Jurnal Biodjati 10(1): 77-89, May 2025

e-ISSN : 2541-4208
p-ISSN : 2548-1606

JURNAL BIODJATI

http://journal.uinsgd.ac.id/index.php/biodjati

Cadmium Accumulation and Tolerance of *Talinum paniculatum* Callus Culture

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Received: 09 August 2024
Revise from: 03 January 2025
Accepted: 23 April 2025

DOI: 10.15575/biodjati.v10i1.38255

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Abstract. *The increasing use of inorganic fertilizers is one of the main factors contributing to increased cadmium (Cd) pollution in the environment. Phytoremediation is one of the strategies that can be used to address the problem of Cd pollution in the environment. The selection of cadmium-tolerant plants can be conducted using an in vitro culture. Talinum paniculatum as an ornamental plant is potentially used as a phytoremediation agent, but limited information is available regarding its accumulation ability and tolerance to cadmium stress. Therefore, this study aims to determine the accumulation ability and tolerance of T. paniculatum callus to cadmium at various concentrations. This study used a completely randomized design (CRD) with six replications of Cd concentration treatment (0, 5, 10, and 20 ppm). Accumulation and tolerance of callus to Cd were assessed based on callus biomass, callus color, tolerance index, heavy metal concentration in callus and media, and bioconcentration factor value. An increase in Cd concentration showed a change in callus color from yellowish green to blackish. In addition, there was no significant difference in dry weight (0.078-0.086 g) and tolerance index (102.631%-113.158%) of callus. However, increasing Cd concentration showed significant differences in callus Cd accumulation from media (5-20 ppm) and Bioconcentration Factor (BCF) ratio (1.282-5.701), indicating the ability of T. paniculatum as an accumulator plant. This study's results support phytoremediation efforts against heavy metal pollution, including cadmium.*

Keywords: *accumulation, cadmium, callus, phytoremediation, Talinum paniculatum*

Citation

Viorenta, M. G., Restiani, R., & Aditiyarini, D. (2025). Cadmium Accumulation and Tolerance of *Talinum paniculatum* Callus Culture. *Jurnal Biodjati*, 10(1), 77-89.

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INTRODUCTION

Environmental pollution caused by heavy metals is a global problem and requires intensive handling because it has an impact on reducing environmental quality, agricultural productivity, and human health. Cadmium (Cd) is one type of heavy metal that widely contaminates soil, water, air, agricultural crops, and has high mobility and toxicity properties (Jan et al., 2023; Sharma et al., 2023). One of the main sources of Cd into the environment is the intensive use of inorganic fertilizers, especially phosphate fertilizers, which contain heavy metal Cd at an average of 138 mg Cd/kg (Kusumaningrum et al., 2012). This has led to cadmium accumulation in the soil, which is then absorbed by agricultural and crop plants and enters the food chain (Nur, 2013). The long-term impacts of Cd contamination are decreased environmental quality (soil, water, and air), decreased agricultural productivity, and chronic diseases for humans, such as cancer, high blood pressure, reproductive problems, nerve damage, organ system damage, and respiratory disorders (Sharma et al., 2023).

Phytoremediation is one of the strategies that can be used to address the problem of Cd pollution in the environment. To date, the implementation of phytoremediation in addressing contaminated environments has increased compared to other decontamination technologies (excavation, precipitation, heating, electromediation, and chemical leaching). Phytoremediation is considered one of the more effective, efficient, and environmentally friendly technologies in decontaminating contaminants as it utilizes the natural ability of plants (plant organ parts) to transform harmful and non-degradable heavy metals into more stable, safe, and degradable compounds (Nedjimi, 2021). However, not all plant species can be used in phytoremediation. Plants that can be used in phytoremediation strategies generally have fast growth rate, short life cycle, high biomass, and strong and numerous roots (Hidayati, 2005; Nur, 2013).

