

Effects of Different Concentrations of FeEDDHA on Callus Induction and Plant Conversion in *Gynura aurantiaca*

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Abstract

Gynura aurantiaca, commonly known as purple passion vine (also called purple velvet plant) is an ornamental species belonging to the Asteraceae family, recognized for its striking purplish foliage. While primarily cultivated as an indoor ornamental plant, its reported antioxidant potential, often linked in the literature to anthocyanin compounds, has increased interest in biotechnological applications. This study evaluated the effects of the alternative iron source FeEDDHA on callus induction and plant conversion from leaf explants under *in vitro* conditions. The standard iron source FeEDTA (100 µM) served as the control. Murashige and Skoog (1962) medium was supplemented with FeEDDHA at 100, 200, 400, and 800 µM. Parameters assessed included callus formation percentage, callus weight, callus area, organogenesis rate, and plant conversion rate. FeEDDHA at 200 µM yielded the highest callus formation, biomass, and area, whereas the highest plant conversion rate was obtained at 100 µM. In contrast, 800 µM markedly reduced callus formation and plant conversion, with complete inhibition in some replicates. Structures obtained during the callus and organogenesis stages were transferred to hormone-free MS medium, where 74% of explants treated with 100 µM FeEDDHA developed into fully rooted plantlets. Overall, precise iron dose optimization is critical: FeEDDHA outperformed FeEDTA at appropriate concentrations, with 200 µM optimal for callus formation/biomass and 100 µM optimal for plant conversion. These findings highlight the importance of iron source selection in micropropagation protocols.

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1. Introduction

Gynura aurantiaca, commonly known as purple passion vine, is particularly notable for its vivid purple foliage. This striking coloration makes it an ideal ornamental plant, often used to add a vibrant accent to mixed containers. While it initially exhibits an upright growth habit, it gradually develops into a trailing, vine-like form as it matures, making it a popular choice for decorative purposes (Chen et al., 2010). Beyond its ornamental appeal, *G. aurantiaca* has attracted attention for its potential health-related benefits, largely attributed to its high anthocyanin content. Phytochemical analyses of *Gynura* species have revealed a diverse array of bioactive compounds, including alkaloids, phenolic compounds (notably derivatives of quinic acid), flavonoids, steroids, glycosides, and terpenoids (Do et al., 2020). Despite its ornamental and phytochemical value, *G. aurantiaca* remains underexplored in terms of *in vitro* regeneration protocols, particularly regarding iron source optimization. Given its high anthocyanin content and decorative potential, establishing an efficient regeneration system is essential for both propagation and future biotechnological applications. FeEDDHA (ferric ethylenediamine di-2-hydroxyphenyl acetate) is a highly stable iron chelate widely used in plant tissue culture due to its superior solubility and bioavailability compared to conventional iron sources. Its application in culture media has been shown to significantly enhance callus formation, shoot regeneration, and overall organogenic efficiency. Although no direct studies have been conducted on *G. aurantiaca*, the effects of FeEDDHA as an iron source on callus induction and plant regeneration have been extensively investigated in comparable tissue culture systems. For instance, optimal concentrations of FeEDDHA in date palm cultures have been reported to promote callus proliferation, increase indirect shoot regeneration rates, improve rooting, and elevate chlorophyll and endogenous auxin levels, factors critical for successful plant regeneration (Al-Mayahi, 2021). In raspberry cultures, FeEDDHA supplementation reduced

