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Plant regeneration of *Ferula assa-foetida* (asafoetida) via root-derived callus from seed-embryo plantlets

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Abstract

Ferula assa-foetida L. (asafoetida), a monocarpic and endangered medicinal-industrial plant native to the cold steppes of Iran and Afghanistan, faces extinction due to overexploitation for its oleo-gum-resin and complex seed dormancy. To develop an efficient micropropagation protocol using root-derived callus from seed-embryo plantlets and to determine optimal plant growth regulator concentrations for callus induction and plant regeneration. Mature embryos excised from surface-sterilized seeds were cultured on half-strength MS medium without hormones. Root segments (1 cm) from 21-day-old plantlets were cultured on half-strength MS medium supplemented with BAP (0, 0.5, 1.0, 1.5 mg/L) and TDZ (0, 0.5, 1.0, 1.5 mg/L). Callus growth parameters (length, width, height, fresh weight) were measured after four weeks. Analyzing the data, embryo culture achieved > 95% germination within 5–7 days. Callus formation from root explants occurred only in four hormone combinations. Treatment T1 (0.5 mg/L BAP + 0.5 mg/L TDZ) produced the highest callus length (4.73 cm) and fresh weight (5.94 g), significantly outperforming all other treatments ($p < 0.01$). The results revealed that TDZ had a stronger effect on callus growth than BAP. Only T1-derived calli regenerated whole plantlets. Regenerated plantlets exhibited severe vitrification on ½ MS medium but recovered completely after transfer to ½ QL medium (lower nitrogen content), followed by successful rooting and > 85% survival under greenhouse conditions. Using only 1 cm of root, approximately 30 healthy plantlets were produced. The optimized protocol provides an efficient, reproducible method for large-scale asexual propagation, conservation, and sustainable cultivation of this endangered medicinal species.

Key message

Successful embryo-root-derived regeneration of ***Ferula assa-foetida***, enabling conservation, mass propagation, and sustainable cultivation of this endangered medicinal-industrial species.

1. Introduction

Ferula assa-foetida L. (Apiaceae), commonly known as asafoetida or heeng, is a perennial herbaceous plant native to the cold continental steppes and semi-arid rangelands of Iran and Afghanistan (Singh et al. 2023; Kumar et al. 2025). *F. assa-foetida* characteristically grows at elevations ranging from 600 to 2,500 m a.s.l, where annual precipitation is typically below 300 mm and winter temperatures can drop to -4°C (Zare Chahouki et al. 2019; Mehrpour et al. 2016). The plant develops a fleshy, carrot-like taproot that can reach up to 15 cm in diameter and stores the oleo-gum-resin for which the species is globally renowned (Omidbeigi et al. 2006). Under natural conditions, *F. assa-foetida* requires approximately five to seven years of vegetative growth before it produces a single flowering stalk, after which the plant senesces and dies, exemplifying its monocarpic reproductive strategy (Punia et al. 2024; Murzakanova 2025).

The oleo-gum-resin exuded from its taproot has been a cornerstone of traditional medicine systems for over two millennia, including Persian, Ayurvedic, and Unani practices (Bagheri et al. 2017; Boghrati and

Iranshahi 2019). Pharmacological studies have validated numerous therapeutic applications, demonstrating anti-inflammatory, antiviral, antidiabetic, and antispasmodic properties (Elarabany et al. 2023; Bagheri et al. 2024). Recent research has also revealed significant anticancer activity against colorectal and breast cancer cell lines (Sirizi et al. 2023; Bahrami-Taghanaki et al. 2024). Beyond human health, asafoetida serves as an important industrial crop; its essential oil exhibits potent fumigant toxicity against stored product pests such as the carob moth (*Ectomyelois ceratoniae*) and is increasingly investigated as a natural alternative to synthetic pesticides (Fotouhi et al. 2024; Karimi et al. 2024). Economically, the collection and trade of asafoetida provide crucial supplementary income for local communities in Iran, Afghanistan, and India, where the resin commands high prices in international markets (Rajabian et al. 2007; Kumari et al. 2025). The growing global demand for natural phytopharmaceuticals and organic pest control agents has further intensified pressure on wild populations (Hashemi et al. 2023).