Java ginseng is one of the multipotential plants as it can be utilized as a vegetable crop, herbal medicinal plant, ornamental plant, and phytoremediation agent. Several previous studies have investigated the accumulation and tolerance of *Talinum* genus, especially *T. paniculatum*, to various types of heavy metals by using ex vitro (environmental) systems. Bukar & Onoja (2020) reported that the leaves of *T. triangulare* have a high value of bioaccumulation of Al and Fe compared to other heavy metals. In addition, dos Reis et al. (2022) also reported that *T. paniculatum* cuttings have a tolerance index (TI) exceeding 70% to lead (Pb) and manganese (Mn), rapid root growth, high accumulation of Pb and Mn and high resistance to increase concentrations of Pb and Mn (0, 10, 50, 100, and 200 mg/L). Gonzales de Souza et al. (2018) also reported that *T. patens* has a high tolerance to increasing Pb concentrations (0, 50, 100, 250, and 500 µM), and the increase of antioxidant enzymatic systems, proline synthesis, increased epidermal tissue thickness, and photosystem efficiency influences this ability.

The effectiveness of phytoremediation is influenced by the selection of tolerant plants that can accumulate heavy metals to high concentrations without impairing their growth (hyperaccumulators). Selection of heavy metal-tolerant plants can be carried out through in vitro culture. This approach is more effective and efficient, since cultures such as callus cultures, cell suspensions, and shoots can be induced in a relatively short time, and the internal capabilities (physiological and molecular) of plant cells in absorbing, accumulating, and tolerating heavy metals in the culture medium can be intensively studied without the intervention of other factors from the environment or soil microorganisms that symbiotize with plant roots (Jaskulak & Grobelak, 2017). In vitro culture has been proven to be applicable in mitigating environmental pollution by heavy metals (Elazab et al., 2023). In addition, the application of in vitro culture in plant selection at various concentrations of heavy metals has the advantage of producing somaclonal variations in the form of tolerant or sensitive plants to heavy metals (Ashrafzadeh & Leung, 2015). This approach is more economical than producing heavy metal-tolerant plants through genetic engineering technology.

Several previous studies have used in vitro culture to select heavy metal tolerant plants, including accumulation and tolerance of chromium (Cr) and lead (Pb) in Jatropha curcas callus cell suspension culture (Bernabé-Antonio et al., 2015), accumulation and tolerance of copper (Cu) in Datura metel callus (Nurchayati et al., 2016), cadmium (Cd) tolerance in *Alyssum montanum* and *Daphne jasminea* shoot cultures (Wiszniewska et al., 2017), accumulation and tolerance of Cu, Cd and Pb in Abies nordmanniana callus (Nawrot-Chorabik, 2017), and tolerance and morphological changes in *Chenopodium ambrosioides* shoot cultures to Cd and Pb stress (Jan et al., 2023). Despite many studies on the selection of various plants tolerant to heavy metals through in vitro culture, studies on the investigation of *T. paniculatum* Cd-tolerant based on the accumulation and tolerance ability through callus culture have not been conducted. Therefore, it is important to conduct this research. The information from this study could provide helpful information for the development of Cd-tolerant Java Ginseng plants as phytoremediators in polluted environments.

MATERIALS AND METHODS

Time and Location of Research

This research was conducted from February to June 2024. The research was conducted at the Biotechnology Laboratory, Faculty of Biotechnology, University of Kristen Duta Wacana Yogyakarta. The analysis of cadmium (Cd) content was conducted at the Integrated Laboratory, Universitas Islam Indonesia (UII), Yogyakarta. This research used an experimental design using the CRD method (Completely Randomized Design) to determine the level of accumulation and tolerance of callus of *T. paniculatum* to Cadmium (Cd) at various concentrations, with six replications.

