Effects of 2,4-Dichlorophenoxyacetic acid and Thidiazuron on Callus Induction and Organogenesis in the Medicinal Plant *Calotropis procera*

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Received: 2025-01-22 Accepted: 2025-04-15

Abstract

Calotropis procera, a medicinally and industrially valuable plant of the Apocynaceae family, faces challenges in propagation due to declining seed germination potential and a lack of set mass production methods. This study evaluates the effects of plant growth regulators, specifically 2,4-Dichlorophenoxyacetic acidichlorophenoxyacetic acid and Thidiazuron, on callus induction, shoot formation, leaf development, and root induction in stem explants of *C. procera*. Tissue culture techniques were employed to address propagation challenges and conserve this species.

The highest callus induction (100%) was achieved using 7.5 mg/L 2,4-Dichlorophenoxyacetic acidichlorophenoxyacetic acid and 7.5 mg/L Thidiazuron, with auxins and cytokinins demonstrating a synergistic effect in promoting cell division. For shoot formation, the optimal combination was 1.25 mg/L 2,4-Dichlorophenoxyacetic acidichlorophenoxyacetic acid and 5 mg/L Thidiazuron, while leaf formation peaked with 1.25 mg/L Thidiazuron alone. Excessive Thidiazuron concentrations, however, inhibited leaf formation, underscoring the importance of hormonal balance. Root induction was most effective with 2.5 mg/L 2,4-Dichlorophenoxyacetic acid and 7.5 mg/L Thidiazuron, whereas treatments with high cytokinin concentrations were found to hinder root growth.

These findings align with prior research on other medicinal plants, such as *Catharanthus roseus* and *Calotropis gigantea*, demonstrating the complementary roles of auxins and cytokinins in plant tissue culture. This study highlights the potential of tissue culture as a scalable and sustainable method for the propagation and conservation of *C. procera*, ensuring the preservation of its medicinal and industrial applications.

This study confirmed that the proper balance between auxin and cytokinin is important for the successful induction of each growth phase in the plant. This study highlights the potential of tissue culture as a scalable and sustainable method for the propagation and conservation of *C. procera*, ensuring the preservation of its medicinal and industrial applications.

Keywords: Plant tissue culture, Plant growth regulators, Hormonal synergy, Callus induction, *Calotropis procera*

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Doi: 10.48308/pae.2025.238473.1104

Introduction

The medicinal plant *Calotropis procera* (commonly known as the giant milkweed or crown flower) is a valuable species in the Apocynaceae family, known for producing important secondary metabolites such as cardiac glycosides and natural latex (Van Quaquebeke et al., 2005). It holds significant pharmaceutical and industrial importance due to its bioactive compounds and applications. Native to various regions of Iran, including South Khorasan, Sistan and Baluchestan, Khuzestan, and Hormozgan, *C. procera* is commonly used for its medicinal and industrial properties (Mohebi, 2021).

The latex of *C. procera* has been reported to possess anthelmintic (Iqbal et al., 2005), anti-inflammatory (Alencar et al., 2006), antioxidant (Chavda et al., 2010), and anticancer properties (Magalhães et al., 2010). Additionally, its fibers are utilized in the textile industry, and its hydrocarbons serve as a potential source for biofuel production (Parić et al., 2011).

Tissue culture techniques, as a branch of advanced biotechnological methods, provide a promising tool for the propagation and conservation of valuable plant species (García González et al., 2010). These methods not only enable the mass propagation of healthy plants but also offer a controlled environment for producing valuable secondary metabolites. However, in a controlled laboratory condition culture of plants like *C. procera*, which secrete latex, faces challenges such as phenolic exudation, which can damage explants. Using explants like immature embryos or hypocotyls, which contain lower phenolic content, has been suggested as a solution to mitigate these issues (Abasi et al., 2017).

Phytohormones play a pivotal role in enhancing various tissue culture stages such as callus induction, shoot formation, and root formation. The auxin 2,4-Dichlorophenoxyacetic acid is a potent promoter of cell division and facilitates callus induction, while Thidiazuron, an effective cytokinin, is efficient in inducing shoot and leaf formation (George et al., 2008). The combination of these two hormones can regulate hormonal balance, optimizing the propagation process under laboratory conditions (Sidik et al., 2024; Rineksane et al., 2021).