chlorosis, enhanced chlorophyll and iron content, and improved regeneration rates and the number of adventitious shoots in certain cultivars (Zawadzka and Orlikowska, 2006). In various plant species such as rice and chickpea, both the source and concentration of iron have been shown to significantly influence callus induction and regeneration success (Mostafiz and Wagiran, 2018). Studies involving alternative iron sources (e.g., FeO-NP, FeSO₄) have also demonstrated that callus induction and regeneration are dependent on the type and dosage of iron applied, with inhibitory effects observed at supra-optimal concentrations (Ullah et al., 2024; Irum et al., 2020). Callus induction typically begins with the culture of plant explants (leaf, root, stem) on media supplemented with appropriate combinations of plant growth regulators, particularly auxins and cytokinins. Under suitable conditions, callus tissue may acquire morphogenic potential, leading either to somatic embryogenesis or to organogenesis (Fehér, 2023; Mostafiz and Wagiran, 2018). While somatic embryogenesis involves embryo formation from callus tissue and is widely used in plant biotechnology for clonal propagation and genetic improvement (Fehér, 2023), the present study focuses specifically on indirect shoot organogenesis in *Gynura aurantiaca*. Organogenesis from callus tissue, especially shoot formation, is achievable through specific hormonal balances, where the auxin-to-cytokinin ratio plays a pivotal role: a high auxin-to-cytokinin ratio favors callus formation, whereas an appropriate cytokinin level promotes shoot induction (Seti, 2022). To date, no published studies have addressed the *in vitro* regeneration of *G. aurantiaca* using FeEDDHA. Given the compound's superior stability and bioavailability, its application may offer a more efficient alternative to traditional iron sources in tissue culture. Therefore, the objective of this study is to evaluate the effects of different concentrations of FeEDDHA on callus induction and plant conversion in *Gynura aurantiaca*, providing the first report of its *in vitro* response to this iron chelate.

2. Material and Methods

2.1. Plant material

Purple-leaved plants maintained *in vitro* at the third subculture stage were used as explants. The *in vitro* stock cultures were originally established from plants grown in the greenhouses of the Biotechnology Research and Application Center at Çukurova University (Adana, Türkiye), ensuring

reproducibility and traceability of the plant material. The culture medium consisted of MS supplemented with 1 mg L⁻¹ BA. Leaves were cut into 1 cm² segments, and wound tissue was induced on all four edges to promote regeneration (Figure 1). Since *in vitro* material was used, no additional sterilization procedure was required.



Figure 1. Preparation of *Gynura aurantiaca* explants for *in vitro* culture. Preparation of leaves for culture by cutting them into 1 cm² pieces.

2.2. Iron source and dose application

In the standard MS medium (Murashige and Skoog, 1962) protocol, 100 µM FeEDTA is commonly used as the iron source. This concentration was adopted as the control treatment in the present study. To evaluate the effects of FeEDDHA, media were supplemented with four different concentrations: 100 µM, 200 µM, 400 µM, and 800 µM (Figure 2). Each FeEDDHA concentration was incorporated into the basal MS medium by replacing FeEDTA, and the

media were prepared separately for each treatment. Plant explants were cultured on these media under identical conditions to assess the influence of FeEDDHA dosage on callus induction and subsequent regeneration responses. The cultured explants were incubated under controlled conditions at 25 °C, with a 16-hour light / 8-hour dark photoperiod. Cultures were illuminated with cool white fluorescent lamps at an intensity of approximately 2500–3000 lux.

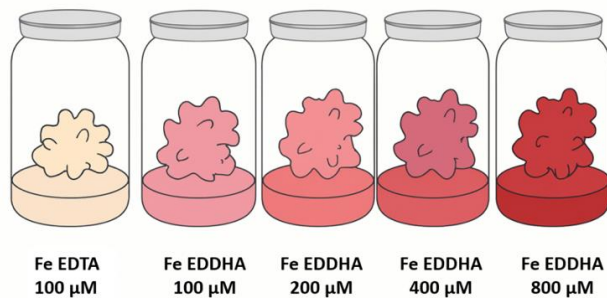


Figure 2. Visual representation of iron source treatments applied in the study. The standard MS medium containing 100 µM FeEDTA was used as the control, while FeEDDHA was tested at four concentrations: 100, 200, 400, and 800 µM

2.3. Nutrient media and hormonal combinations

The nutrient media and hormonal treatments were applied in three distinct stages. In the first and second stages, hormonal combinations were adopted from the protocol described by Cui et al. (2019). The iron source in each medium was modified according to the FeEDDHA concentrations tested in this study. In the third stage, explants derived from previous steps were transferred to hormone-free media to promote rooting and the development of fully regenerated plantlets. The specific media compositions used in each stage are as follows:

- Step 1 – Callus induction

MS medium (Murashige and Skoog, 1962) supplemented with 2 mg L⁻¹ thidiazuron (TDZ) and 0.5 mg L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g L⁻¹ sucrose, 7 g L⁻¹ agar, pH adjusted to 5.8.

