Additionally, the presence of Cd affects mineral nutrient uptake in plants and their growth. This is associated with growth inhibition and low dry matter yield (Meena *et al.*, 2018; Fattahi *et al.*, 2019), photosynthesis and respiration inhibition (Navarro-León *et al.*, 2019), and chlorosis (Chun *et al.*, 2020).

There are various methods, including physical, chemical, and biological approaches, being used and developed for remediation of HMs. One promising method that has the potential to save energy and cost is called 'phytoremediation' which involves using plants and plant processes to remove, contain, or reduce pollutants in the environment (Berti & Cunningham, 2000).

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The mechanism of HMs tolerance of plants has been the subject of numerous studies aimed at improving phytoremediation performance (Yan *et al.*, 2020). Tissue culture-based *in vitro* breeding technique is a practical and economical way to improve plants. During the tissue culture process, callus cells can change due to mutagenic conditions (Phillips *et al.*, 1994; Wang & Wang, 2012), leading to somaclonal variation (Skirvin *et al.*, 1993). The culture stress process can also induce genetic and epigenetic changes (Gao *et al.*, 2010), ultimately improving the genetics of the plant.

The application of ornamental plants (OPs) for phytoremediation of HMs as well as beautification may be an attractive option (Khan *et al.*, 2021). *Tagetes erecta* (locally called 'Sayapatri') and *Gomphrena globosa* (locally called 'Makhamali') are culturally important OPs in Nepal. These two plant species possess industrial value with extensive markets during 'Tihar festival' (i.e. festival of lights observed in Oct/Nov) in Nepal. It has been demonstrated that these plants can effectively accumulate Chromium (Coelho *et al.*, 2017) and Arsenic (Signes-Pastor *et al.*, 2015). *In vitro* testing systems based on OPs can be a quintessential tool for the characterization of possible phytoremediation of different HMs (Khan *et al.*, 2021). In light of this knowledge, this study aimed to evaluate the potential phytoremediation properties of *in vitro* calli developed from *T. erecta* and *G. globosa* (see Figure 1).

Materials and methods

In vitro seed germination and callus culture

The seeds of *T. erecta* and *G. globosa*, obtained from the Puspa Bikash Kendra, Godawari, Lalitpur, Nepal, were separated from other floral parts and cleaned under running water with added 'Tween 20 solution' for 30 minutes. Sterilization was performed inside a laminar flow cabinet using 1% Sodium hypochlorite (NaOCl) solution and 70% ethanol (CH_3CH_2OH). The seeds were then germinated and grown on a hormone-free MS medium as described by Murashige & Skoog (1962). Later on, the leaves from the seed-grown plants (size: 1cm × 0.5cm) were removed and used as explants for growing calli in culture. The hormones 6-Benzylaminopurine (BAP), Naphthaleneacetic acid (NAA), and 2, 4-Dichlorophenoxyacetic acid (2, 4-D) were added to the MS medium for callus culture (Belarmino *et al.*, 1992; Bodhipadma *et al.*, 2017).

Callus growth in Cd-enriched media

The type of medium used for growing callus was selected based on the percentage of callus induction and the average weight of the callus. The selected medium was enriched with different quantities of Cd (50, 100, 150, 200, and 250 μM) by adding Cadmium chloride ($CdCl_2$) solution as described by Nehnevajova *et al.* (2007). The
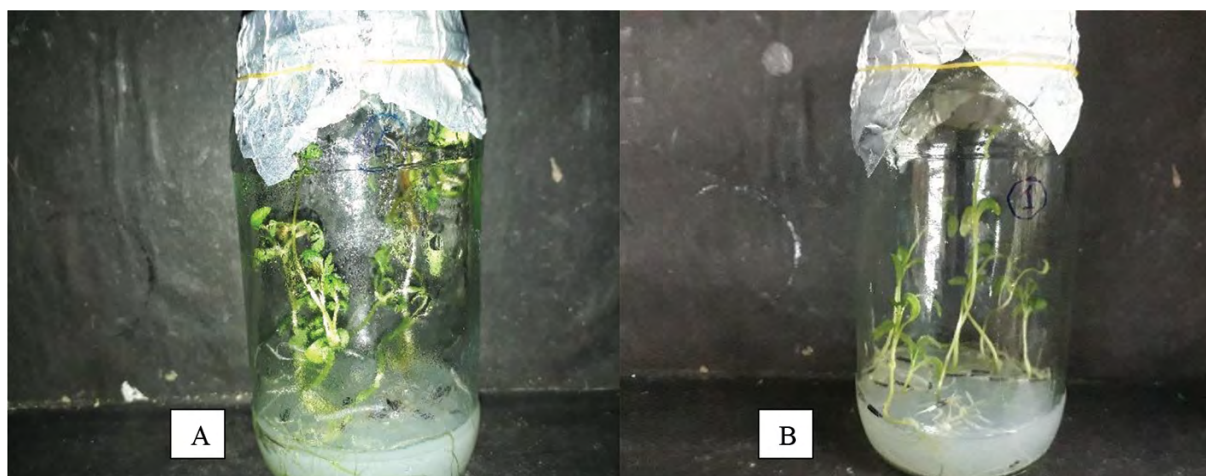