Therefore, this study aims to evaluate the effects of 2,4-Dichlorophenoxyacetic acid and Thidiazuron on callus induction and organogenesis in *C. procera*. Achieving optimal results can contribute to the mass propagation and conservation of this valuable species, preventing its potential extinction.

Material and Methods

Plant material

Seeds of *C. procera* were collected from the Kahnuj region in Kerman, Iran (27.9514° N, 57.7002° E; elevation ~435 m above sea level).

Seed sterilization

The seeds were washed with distilled water and then immersed in 5% sodium hypochlorite solution for 15 minutes. They were later rinsed in autoclaved distilled water for 5 minutes, followed by 2 minutes in 70% ethanol. Afterward, they were rinsed three more times in autoclaved distilled water, each time for 5 minutes (Lindsey et al., 2017).

Seed cultivation and culture conditions

To induce germination and obtain sterile seedlings, seeds were cultivated on basal Murashige & Skoog medium (Murashige and Skoog, 1962) containing 1.25 mg/L 2,4-Dichlorophenoxyacetic acid and 1.25 mg/L Thidiazuron. Cultures were maintained in a growth room under a 16-hour light and 8-hour dark photoperiod at 23 ± 2°C. After three weeks, the aerial parts of sterile seedlings were used as explants for hormonal treatments.

Hormonal treatments for explants

A factorial experiment was conducted in a completely randomized design to evaluate the effects of 2,4-Dichlorophenoxyacetic acid and Thidiazuron on callus, root, and shoot induction. The treatments included five concentrations of 2,4-Dichlorophenoxyacetic acid (0, 1.25, 2.5, 5, and 7.5 mg/L) and five concentrations of Thidiazuron (0, 1.25, 2.5, 5, and 7.5 mg/L), with at least three replicates for each treatment. Explants measuring 2–3 cm were excised from the aerial parts of sterile seedlings under a laminar flow hood. Explants were placed on different media and maintained under the same growth room conditions. After four weeks, parameters such as callus induction percentage, root induction percentage, root number per explant, shoot induction percentage, shoot number per explant, leaf induction percentage, and leaf number per explant were evaluated.

Data Analysis

The data were analyzed using SPSS software (version 22, Allen et al., 2014). Duncan's multiple range test was employed to compare the means.

Results

Ten days after culturing the explants on media with different hormonal treatments, we observed callus formation, shoot induction, and root induction.

Callus Induction

The highest callus induction rate (100%) occurred with 7.5 mg/L Thidiazuron and 7.5 mg/L 2,4-Dichlorophenoxyacetic acid, highlighting the synergistic effect of these hormones (Figure 2). At lower concentrations of 2,4-Dichlorophenoxyacetic acid (0 and 1.25 mg/L), callus induction was significantly reduced.

Shoot Induction

Optimal shoot formation was achieved with a combination of 1.25 mg/L 2,4-Dichloro-

Fig. 1. Induction of callus, shoots, leaves, and roots in stem explants of *C. procera* cultured on Murashige & Skoog medium with varying concentrations of 2,4-Dichlorophenoxyacetic acid and Thidiazuron

phenoxyacetic acid and 5 mg/L Thidiazuron. Higher concentrations of 2,4-Dichlorophenoxyacetic acid negatively affected shoot formation, while low concentrations were more effective (Figure 3).

Leaf Induction

We observed the highest percentage of leaf formation and the maximum number of leaves at 1.25 mg/L Thidiazuron (Figure 4)—increasing Thidiazuron concentrations above 2.5 mg/L reduced leaf formation, indicating an inhibitory effect at higher concentrations.

Root Induction

We observed root formation only with the combined treatment of 2.5 mg/L 2,4-Dichlorophenoxyacetic acid and 7.5 mg/L Thidiazuron, achieving a 90% success rate. On average, this condition produced 2.5 roots per explant.

Discussion

The native Iranian plant *Calotropis procera*, a notable member of the family Apocynaceae, holds special importance. One of the challenges in propagating the medicinal species *C. procera* is the decline in seed germination potential over time as seeds are stored longer (Galal et al., 2015). Furthermore, no set method exists for the mass production of this plant, which is nearing extinction. In this context, research and modern tissue culture techniques have gained attention as effective solutions to address these challenges. Tissue culture can be employed for the propagation of these plant species, whose populations are dwindling, enabling production in an aseptic environment free from environmental contaminants. Plant growth and differentiation are notably influenced by the type and concentration of plant growth regulators and culture media composition. Auxins and cytokinins, the most commonly used plant growth regulators in plant tissue culture, play crucial roles in regulating developmental pathways. These substances can improve the plant's growth responses. For example, in the case of *C. procera*, data indicates that the use of 2,4-Dichlorophenoxyacetic acid is notably more effective for callus induction than Naphthaleneacetic acid or Indole-3-acetic acid. Moreover, the cytokinin-to-auxin ratio in this process is crucial and influences callus growth and plant regeneration (Tripathi et al., 2013).