- Step 2 – Organogenesis

MS medium supplemented with 2 mg L⁻¹ benzyladenine (BA) and 0.5 mg L⁻¹ 2,4-D, 30 g L⁻¹ sucrose, 7 g L⁻¹ agar, pH adjusted to 5.8.

- Step 3 – Plant conversion

Hormone-free MS medium containing 30 g L⁻¹ sucrose and 7 g L⁻¹ agar, pH adjusted to 5.8.

2.4. Experimental design and statistical analysis

The experiments were conducted in a completely randomized design with three replications. In each treatment, five explants were placed per Petri dish, and ten Petri dishes constituted one replicate, resulting in three replicates per treatment. The collected data were subjected to analysis of variance (ANOVA), and differences between statistically significant means were determined using the Least Significant Difference (LSD) test at $p < 0.01$. Statistical analyses were performed using JMP Pro (version 17.0.0)

software. Data visualization and illustrative graphics were generated using Python 3.11.5, along with relevant libraries (matplotlib 3.8.0, seaborn 0.13.0, numpy 1.26.0). To assess the effects of iron sources on callus formation and regenerative response, the following five key parameters were evaluated:

Callus formation percentage (%): Ratio of explants that developed callus to the total number of explants

Callus weight (g): Fresh weight of callus tissue per replicate

Callus area (cm²): Surface expansion of callus measured via visual analysis. Callus area was measured using the Petiole Pro Android application (calibration pad no. 5), which provides a rapid estimation of callus surface area based on calibrated image analysis. Images were acquired at a fixed distance and lighting, using the app's calibration pad no. 5 prior to each session.

Organogenesis rate (%): Percentage of callus-bearing explants that produced shoots (indirect organogenesis).

Plant conversion rate (%): Success rate of shoots developing into complete plants.

3. Results

The effect of different FeEDDHA concentrations on callus formation was clearly observed (Figure 3). The highest callus formation rate reached 100% at 200 µM FeEDDHA, which was statistically distinct from all other groups (LSD: 6.2; $p < 0.01$). In the control group treated with 100 µM FeEDTA, callus formation was 86%, decreasing to 80% at 400 µM FeEDDHA and further to 58% at 800 µM. These findings indicate that FeEDDHA supports callus induction more effectively than FeEDTA when applied at appropriate concentrations. In particular, the 100–200 µM range appears optimal for callus induction (Figure 4).

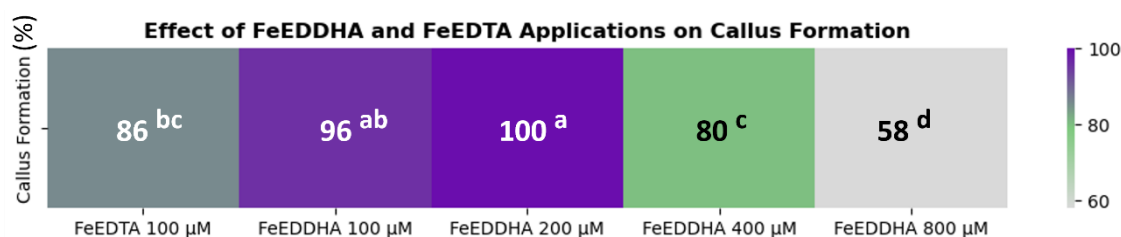


Figure 3. Effect of FeEDDHA and FeEDTA applications at different concentrations on callus formation percentage in *Gynura aurantiaca*