Figure 1: **The *in vitro* germinated plantlets from seeds: (A) *T. erecta* and (B) *G. globosa*.**

effects of Cd on callus growth were monitored by measuring the diameter of the callus using the *Diameter = √Length×√Width* (Compton, 1994; Chenar et al., 2016). formula:

The callus growth tolerance index (GTI) was used to select calli that are tolerant to HMs exposure (Samantaray *et al.*, 2001). It was calculated using the formula:

$$GTI = \frac{Mean\ callus\ growth\ in\ media\ with\ HMs}{Mean\ callus\ growth\ in\ media\ without\ HMs} \times 100\%$$ (Rout *et al.*, 1998).

Catalase activity

Catalase activity was evaluated from the callius developed on a Cd-enriched medium as described by Zhang *et al.* (2007). To accomplish this, 1.0 gram of callus was combined with 3 ml of a buffer solution (50 mmol/L sodium phosphate buffer at pH 7.8 with 1.0 mmol/L Ethylene diamine Tetra acetic Acid (EDTA) and 2% polyvinylpyrrolidone) and then centrifuged for 10 minutes at 5000g (relative centrifugal force). The resulting enzyme extract was added to a reaction mixture containing phosphate buffer (pH 7.0), 0.1 mmol/L EDTA and 20 mmol/L hydrogen peroxide (H_2O_2), and the reaction was monitored by measuring the depletion of H_2O_2 at 240 nanometers applying the Beer-Lambert law and using the Molar extinction coefficient (ε) of 36 mol/L cm. As per the Beer-Lambert law, the absorbance (A) of H_2O_2 was calculated as:

$A = \varepsilon Lc,$

Where, ε = Molar extinction coefficient; L = Length of light path; and c = Concentration of sample.

The results were reported as the amount of enzyme activity per milliliter of sample per gram of callus as described by Swinehart (1962).

Statistical analysis

The study collected data on the growth parameters (percentage callus induction, diameter, and GTI) from five different samples, and data on the activity of the catalase enzyme from three different samples. These were measured in the form of 'mean' and 'Standard Error'. A statistical test called 'Spearman's Correlation Test' was used to investigate the relationship between the concentration of Cd in the growth media and the activity of the catalase enzyme.

Results

Selection of callus induction media

The *T. erecta* leaf explants showed the earliest callus initiation on medium supplemented with 8 μM BAP + NAA, with 80% of the explants forming callus in the first week as shown in Table 1.

Table 1: Effects of BAP, NAA, and 2, 4-D supplemented MS media on callus initiation of *T. erecta* observed during a four-week period

BAP (μM)	NAA (μM)	2, 4-D (μM)	Week 1	Week 2	Week 3	Week 4	Weight measured in Week 4 (gm)
0	0	0	0%	0%	0%	0%	0
2	2	0	0%	0%	40%	100%	0.85±0.12
4	4	0	0%	0%	0%	20%	0.33±0.14
6	6	0	0%	0%	0%	80%	0.77±0.27
8	8	0	80%	80%	80%	100%	1.03±0.20
10	10	0	0%	80%	80%	100%	1.25±0.35
0	0	10	0%	40%	60%	60%	0.82±0.32
0	0	11	40%	60%	60%	80%	0.64±0.33
0	0	12	0%	40%	40%	60%	0.91±0.40
0	0	13	0%	60%	80%	80%	0.67±0.29
0	0	14	60%	60%	80%	80%	0.83±0.3

After four weeks, 2 μM, 8 μM, and 10 μM BAP + NAA showed 100% callus formation, but none of the concentrations of 2, 4-D showed 100% callus formation. After four weeks, calli were weighed where 10 μM BAP + NAA gave the highest final weight. All the calli obtained from the combination of BAP and NAA were greenish-yellow (see Figure 2A), whereas the calli added to the 2, 4-D supplemented medium were brownish and granular. Since the search was directed towards rapid callus initiation, the MS medium supplemented with 8 μM BAP + NAA was selected as a controlled growth medium for *T. erecta* calli.