Callus Induction

Callus was induced from explants obtained from young leaves of *T. paniculatum* at the second to third position from the shoot. Explants were taken from healthy and non-wilted plants. Explants were washed under running water and soaked with detergent mixed with three drops of tween 80 and then rinsed with sterile distilled water followed by sterilization in Laminar Air Flow (LAF). Explants were soaked with 70% alcohol for three minutes and rinsed thrice with sterile distilled water. Sterile leaf explants were cut with a size of ±1x1 cm and inoculated on Murashige and Skoog (MS) media combined with the addition of hormones 2,4-D 2 mg/L (auxin) and kinetin 2 mg/L (cytokinin). Cultures were incubated at 20-24°C under 24-hour light conditions using fluorescent lamps (Restiani et al., 2024).

Cadmium (Cd) Treatment

The callus cultured for 30 days was subcultured into the treatment media containing Cd at various concentrations of

0, 5, 10, 20 ppm with a combination of plant growth regulators 2,4-D 2 mg/L (auxin) and kinetin 2 mg/L (cytokinin). This research used these concentrations to represent a gradient of low to moderately high levels of Cd stress commonly found in contaminated soils and wastewaters (Kubier et al., 2019). The various Cd concentrations are sufficient to evaluate the tolerance threshold and accumulation potential of *T. paniculatum* callus without causing necrosis. Previous studies have used similar Cd concentrations to evaluate the accumulation, tolerance, and regeneration of plant callus (Nawrot-Chorabik, 2017; Suman & Kalpana, 2013). Cultures were incubated at 20-24°C with 24-hour light conditions using fluorescent lamps for ten days.

Callus Morphology and Growth

The observation of callus morphology and biomass is one of the parameters that can be used to determine the tolerance of callus to Cd stress. Adding heavy metal cadmium will respond to the texture and color of the callus. Callus characteristics include friable, compact, and intermediate. Determination of callus biomass is done by weighing the dry weight of callus. Determination of callus dry weight is conducted by weighing the callus on day 10, which has been dried using an oven at 60°C until it reaches a constant weight (Eddijanto et al., 2022).

Bioconcentration Factor (BCF) and Tolerance Index (TI)

The measurement of Bioconcentration Factor (BCF) aims to determine the ability of T. paniculatum callus to accumulate and absorb heavy metal Cd. BCF ratio was obtained by analyzing using flame Atomic Absorption Spectrophotometer (AAS) from callus and medium. Calculation of the tolerance index (TI) aims to determine the callus's ability to tolerate heavy metal Cd, which is done by measuring its dry weight. The TI value was calculated based on the callus biomass value with the addition of Cd to the callus biomass in the control medium (without Cd) and the BCF ratio was calculated based on the heavy metal Cd accumulated by the callus to the heavy metal Cd accumulated by the medium (Bernabé-Antonio et al., 2015).

Data Analysis

Callus morphology was analyzed descriptively, and the TI value, BCF ratio, and biomass (dry weight) were analyzed statistically using IBM SPSS Statistics with an ANOVA test at a 5% significance level. If the obtained significance value is less than 5%, then the results are significant. A Tukey post hoc test followed the significant ANOVA test results.

RESULTS AND DISCUSSION

The Effect of Cadmium Concentration on *T. paniculatum* Callus Morphology

In this study, *T. paniculatum* callus cultured for 30 days had a friable texture and greenish yellow color. The appearance of callus can be caused on the injured part, so there is stimulation from the tissue in the explant to cover the wound (Ikeuchi et al., 2013). Leaf explants were grown on MS media with the addition of kinetin and 2,4-D. Combining two ppm auxin (2,4-D) and two ppm cytokinin (kinetin) can induce callus formation, producing optimal callus. Auxin plays a role in stimulating the growth of explant cells, so auxin tends to form callus that begins with cell division in the meristematic region. Furthermore, the use of cytokinin is proved to affect callus formation by reducing cell wall lignification to increase the initiation and development of callus in vitro so that combining cytokinin and auxin

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produces optimum results in callus growth (Sanchez-Ramos et al., 2022). Subsequently, callus produced from induction media were used for Cd treatment. Observation of callus morphology after cadmium treatment indicates an effect of Cd concentration on callus growth.