Figure 4. Morphological appearance of callus tissues obtained from *Gynura aurantiaca* leaf explants cultured on MS media supplemented with different iron sources and concentrations. Treatments include FeEDTA (100 µM) and FeEDDHA at 100, 200, 400, and 800 µM. (Ruler: 5 cm)

The effect of different concentrations of FeEDDHA on callus biomass was clearly evident (Figure 5). The highest callus weight was recorded at 200 µM FeEDDHA, reaching 3.96 g, and this group was statistically distinct from all other treatments (LSD: 0.31; $p < 0.01$). This concentration not only supported callus induction but also promoted substantial biomass accumulation. In the control group

treated with 100 µM FeEDTA, callus weight was 2.81 g, while at 800 µM FeEDDHA the value dropped sharply to 1.1 g, indicating a significant reduction in callus growth. These findings suggest that FeEDDHA enhances callus biomass more effectively than FeEDTA when applied at optimal levels. The 100–200 µM range appears to be ideal for promoting callus growth.

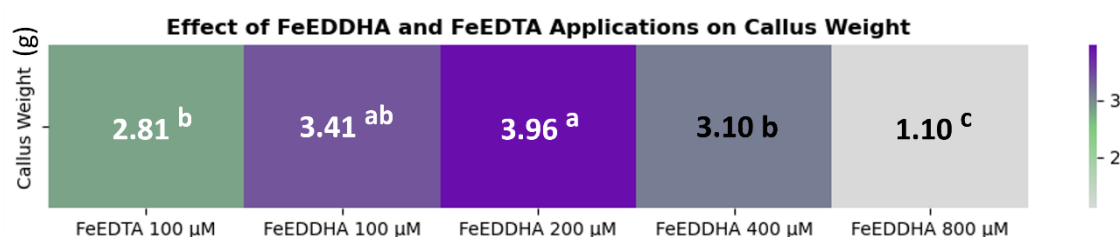


Figure 5. Effect of FeEDDHA and FeEDTA applications at different concentrations on callus fresh weight (g) in *Gynura aurantiaca*. Bars represent mean values with statistical groupings (a–c) based on LSD test ($p < 0.01$)

The effect of different concentrations of FeEDDHA on callus area provides valuable insights into the spatial expansion of callus tissue (Figure 7). Using a fixed 1 cm² leaf

explant, the measurements reflect how extensively the callus spreads across the surface. The highest callus area was observed at 200 µM FeEDDHA, reaching 5.48 cm², and

this group was statistically distinct from the others (LSD: 0.45; $p < 0.01$). The 100 μM FeEDDHA treatment resulted in 5.34 cm^2 , while the control group (FeEDTA 100 μM) showed 4.04 cm^2 . At 400 μM FeEDDHA, the area was 4.48 cm^2 , whereas the 800 μM FeEDDHA treatment led to a sharp decline, with callus area dropping to 2.68 cm^2 , indicating pronounced inhibition of spatial proliferation. To facilitate a clearer understanding of spatial proliferation, callus

area expansion from 1 cm^2 explants was visualized comparatively (Figure 6). These findings highlight the importance of callus area as a parameter that goes beyond biomass, offering a direct measure of surface-level regenerative capacity. The 200 μM FeEDDHA dose appears optimal for promoting both volumetric and spatial callus development, while excessive iron may severely restrict tissue expansion.