On the other hand, *G. globosa* showed callus initiation in the first week, with all the concentrations of 2, 4-D; however, 100% initiation was shown only in the media supplemented with 2 and 8 μM 2, 4-D. All the calli were pale yellow (see Figure 2C). As shown in Table 2, the MS medium containing 2 μM 2, 4-D showed the fastest callus initiation and better growth, and was thus used as a controlled growth medium for *G. globosa* calli.
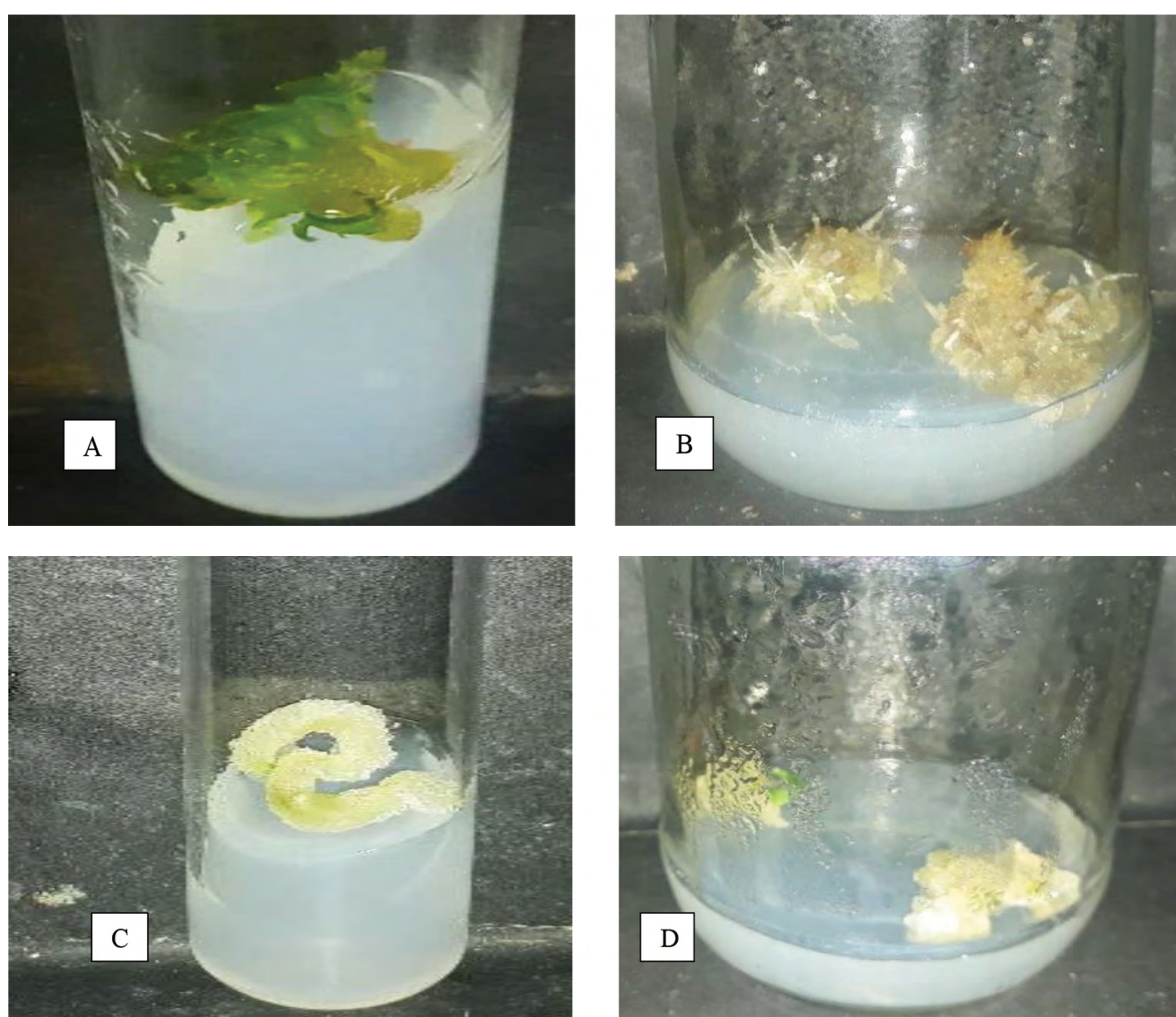