Table 1. Callus morphology after 10 days Cd treatment

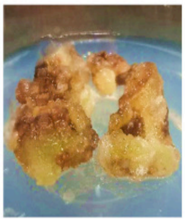
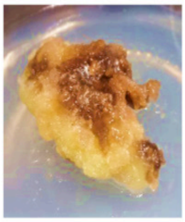
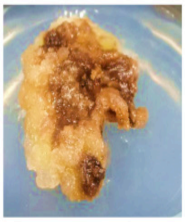
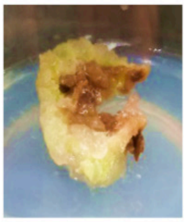
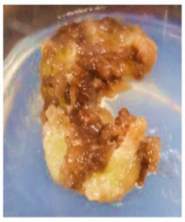
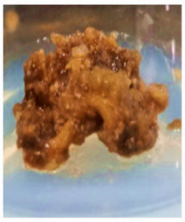
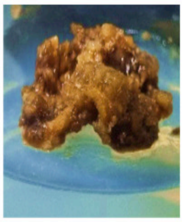
Cd Concentration	Days		
	0	7	10
Control (0 ppm)			
5 ppm			
10 ppm			
20 ppm			

Table 1 shows the morphology of callus to identify changes in callus from the color and texture of the callus. Based on observations for 10 days, all callus cultures showed normal growth in the control medium. In addition, callus in the control medium did not show additional browning/browning, resulting in a friable texture. This might be because the control is not supplemented with heavy metals; thus, it does not affect the callus growth. At a concentration of 5 ppm, the callus can still survive the exposure to cadmium without showing different changes in the intensity of the brownish color after incubation for 10 days. This indicates that the callus can maintain its normal morphology at this concentration. At this concentration, it has not caused toxicity for *T. paniculatum* callus, thus the callus can continue its growth. Anthocyanins are the dominant secondary metabolites that protect against oxidative stress by neutralizing free radicals and protecting against abiotic stress (Kumar et al., 2013). This is supported by the research of Kumar et al. (2013), which showed that *T. triangulare* accumulated greater concentrations of anthocyanins in response to heavy metal exposure.

Callus in Cd concentration of 10 ppm still survives after 10 days of exposure to cadmium. However, some parts of the callus begin to change to a brownish color. This might be due to toxicity of Cd that causes chlorosis, inhibition of photosynthesis, low biomass accumulation, growth retardation, changes in osmoregulation, and changes in nutrient assimilation (Singh et al., 2016). The 20 ppm Cd-treated callus showed a more extensive browning response on the callus surface and changed its texture. This condition indicates that exposure to cadmium at this concentration can cause necrosis of callus morphology. This is because toxic concentrations of cadmium are reported to interfere with most plant biological processes (Tahtamouni et al., 2020) and callus tissue is dehydrated and does not undergo morphogenesis. Hence, there are no green fragments, meaning chlorophyll content is absent. Differences in the growth response of some callus to increasing heavy metal concentrations in in vitro culture media can be influenced by several factors, namely the type and concentration of heavy metals, the type and genotype of plants, and the type of culture (Jaskulak & Grobelak, 2017).

Effect of Cadmium Concentration on Growth and Tolerance Index (TI) of *T. paniculatum*

Callus biomass is one of the parameters used to indicate callus growth. One of the measured biomasses is dry weight, which results from drying the callus using an oven after 10 days of subculture to cadmium treatment. Figure 1 shows that the callus at 0 ppm has the lowest weight of 0.076 g compared to other concentrations. Callus treated with cadmium is at a concentration of 5 ppm (0.086 g) compared to concentrations of 10 ppm (0.084 g) and 20 ppm (0.078 g). The presence of Cd affects the absorption of mineral nutrients in plants and their growth. This is associated with growth inhibition, low dry weight yield, inhibition of photosynthesis and respiration, and chlorosis (Lama et al., 2024). The results showed that Cd treatment at various concentrations did not show significant differences (p>0.05) with a significance value of 0.555. These results indicate that Cd concentrations of 5, 10, and 20 ppm are still tolerated by *T. paniculatum* callus. The results do not show much difference between 5 ppm cadmium concentration and 20 ppm concentration. Cadmium has a damaging effect that causes a decrease in dry weight through a reducing effect on morphological and physiological traits related to photosynthesis. Therefore, plants become weak and their tolerance to biotic and abiotic stresses decreases (Aghaz et al., 2013).