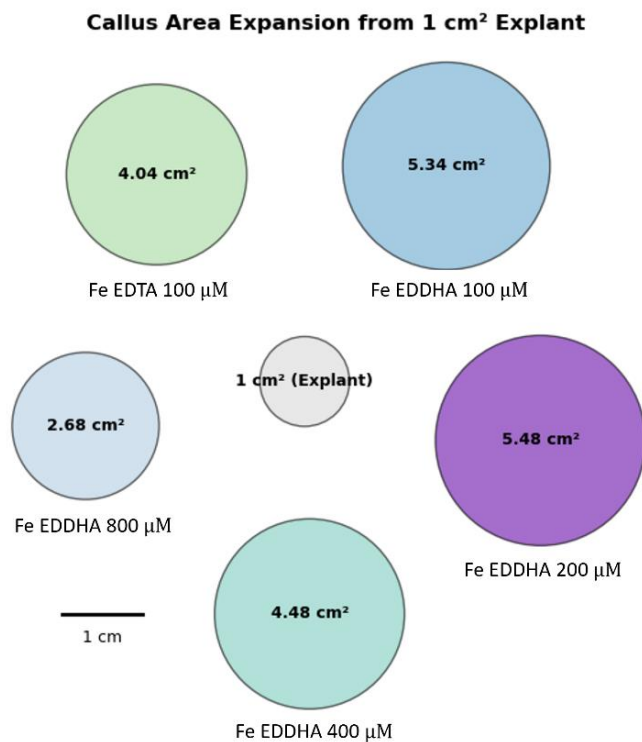


Figure 6. Comparative visualization of callus area expansion from 1 cm^2 leaf explants of *Gynura aurantiaca* cultured on MS media supplemented with different iron sources and concentrations

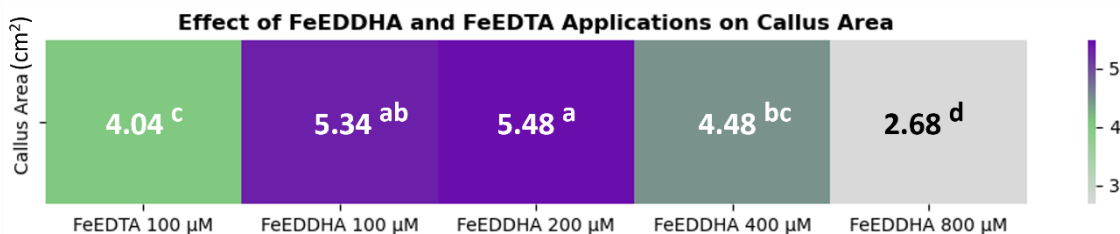


Figure 7. Effect of FeEDDHA and FeEDTA applications at different concentrations on callus area (cm^2) in *Gynura aurantiaca*. Bars represent mean values with statistical groupings (a–d) based on LSD test ($p < 0.01$)

The hormone combinations used for callus induction from leaf tissues in the first stage

also triggered indirect organogenesis. The resulting structures are presented in Figure 8.

The effect of different concentrations of FeEDDHA and FeEDTA on indirect organogenesis was clearly observed (Figure 9). The highest rate was recorded at 200 μM FeEDDHA, reaching 23.2%, and this group was statistically distinct from the others (LSD applied; $p < 0.01$). The control group (FeEDTA 100 μM) showed 13.6%, while FeEDDHA 100

μM yielded 6.4%, 400 μM resulted in 4.0%, and 800 μM showed complete inhibition (0.0%). These findings suggest that FeEDDHA promotes indirect shoot regeneration at optimal concentrations, but suppresses it at higher doses. The 200 μM FeEDDHA treatment stands out as the most effective condition for enhancing regenerative potential.



Figure 8. Regeneration shoots obtained from *Gynura aurantiaca* explants cultured in Callus Induction medium (MS + 2 mg L⁻¹ TDZ + 0.5 mg L⁻¹ 2,4-D) supplemented with different iron sources and concentrations

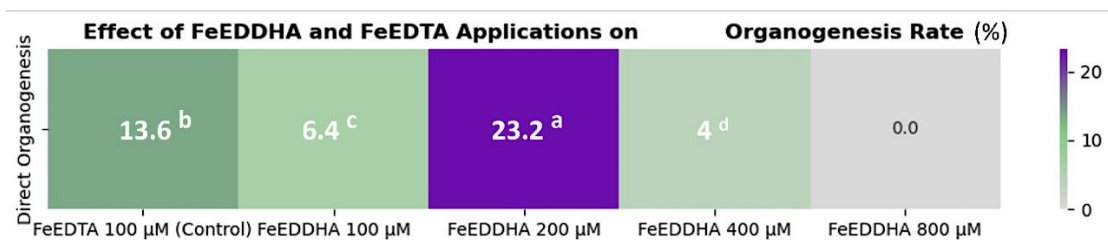


Figure 9. Indirect organogenesis rates (%) of *Gynura aurantiaca* leaf explants cultured on MS media supplemented (Step 1) with FeEDTA (100 μM) and FeEDDHA at 100, 200, 400, and 800 μM . Statistical groupings (a–d) indicate significant differences among treatments (LSD test, $p < 0.01$)