Figure 2: **Callus culture of *T. erecta* and *G. globosa*: (A) callus developed from *in vitro* leaf explants of *T. erecta* in MS medium supplemented with 8 μM BAP+NAA; (B) *T. erecta* callus developed in 150 μM Cd enriched MS medium supplemented with 8 μM BAP+NAA; (C) Callus developed from *in vitro* leaf explants of *G. globosa* in MS medium supplemented with 2 μM 2,4-D; and (D) *G. globosa* callus developed in 150 μM Cd enriched MS medium supplemented with 2 μM 2,4-D.**

Table 2: **Effects of 2, 4-D supplemented media on callus growth of *G. globosa* observed during a four-week period**

| 2, 4-D (µM)	Week 1	Week 2	Week 3	Week 4	Weight measured in Week 4 (gm)
2	100%	100%	100%	100%	0.93±0.4
4	80%	80%	80%	80%	1.08±0.26
6	80%	100%	100%	100%	0.31±0.1
8	100%	100%	100%	100%	0.65±0.21
16	80%	100%	100%	100%	0.65±0.2

Callus diameter and GTI in Cd-enriched media

In the first week, all of the *Tagetes erecta* calli samples grown in the controlled medium and those containing 100 and 150 µM of $CdCl_2$ were successfully initiated. After two weeks, the calli grown in a medium with 100 µM $CdCl_2$ had the greater diameter, followed by those with 50 µM $CdCl_2$ (see Figure 3). In the third week too, the calli grown in a medium with 100 µM $CdCl_2$ had the largest diameter. The highest increase in overall diameter was seen in the controlled calli, followed by those with 200 and 150 µM $CdCl_2$. The *T. erecta* calli turned brown when exposed to all concentrations of Cd treatment, with the most severe browning observed with 250 µM $CdCl_2$.
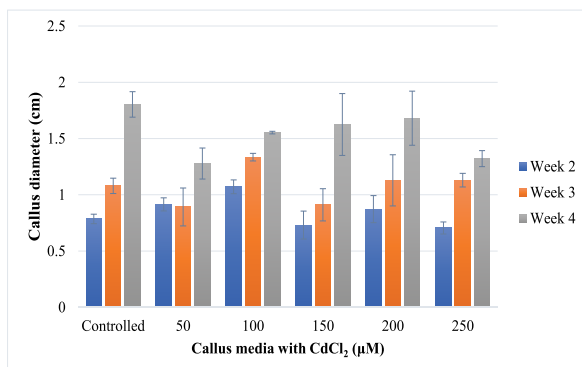
Figure 3: Effects of $CdCl_2$ concentrations (50, 100, 150, 200, and 250 µM) on the callus diameter of *T. erecta*.

In the case of *G. globosa*, the calli grown on the controlled medium were found to have the highest diameter followed by the medium supplemented with 150 µM $CdCl_2$ throughout all the four weeks of callus growth (Figure 4). The browning of the calli was observed at the concentration of 150 µM $CdCl_2$ and above.
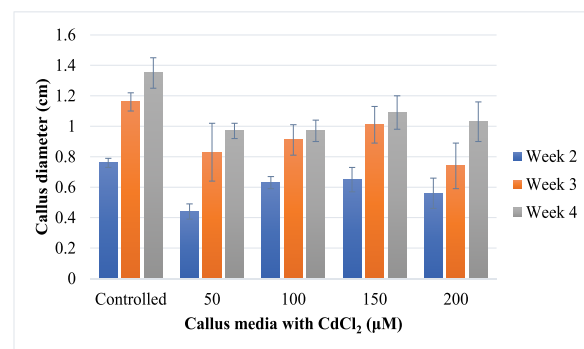
Figure 4: Effects of $CdCl_2$ concentrations (50, 100, 150, 200, and 250 µM) on the callus diameter of *G. globosa*.