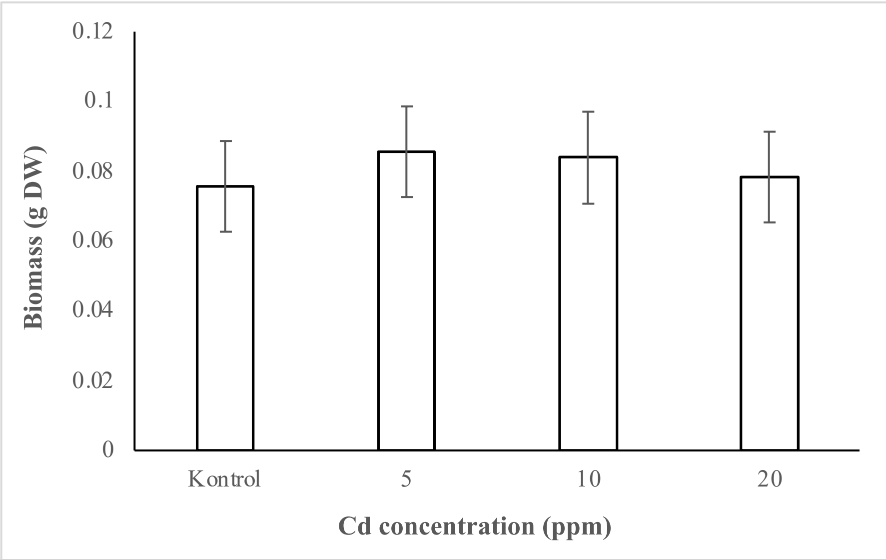


Figure 1. The effect of Cd concentration on *T. paniculatum* callus biomass at 10 days after treatment

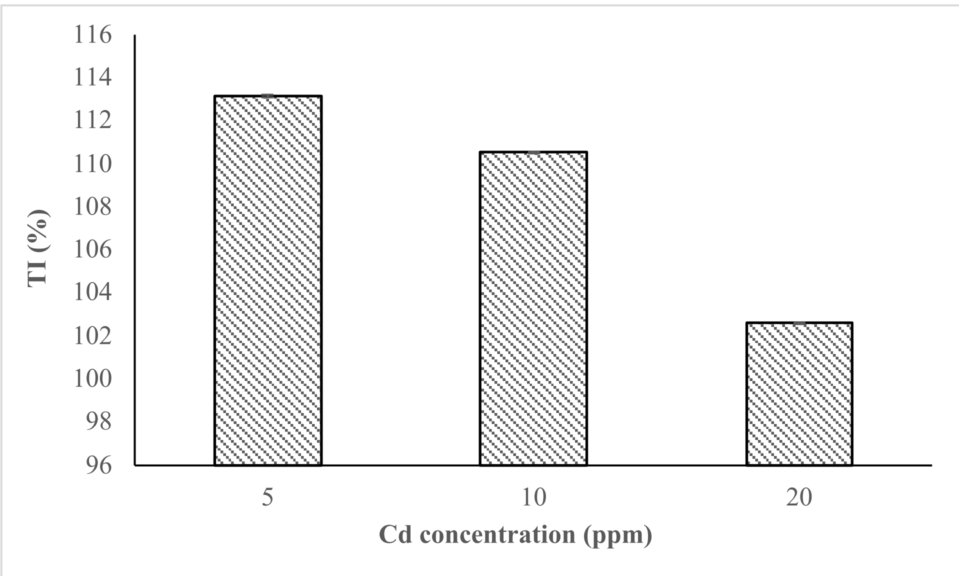


Figure 2. The effect of Cd concentration on Tolerance Index (TI) of *T. paniculatum* callus at 10 days after treatment