Regenerated shoots and plant structures of *Gynura aurantiaca* obtained from the organogenesis medium applied in the second stage are presented in Figure 10. For organogenesis, MS medium was supplemented with 2 mg L⁻¹ BA and 0.5 mg L⁻¹ 2,4-D, along

with different iron sources (FeEDDHA and FeEDTA). The visual differences in shoot formation, pigmentation, and tissue density reflect the impact of iron treatments on morphogenic responses.



Figure 10. Morphological appearance of regenerated shoots and plant structures obtained from *Gynura aurantiaca* explants cultured on organogenesis medium (MS + 2 mg L⁻¹ BA + 0.5 mg L⁻¹ 2,4-D) supplemented with different iron sources and concentrations

The chart illustrates the effect of FeEDTA and FeEDDHA applications at different concentrations on plant conversion rate in *Gynura aurantiaca* (Figure 11). The highest conversion rate (74%) was observed at 100 μM FeEDDHA, indicating its strong promotive effect on regeneration and significantly outperforming all other treatments. As the FeEDDHA concentration increased: 200 μM resulted in a moderate conversion rate (54%),

400 μM showed a sharp decline (18%), 800 μM completely inhibited regeneration (0%), suggesting possible toxicity or suppression of organogenic potential. The control treatment (100 μM FeEDTA) yielded a 43% conversion rate, confirming that FeEDDHA at optimal dose enhances regeneration more effectively than standard iron sources. These findings highlight the importance of precise iron dose optimization in *in vitro* protocols.

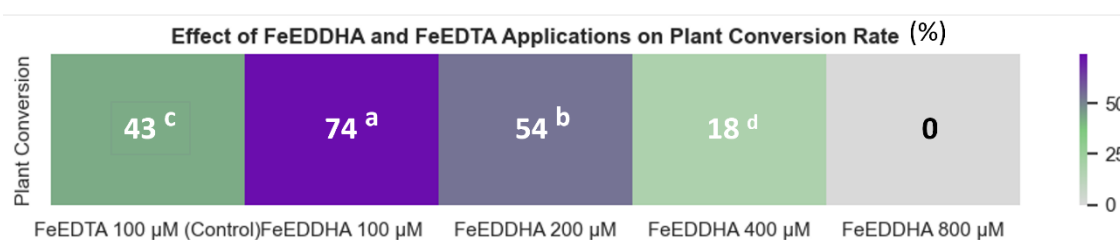


Figure 11. Effect of FeEDDHA and FeEDTA applications at different concentrations on plant conversion rate (%) in *Gynura aurantiaca*. Statistical groupings (a–d) indicate significant differences among treatments (LSD test, $p < 0.01$)

In the final stage of *in vitro* culture, explants transferred to hormone-free MS medium successfully developed into complete plantlets, exhibiting both root formation and shoot

elongation (Figure 12). These results confirm the regenerative potential of *Gynura aurantiaca* under optimized FeEDDHA treatments.



Figure 12. Full plant development of *Gynura aurantiaca* explants following transfer to hormone-free MS medium during the third stage of *in vitro* culture. Root formation and shoot elongation indicate successful conversion into complete plantlets