This study demonstrated the synergistic effect of 2,4-Dichlorophenoxyacetic acid and Thidiazuron on callus induction and organogenesis in *C. procera*. The combination of 7.5 mg/L 2,4-Dichlorophenoxyacetic acid and 7.5 mg/L Thidiazuron achieved the highest callus induction (100%). The combined use of these hormones increased the expression of genes related to cell division, supporting previous findings (Kumar & Reddy, 2011). These findings align with previous studies that demonstrate the complementary roles of auxins and cytokinins in callus induction (George et al., 2008). The hormone 2,4-Dichlorophenoxyacetic acid, a strong auxin, promotes increased cell division, while Thidiazuron, a phenylurea-type cytokinin, acts as a regulator of cellular development. According to prior research, explants of *Calotropis gigantea* cultured on media containing a combination of BAP and 2,4-Dichlorophenoxyacetic acid present-

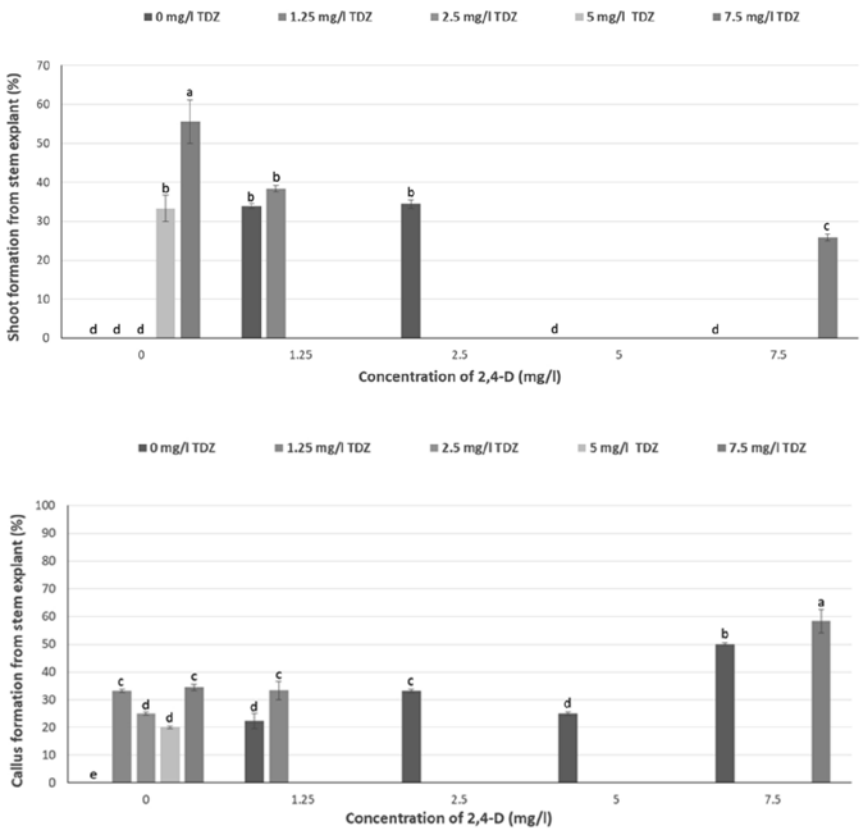

Fig. 3. The effect of different concentrations of the hormones 2,4-Dichlorophenoxyacetic acid and Thidiazuron on the percentage of shoot formation (A) and the number of shoots (B) in stem explants of *C. procera* after 4 weeks. Data represent the mean values of at least three replicates ± standard error. Different letters indicate significant differences between means at a probability level of $P \leq 0.05$