In addition to measuring biomass, selecting heavy metal-tolerant plants is often based on the tolerance index (TI) value. A TI value ≥ 100% indicates that the plant's tissue (callus) can sustain growth under heavy metal stress and is therefore categorized as a tolerant species (Kozminska et al., 2018). Based on Figure 2, TI values at 5 ppm (113.216%), 10 ppm (111.013%), and 20 ppm (103.524%), suggest a strong tolerance of *T. paniculatum* callus. Although the callus remained viable and able to grow, the gradual decline in TI values and the increasing Cd concentration reflect an accumulating physiological stress effect on callus growth. The ability of *T. paniculatum* callus to tolerate high Cd concentration at 20 ppm might be attributed to several mechanism such as Cd storage and sequestration in the callus vacuole (Raza et al., 2020), heavy metals chelation by phytochelatins or methallothioneins inside the plant callus (Nurchayati et al., 2016; Restiani et al., 2024), activation of antioxidant and enzymatic antioxidant to prevent/mitigate reactive oxidation induced by Cd stress (Fan et al., 2023), and changes in cell membrane as well as cell wall composition that reduce Cd uptake (Fan et al., 2023; Raza et al., 2020). Despite these several possible strategies, the reduction of TI values reflects that the callus cellular energy is increasingly directed towards defense rather than growth. However, ANOVA statistical analysis showed that Cd treatment at various concentrations did not show significant differences ($p>0.05$) with a significance value of 0.526. These results confirm *T. paniculatum* callus can tolerate moderate Cd stress without significant impairment, thus supporting its potential in phytoremediation strategies, according to Restiani dkk. (2024) tolerant plants are those capable of surviving and adapting to high heavy metal concentrations, aligning with the findings of this research.

Effect of Cadmium Concentration on Bioconcentration Factor (BCF) Ratio of *T. paniculatum*

The supplementation of Cd in the culture medium can cause an increase in somaclonal variation and natural plant genetic changes in some callus cells resulting in regeneration with changes in heavy metal (Lama et al., 2024). Figure 3 shows that the Cd content in the callus and media increased in line with the increase in concentration. At a concentration of 5 ppm, the content of Cd in the media and callus accumulated less compared to 10 ppm and 20 ppm which accumulated more. The highest increase in Cd accumulation in the media and callus was at a concentration of 20 ppm, where the Cd content in the media and callus reached its peak. This increase indicates that the callus can absorb and accumulate Cd from the medium very effectively. This increase also indicates that the callus has adapted itself to the presence of Cd in the medium very well, so it can absorb and accumulate more Cd. In callus, Cd accumulates with increasing Cd concentration in the medium (Bajji & Druart, 2012).

The results of ANOVA statistical analysis showed that the treatment of various concentrations of Cd in the media and callus after being subcultured for 10 days showed significant differences ($p<0.05$) with significance values of 0.001 and 0.016 which were then continued with the Tukey test. These results indicate that Cd concentrations of 5, 10, and 20 ppm significantly affect the content of Cd accumulated in the media and callus of *T. paniculatum* and the ability of callus to absorb cadmium up to 20 ppm. Based on the Tukey test results, there is a significant difference in each concentration in the media, indicating that the concentration variation on the accumulation of Cd in the media has a real effect. In contrast, the Tukey test results on the callus, the concentration of 5 ppm has a real effect compared to the concentrations of 10 and 20 ppm. However, at concentrations of 10 and 20 ppm, the accumulation of Cd at both concentrations had no significant effect. The content of Cd accumulated by the media increases with increasing Cd concentration because it can be caused by the use of growth regulators such as 2,4-D and kinetin which increase callus survival under Cd stress, prevent biomass decline, increase photosynthesis and stimulate antioxidant activity so that it can be used to increase Cd accumulation in plants and has an effective role in reducing Cd toxicity (Elazab et al., 2023).