4. Discussion

The present study demonstrated that FeEDDHA, when applied at appropriate concentrations, significantly enhances callus induction and plant conversion in *Gynura aurantiaca*, outperforming the standard FeEDTA treatment. These findings are consistent with previous reports in other species, where FeEDDHA has been associated with improved *in vitro* morphogenesis, likely due to enhanced iron bioavailability and possible mitigation of oxidative stress (as reported in the literature). In date palm (*Phoenix dactylifera*), Al-Mayahi (2021) reported that FeEDDHA supplementation led to enhanced callus growth, shoot regeneration, and chlorophyll accumulation, with optimal effects observed at 93.5–187.0 mg L⁻¹. Similarly, in *Carlina onopordifolia*, FeEDDHA increased chlorophyll content and shoot elongation, although excessive concentrations inhibited shoot growth (Trejgell et al., 2012). These dose-dependent responses align with the current findings, where 100–200 µM FeEDDHA promoted callus proliferation and plant conversion, while 800 µM resulted in significant inhibition. The observed increase in callus area and biomass at 200 µM FeEDDHA suggests that iron availability plays a critical role not only in cellular proliferation but also in spatial expansion of regenerative tissue. This is supported by studies in *Prunus* rootstocks, where elevated iron levels enhanced somatic

embryo development (Pesce and Rugini, 2004), and in *Hibiscus rosa-sinensis*, where FeEDDHA improved chlorophyll content and shoot quality (Christensen et al., 2008). Interestingly, the highest plant conversion rate was observed at 100 µM FeEDDHA, indicating that lower concentrations may favor organogenic transition. This mirrors findings in *Rosa damascena* and pineapple (*Ananas comosus*), where FeEDDHA-enriched media improved shoot quality and acclimatization success (Khajeh et al., 2021; Alavijeh, 2025). The decline in regeneration at higher doses is consistent with previous reports where high iron levels were associated with oxidative stress or hormonal imbalances, as observed in *Cicer arietinum* and *Oryza sativa* (Irum et al., 2020; Ullah et al., 2024). The hormonal combinations used in this study were adapted from Cui et al. (2019), who successfully regenerated *G. aurantiaca* via indirect shoot organogenesis using TDZ or BA with 2,4-D. Our results confirm the effectiveness of this protocol and further demonstrate that iron source optimization can significantly modulate its efficiency. While Cui et al. focused on shoot proliferation, our study extends this by quantifying callus area and biomass, providing a more comprehensive view of regenerative potential. In related *Gynura* species, such as *G. procumbens*, callus induction and shoot regeneration have been achieved using various auxin–cytokinin combinations (Nurokhman et al., 2019; Azad and Amin, 2017). However,

none of these studies evaluated the role of iron chelates, highlighting the novelty of our approach. Moreover, the reddish-purple callus observed in *G. aurantiaca* has been associated with high anthocyanin content (Cui et al., 2019). In the present study, anthocyanin content was not measured; therefore, the possible influence of FeEDDHA on secondary metabolite accumulation should be considered only as a hypothesis to be tested in future work. Overall, our findings underscore the importance of precise iron dose optimization in tissue culture protocols. FeEDDHA at 200 μ M was optimal for callus formation and biomass, whereas the highest plant conversion rate was obtained at 100 μ M. Plant regeneration rates declined at higher concentrations (≥ 400 μ M). These results highlight FeEDDHA as a promising alternative to FeEDTA in *Gynura aurantiaca*. Further studies involving histological, biochemical, and molecular analyses are needed to elucidate the underlying mechanisms and to assess the broader applicability of these findings across other ornamental and medicinal species.

5. Conclusion and Recommendations

FeEDDHA treatments showed distinct effects on callus formation and expansion compared to FeEDTA. FeEDDHA at 200 μ M was optimal for callus formation and biomass, whereas the highest plant conversion rate was obtained at 100 μ M. Plant regeneration rates declined at higher concentrations (≥ 400 μ M). These findings highlight the need for careful optimization of chelated iron forms in *in vitro* conversion protocols.

Future studies may consider:

- Testing **nearby concentrations** around the identified optimal dose
- Exploring interactions with different **plant species** and **hormonal combinations**
- Conducting **histological analyses** to examine the structure of induced tissues
- Performing **genetic stability assessments** on regenerated plants

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Ethical Committee Approval

This study did not involve human or animal subjects; therefore, ethical approval was not required.

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