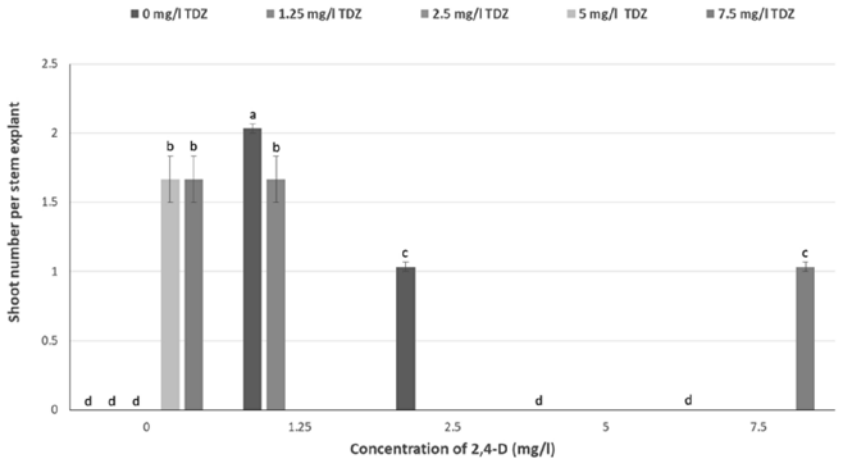

Fig. 4. The effect of different concentrations of the hormones 2,4-Dichlorophenoxyacetic acid and Thidiazuron on the percentage of leaf formation (A) and the number of leaves (B) in stem explants of *C. procera* after 4 weeks. Data represent the mean values of at least three replicates ± standard error. Different letters indicate significant differences between means at a probability level of $P \leq 0.05$

ed callus formation, whereas explants on media lacking 2,4-Dichlorophenoxyacetic acid failed to form calluses (Muthi'ah et al., 2023). These findings are consistent with the results of our experiments and underscore the complementary and sometimes antagonistic roles of auxins and cytokinins in regulating growth processes. Furthermore, the demonstrated ability to manipulate hormone concentrations for optimal outcomes emphasizes the potential of tissue culture as a scalable method for the conservation and propagation of *C. procera*. Our data show that Thidiazuron alone cannot induce callus formation but enhances its performance in the presence of auxin.

For shoot formation, the optimal combination was 1.25 mg/L 2,4-Dichlorophenoxyacetic acid with 5 mg/L Thidiazuron, while leaf formation peaked at 1.25 mg/L Thidiazuron. However, higher Thidiazuron concentrations inhibited leaf formation, emphasizing the importance of hormonal balance. These findings align with studies on *Catharanthus roseus* and *Calotropis gigantea* (Talitha et al., 2023; Dhandapani et al., 2008). Cytokinins such as Thidiazuron play a key role in this process by stimulating signaling pathways associated with stem meristem proliferation (Malik et al., 2023).

The findings reported by Rout et al. (2000) underscore the critical role of cytokinins in enhancing leaf formation. Leaf formation occurred at a concentration of 5 mg/L 2,4-Dichlorophenoxyacetic acid when we used only 2,4-Dichlorophenoxyacetic acid as the hormonal treatment in the culture medium. According to studies (Talitha et al., 2023) on *Calotropis gigantea*, leaf formation is influenced by specific concentrations of auxins and cytokinins. Among hormonal treatments, the one containing only Indole-3-butyric acid in the culture medium was found to be optimal for increasing leaf numbers compared to other treatments. This is because auxins stimulate the action of gibberellin hormones in increasing internode length, which later enhances the number of nodes and leaves. Agustina et al. (2020) have noted that both auxins and cytokinins have long been recognized to work synergistically and antagonistically in regulating various key growth processes.

Root induction required the combined treatment of 2.5 mg/L 2,4-Dichlorophenoxyacetic acid and 7.5 mg/L Thidiazuron. Similar results were reported for *Zingiber officinale*, where Thidiazuron combined with low auxin concentrations was most effective (Lincy & Sasikumar, 2010). Research findings (Talitha et al., 2023) have shown that in *Calotropis gigantea*, low concentrations of BAP increased the number of roots formed. However, there was an inverse relationship between increased BAP concentration and the number of roots formed. Optimal root formation occurred in the absence of BAP treatment in the culture medium. This is supported by prior research by Chen et al., which states that high concentrations of cytokinins can hinder root growth (Chen et al., 2020).

In conclusion, modern tissue culture techniques, guided by a deeper understanding of plant growth regulator interactions, hold promise not only for the sustainable production of *C. procera* but also for broader applications in conserving other endangered

medicinal plants.

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