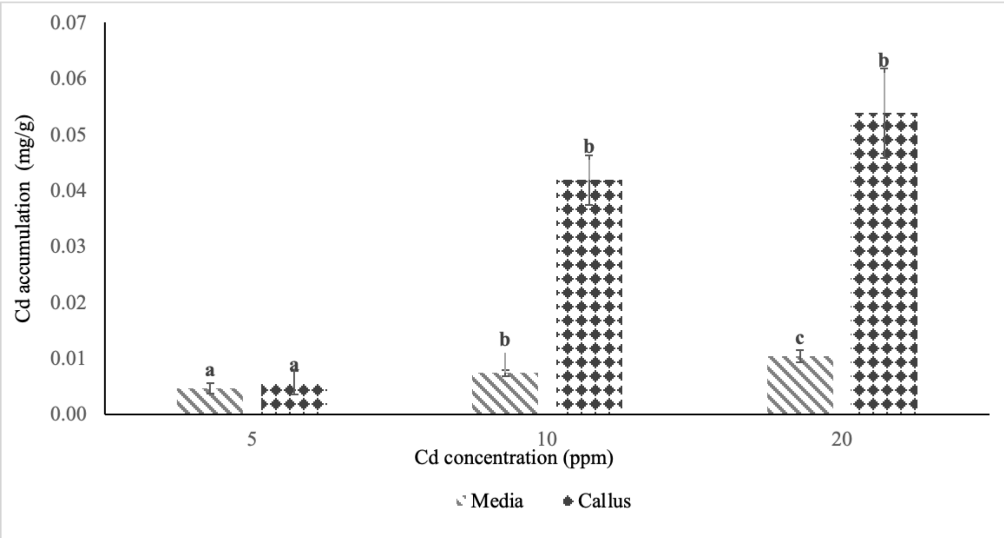


Figure 3. The effect of Cd concentration on Cd accumulation of *T.paniculatum* callus at 10 days after treatment

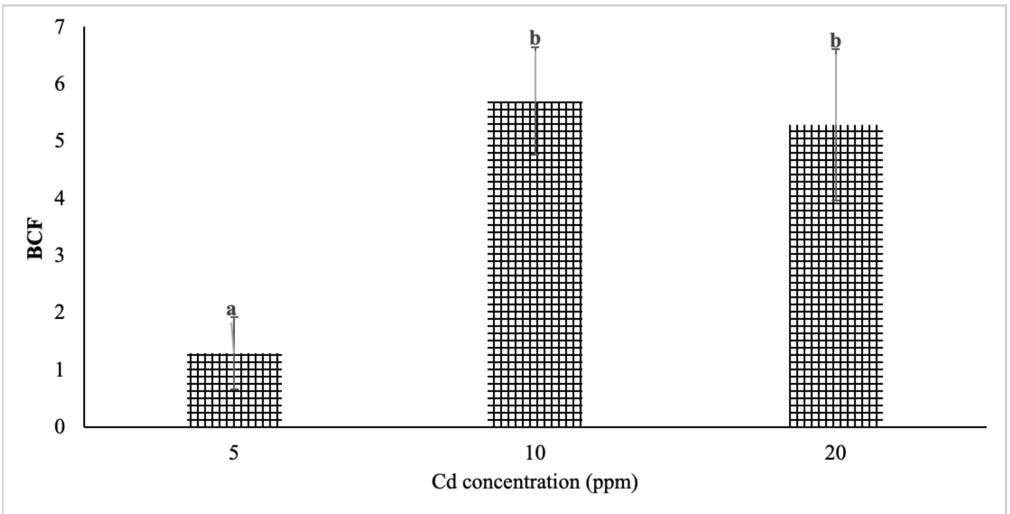


Figure 4. The effect of Cd concentration on BCF value of *T.paniculatum* callus at 10 days after treatment