The GTI of both *G. globosa* and *T. erecta* calli were found to be the highest when the medium contained 150 µM of $CdCl_2$ (Figure 5). Similarly, the second highest growth rate was observed in the medium containing 200 µM of $CdCl_2$. The GTI for G. *globosa* was 149.32% while it was 130.95% for *T. erecta*, suggesting a potential tolerance level to Cd stress of calli in both the plants.
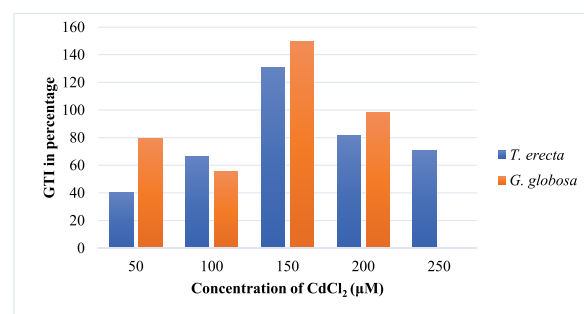
Figure 5: GTI of *T. erecta* and *G. globosa* in media enriched with 50 µM, 100 µM, 150 µM, 200 µM, and 250 µM $CdCl_2$.

Catalase activity

The CAT activity in the *T. erecta* calli were found to have increased with the increase in the concentration of $CdCl_2$ in the medium, reaching a peak (nearly 60) at a concentration of 150 µM, before decreasing as shown in Figure 6. In contrast, the highest level of CAT activity (nearly 45) in *G. globosa* callus was observed at a concentration of 50 µM $CdCl_2$. A positive correlation was seen between the concentration of $CdCl_2$ and CAT activity in *T. erecta* callus up to 150 µM $CdCl_2$ [Spearman's rank correlation coefficient (1) = 0.54 & p = 0.18]. In contrast, *G. globosa* callus showed a significant negative correlation between the concentration of $CdCl_2$ and CAT activity at 50 µM $CdCl_2$ [Spearman's rank correlation coefficient (1) = -0.51 & p = 0.5].
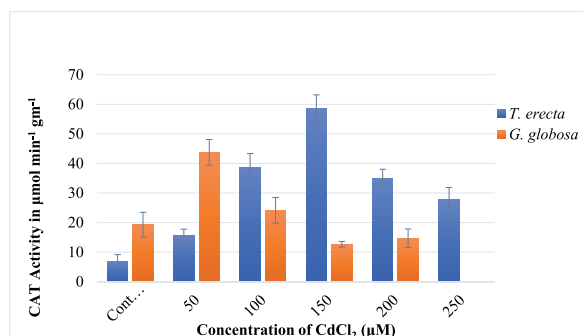

Figure 6: **CAT activity of calli of both *T. erecta* and *G. globosa* in controlled medium 'C' and the media supplemented with 50 µM, 100 µM, 150 µM, 200 µM, and 250 µM $CdCl_2$.**

Discussion

The *T. erecta* callus were successfully induced with a combination of BAP and NAA at a concentration of 8 µM, similar to the results of previous studies by Benítez-García *et al.* (2014) and Munshi *et al.* (2021), although Belarmino *et al.* (1992) reported difficulty in regenerating callus from leaf explant. The browning of *T. erecta* callus in media supplemented with 2, 4-D could be caused by the oxidation of phenolic compounds, leading to cell death (Khosroushahi *et al.*, 2011; Vijayalakhsmi & Shourie, 2017). In contrast, the *G. globosa* callus formation was the most effective at a low concentration of 2, 4-D, similar to the findings of Vieira *et al.* (1994) and Bodhipadma *et al.* (2017).