Despite its immense value, *F. assa-foetida* is currently classified as an endangered species in the Red Data Book of Iran, primarily due to decades of unsustainable harvesting (Jalili and Jamzad 1999; Zare Chahouki et al. 2019). The resin extraction process involves wounding the taproot, which often kills the plant or severely weakens it, and the collection of wild specimens has far exceeded the species' natural regeneration capacity (Tavili and Saberi 2020; Karimzadeh and Mahmoudi 2022). This overexploitation is exacerbated by the plant's monocarpic life cycle, which dictates that each individual reproduces only once after years of vegetative growth (Omidbeigi et al. 2006). Consequently, seed production is the sole mechanism for natural population renewal, yet asafoetida seeds exhibit complex morphophysiological dormancy (Punia et al. 2024). Specifically, the seeds require a period of cold stratification at 4°C for 6–8 weeks followed by treatment with gibberellic acid to break dormancy, and even under optimal conditions, germination rates rarely exceed 40–50% (Tavili and Saberi 2020; Karimzadeh and Mahmoudi 2022; Kumar et al. 2025). This combination of overharvesting, monocarpic reproduction, and poor seed germination has created a critical conservation crisis, necessitating urgent intervention through advanced propagation technologies (Rajasekharan and Wani 2020; Murzakanova 2025).

Plant tissue culture, founded on the principle of cellular totipotency, offers a powerful alternative to conventional propagation methods for endangered medicinal species (Zhou and Wu 2006; Thorpe 2007). This technique enables the rapid production of genetically identical, disease-free plantlets from minimal starting material under controlled environmental conditions, independent of seasonal constraints (Rajasekharan and Wani 2020). Over the past two decades, successful micropropagation protocols have been developed for numerous threatened Apiaceae species, including *F. gummosa*, *F. ovina*, and *F. sinkiangensis* (Zare et al. 2020; Roozbeh et al. 2021; Hashemi et al. 2023). However, research on *F. assa-foetida* remains limited and has encountered significant obstacles. Previous studies have primarily relied on stem or leaf explants collected from mature plants, but the prolonged dormancy period of this species (lasting 8–10 months annually) renders these explants available for only a short window of 4–6 weeks in early spring (Zare et al. 2020; Roozbeh et al. 2021). Furthermore, field-collected explants are frequently contaminated with endophytic microorganisms, leading to high rates of loss during surface sterilization procedures (Karimzadeh and Mahmoudi 2022). These limitations highlight the pressing

need for an alternative, season-independent explant source that can support year-round micropropagation.

Seeds of *F. assa-foetida*, despite their dormancy issues, offer a practical solution because they can be collected during the brief fruiting period and stored for extended periods (up to 3–5 years) under cool, dry conditions without significant loss of viability (Punia et al. 2024; Kumar et al. 2025). An innovative strategy to bypass the germination bottleneck is the “in vitro” culture of isolated mature embryos, a technique commonly termed "embryo rescue" or "seed cesarean" (Hashemi et al. 2023). When aseptically excised from the seed coat and endosperm and placed on a suitable culture medium such as half-strength Murashige and Skoog (MS/2), the embryos can germinate within 5–7 days, producing healthy, uniform plantlets at nearly 100% efficiency (Zare et al. 2020; Hashemi et al. 2023). The root segments of these plantlets represent a novel and abundant explant source that has received little attention in Ferula biotechnology. Roots are highly meristematic, exhibit robust callus formation in response to appropriate plant growth regulators, and can be obtained in large numbers from a single embryo culture event (Roozbeh et al. 2021; Kumari et al. 2025). Previous studies on other medicinal plants, including *Glycyrrhiza glabra* and *Panax ginseng*, have demonstrated that root-derived callus possesses superior regenerative capacity compared to leaf or stem-derived callus (Rajasekharan and Wani 2020). However, no published report has systematically investigated the potential of root explants derived from the main plant stand or seed embryo plantlets for micropropagation of *F. assa-foetida*.