Another important indicator of plant response to heavy metals and a direct indicator of plant phytoremediation potential is the Bioconcentration Factor (BCF). BCF is calculated as the ratio of heavy metal concentration in the callus to the concentration in the media. Based on Figure 4, the BCF results of *T. paniculatum* callus at concentrations of 5, 10, and 20 ppm generally increased as the concentration of Cd increased. Concentrations of 5 ppm (1.282), 10 ppm (5.701), and 20 ppm (5.279) are included in the accumulator category (>1) which indicates that *T. paniculatum* callus is a potential Cd accumulator (Bernabe-Antonio et al., 2015). The increased BCF of *T. paniculatum* callus and the increased Cd concentration indicate a positive response to Cd. At a concentration of 5 ppm, the ability of callus to accumulate Cd is proportional to the concentration of Cd given, so that at low concentrations, the ability of callus to accumulate Cd is also small. This indicates that *T. paniculatum* has an adaptation mechanism affecting low Cd exposure. At 10 ppm concentration, the BCF reflected a drastic increase in the ability of callus to accumulate Cd. This indicates that *T. paniculatum* at this concentration can be considered a potential Cd accumulator. An increase in concentration showed greater accumulation of Cd. This can occur by increasing the production of ROS, Cd can stimulate the production of antioxidant enzymes which include SOD, catalase, and GPX to protect against oxidative effects in tolerant plants (Kumar et al., 2013).

The concentration of 20 ppm, BCF decreased to 5.279 compared to the concentration of 10 ppm, this value still indicates that the callus of *T. paniculatum* can still accumulate Cd. The results of ANOVA statistical analysis showed that the treatment of Cd at various concentrations showed significant differences (p>0.05) with a significance value of 0.003, which was then continued with the Tukey test. These results indicate that Cd concentrations of 5, 10, and 20 ppm significantly affect *T. paniculatum* callus as an accumulator, so that the highest concentration of 20 ppm does not have a significant effect and can still be accumulated. Based on the Tukey test results, there is a significant difference in the concentration of 5 ppm, 10 ppm, and 20 ppm on the accumulated Cd, which indicates that the variation of concentration on the accumulation of Cd in that concentration has a real effect. The decrease in the ability to accumulate Cd is caused by the reduced ability of callus to absorb Cd (Zafar & Javed, 2016). The decrease in accumulation ability can be characterized as a sign of stress caused by Cd that can inhibit the ability of callus to accumulate Cd. Prolonged treatment with high concentrations of Cd results in tissue death, and the effect of Cd also affects the decrease in chlorophyll content (Chaitanya et al., 2023).

CONCLUSION

In conclusion, the callus culture of *T. paniculatum* can accumulate and tolerate heavy metal Cd at various concentrations, including 5 ppm, 10 ppm, and 20 ppm. This is due to the BCF ratio of this study has increased along with the increase in Cd concentration that shows a positive response to Cd and the resulting BCF >1 from 5 ppm treatment (1.282), 10 ppm (5.701), and 20 ppm (5.279) which indicates its potency as an accumulator. *T. paniculatum* callus could also tolerate Cd since the TI value at a concentration of 5 ppm (113.216%), 10 ppm (111.013%), and 20 ppm (103.524%) is tolerant to Cd and its growth is not affected because the callus morphology at various

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concentrations can maintain physiological functions and callus can survive.

AUTHOR CONTRIBUTION

The contributions of all authors in this research: **M.G.V.** designed the research, performed the research, analyzed the data, and wrote the manuscript; **R.R.** designed the research, supervised the research, analyzed the data, and wrote, reviewed, and edited the manuscript; and **D.A.** supervised the research and reviewed the manuscript.

ACKNOWLEDGMENTS

This research was supported by the Institute for Research and Community Service of Universitas Kristen Duta Wacana (LPPM UKDW) through The Riset Unggulan Universitas scheme with contract number 99/D.01/LPPM/2024.

CONFLICT OF INTEREST

There is no conflict of interest in this research.

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