The *T. erecta* callus grown in media containing 200 µM $CdCl_2$ had the greatest diameter among all treatment groups, but it did not have the highest fresh weight. This phenomenon might be caused by the reduction in cell division and an increase in cell growth caused by the toxic effect of Cd (Zou *et al.*, 2012). Labancová *et al.* (2020) found that poplar callus entered a linear phase characterized by decreased cell division rate and increased cell growth when exposed to 10 µM Cd. Additionally, the presence of chloride in the Cd source (i.e. $CdCl_2$) has been reported to promote cell elongation, as it has a better ability to regulate osmosis and generate turgor pressure (Colmenero-Flores *et al.*, 2019). In the case of *G. globosa*, both the growth in length and biomass were at the highest when the media contained 150 µM of $CdCl_2$. This indicated that the growth in length and biomass of the callus had a similar pattern. Namjooyan *et al.* (2012) and Israr *et al.* (2006) both found that Cd reduced callus growth in *Carthamus tinctorius* and *Gomphrena globosa*, respectively, with the most significant reduction at higher concentrations (75 µM, 100 µM, and 250 µM, respectively). Our study also found that the callus growth was highest in the explants exposed to 150 µM Cd, which is suggested to be due to Cd accumulation.

The GTI was higher in the calli exposed to 150 µM of $CdCl_2$ for both *T. erecta* and *G. globosa* plants, indicating that these calli had a mechanism for tolerance to Cd (Bernabé-Antonio *et al.*, 2015). However, after a positive response to 150 µM $CdCl_2$, the biomass declined again, which may be due to the phenomenon of hormesis which is an adaptive response where low levels of stress activate cellular and molecular pathways that enhance the ability of the cell and organism to withstand more severe stress (Calabrese *et al.*, 2007; Bernebe-Antonio *et al.*, 2015).

A high level of antioxidant enzymes can improve tolerance to stress caused by heavy metals (Gechev *et al.*, 2006). Tolerant calli have been found to have significantly higher CAT activity than non-tolerant calli (Rout & Sahoo, 2007). This study observed that as the concentration of $CdCl_2$ increased, the activity of CAT initially increased, but then decreased at toxicity levels

of CdCl$_2$. This decline in CAT activity was accompanied by the browning of the calli, which is a sign of cell necrosis caused by the production of phenolic compounds (Sandalio *et al.*, 2001; Shekhawat *et al.*, 2010). Browning was more severe when CAT activity decreased, suggesting that phenolic compounds were replacing the scavenging role of Catalase on Hydrogen peroxide (Michalak, 2006). Phenolic compounds can act as antioxidants in their reduced form, but are cytotoxic in their oxidized form (Michalak *et al.*, 2006; Khosroushahi *et al.*, 2011; Vijayalakshmi & Shourie, 2017).

It was suggested that the variation in the activity of the calli in different media with and without Cd was caused by mutation. The presence of Cd in culture media can lead to increased somaclonal variation and spontaneous mutations in some callus cells, resulting in regeneration with altered metal accumulation (Nehnevajova & Herzig, 2007). The use of phytohormones such as 2, 4-D and NAA can also lead to mutation through an increase in cytosine methylation in plant tissue cultures (Phillips *et al.*, 1994). However, the use of constant hormone concentration across all Cd treatments suggested that the mutation is a result of changes in Cd concentration rather than hormone.

Conclusion

This study presents an initial step in the form of a callus culture to develop tolerant plants for phytoremediation of Cadmium. The results suggest that callus culture in MS media supplemented with 150 μM Cd is appropriate for developing Cd-tolerant calli for *T. erecta*. The toxicity symptoms could be profound above this level as detected in our study. Although the results for the tolerant calli of *G. globosa* did not materialize, the outcome obtained has given a toxicity level for its callus. The toxicity levels for the calli of these plants indicated that the calli of *T. erecta* were more tolerant than that of *G. globosa*. Our study suggests that the calli of *T. erecta* treated with 150 μM Cd can be used to develop its plantlets. Thus, the selection of tolerant calli and the toxicity level of HMs in ornamental plants can be determined

by understanding the stress responses of their calli. This method of selection can be used in other plants as well, but its use, especially, in selecting tolerant ornamental plants can be beneficial as they are less likely to end up in their food-chain process and since their flower parts have an insignificant accumulation of heavy metals. Besides, the cut-flowers of these plants could still be used, and hence could be idle for phytoremediation.

Acknowledgments

Author's contribution

The concept and design were developed by GL. KKP and PRG polished the designs whereas BS, SL and GL carried out experimentation.

Data availability

The data that support the findings of this study are available on request from the corresponding author.

Conflict of interest

The authors declare no conflict of interest

Funding

Ethical approval and consent

No harm to any plants or animals was done.

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