According to the above mentioned, the present study was undertaken with the primary objective of developing an efficient and reproducible micropropagation protocol for *F. assa-foetida* using root-derived callus obtained from plantlets regenerated from isolated seed embryos. Specifically, we aimed to: (1) establish a reliable method for rapid production of sterile plantlets from seed embryos on MS/2 medium; (2) evaluate the capacity of root explants from these plantlets for callus induction and shoot regeneration across a range of concentrations and combinations of plant growth regulators, including benzylaminopurine (BAP) and thidiazuron (TDZ); and (3) determine the optimal treatment for maximizing the number of regenerated plantlets per explant. This study represents the first report on regeneration of *F. assa-foetida* via root-derived callus from seed embryo plantlets. We anticipate that the developed protocol will serve as a valuable tool for the conservation, commercial cultivation, and genetic improvement of this endangered but industrially important medicinal species.

2. Materials and Methods

2.1. Plant Material and Seed Collection

Mature seeds of *F. assa-foetida* were collected from natural rangeland populations located in the steppe regions of Kohgiluyeh and Boyer-Ahmad Province, Iran (30°28'7.04" N, 51°49'42" E; altitude 2,301 m above sea level) during the fruiting period (late July to early August) of 2023 (Fig. 1). The region is characterized by a cold semi-arid climate with mean annual precipitation of 832.3 mm and mean annual temperature of 15.4°C. Seeds were manually harvested from at least 50 healthy, mature plants to ensure

genetic diversity. After collection, seeds were cleaned of debris, surface-dried at room temperature (25 ± 2°C) for 48 h, and stored in sealed paper bags at 4°C for up to three months until use to complete physiological seed maturation (Karimian et al. 2018; Punia et al. 2024). Then, only plump, undamaged seeds of uniform size (approximately 8–10 mm in length) were selected for subsequent experiments. Botanical identification of the plant material was performed, and a voucher specimen has been deposited and preserved under the accession number MPH-2316 at the Herbarium of the Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran. (Karimian et al. 2020)

2.2. Seed Surface Sterilization and Embryo Excision

Selected seeds were thoroughly washed under running tap water for 72 h to remove surface dust and debris. Subsequently, seeds were transferred to a laminar flow hood at the Plant Tissue Culture Laboratory of the Academic Center for Education, Culture and Research (ACECR), University of Tehran, Karaj, Iran, for surface sterilization. The sterilization protocol consisted of immersing seeds in 70% (v/v) ethanol for 3 min, followed by soaking in 1.5% (v/v) sodium hypochlorite (NaOCl) solution containing two drops of Tween® 20 per 100 mL for 20 min with occasional agitation (Zare et al. 2020; Hashemi et al. 2023). Seeds were then rinsed eight times (2 min each) with sterile distilled water to remove residual sterilant. Under aseptic conditions, the softened seed coat and endosperm were carefully removed using a sterile scalpel and forceps to excise the intact mature embryos. Embryos that were damaged, discolored, or abnormally small were discarded (Kumar et al. 2025).

2.3. Embryo Culture and Plantlet Establishment

Excised embryos were cultured individually in glass culture tubes (25 × 150 mm) containing 15 mL of Murashige and Skoog (MS) medium (Murashige and Skoog 1962) at half-strength macro- and micro-nutrients (MS/2). The medium was supplemented with 3% (w/v) sucrose as a carbon source and solidified with 0.8% (w/v) plant agar (Merck, Germany). The pH of the medium was adjusted to 5.75 ± 0.05 using 0.1 N NaOH or 0.1 N HCl prior to autoclaving at 121°C for 20 min (Wetzstein et al., 2019). No plant growth regulators were added to this initial culture medium. All cultures were maintained in a growth chamber at 25 ± 2°C under a 16/8 h (light/dark) photoperiod with a light intensity of 50 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps. The percentage of embryos that germinated (radicle emergence ≥ 2 mm) was recorded after 7 and 14 days. After 21 days of culture, the resulting plantlets (with developed roots and shoots) were used as the source of root explants for callus induction experiments (Roozbeh et al. 2021; Kumari et al. 2025) (Fig. 2).

2.4. Preparation of Root Explants

Healthy, uniform plantlets (3–4 cm in height with 2–3 fully expanded leaves and well-developed root systems) were selected after 21 days of embryo culture. The plantlets were gently removed from culture tubes, and their roots were separated from the shoot using a sterile scalpel. The roots were cut into segments of approximately 1.0 cm in length. Each root segment served as one explant. Approximately

30 root explants were obtained from a single plantlet. The explants were immediately placed onto callus induction medium without any pre-treatment (Zare et al. 2020; Hashemi et al. 2023).

2.5. Callus Induction and Plant Regeneration

The root explants were cultured on half-strength MS medium supplemented with various concentrations and combinations of plant growth regulators (PGRs) to optimize callus induction and subsequent shoot regeneration. Basal ½ MS medium was prepared with full-strength macro- and micro-nutrients, 3% (w/v) sucrose, 0.8% (w/v) plant agar, and pH adjusted to 5.75 ± 0.05 (Bahramali et al. 2023). The following PGR treatments were evaluated (Table 1).

Table 1
Different concentrations and combinations of BAP and TDZ tested for callus induction from root, stem, and leaf explants of *F. assa-foetida*.

		Benzylaminopurine (mg/L)			
Thidiazuron (mg/L)		0	0.5	1.0	1.5
	0	B_0T_0	$B_{0.5}T_0$	B_1T_0	$B_{1.5}T_0$
	0.5	$B_0T_{0.5}$	$B_{0.5}T_{0.5}$	$B_1T_{0.5}$	$B_{1.5}T_{0.5}$
	1.0	B_0T_1	$B_{0.5}T_1$	B_1T_1	$B_{1.5}T_1$
	1.5	$B_0T_{1.5}$	$B_{0.5}T_{1.5}$	$B_1T_{1.5}$	$B_{1.5}T_{1.5}$

For each treatment, 9 root explants (three explant per culture vessel) were used (all treatments were replicated three times). Explants were cultured in 9 cm Petri dishes containing 25 mL of medium or in glass jars (six explants per jar). Cultures were maintained under the same growth chamber conditions described in Section 2.3. After 4 weeks, the percentage of explants producing callus (callus induction frequency) was recorded, and the callus fresh weight (mg) was measured. Callus was subcultured onto fresh medium of the same composition every 4 weeks (Roozbeh et al. 2021).

For shoot regeneration, calli (approximately 200 mg fresh weight) were transferred to regeneration medium containing the same PGR combinations as above with TDZ (0.5 mg/L) and BAP (0.5 mg/L). After 6–8 weeks, the number of regenerated shoots per callus explant was recorded. (Karimzadeh and Mahmoudi 2022; Kumari et al. 2025).

2.6. Acclimatization and Transfer to Greenhouse

Regenerated plantlets with well-developed roots (after 4 weeks on rooting medium) were removed from culture vessels and their roots were gently washed under running tap water to remove adhering agar. Plantlets were then transferred to plastic pots (10 cm diameter, 15 cm height) containing a sterile substrate mixture of peat moss, perlite, and vermiculite in a 2:1:1 (v/v/v) ratio (Nekrasova et al. 2021; Hashemi et al. 2023). The pots were covered with transparent polyethylene bags to maintain high humidity (approximately 90% relative humidity) and placed in a growth chamber at 25 ± 2°C under a 16/8

h photoperiod for 2 weeks. The polyethylene bags were gradually opened over the subsequent 2 weeks to acclimate plantlets to ambient humidity. After 4 weeks of acclimatization, plantlets were transferred to a greenhouse under natural light conditions (temperature 25 ± 5°C, relative humidity 60–70%) and irrigated with tap water every 3 days. Survival percentage was recorded after 8 weeks of greenhouse transfer (Murzakanova 2025) (Fig. 6).

2.7. Experimental Design and Statistical Analysis

The experiment was arranged as a completely randomized design (CRD) with three replications per treatment. For callus induction experiments, each replication consisted of 3 explants, resulting in a total of 9 explants per treatment (n = 9). For regeneration experiments, each replication consisted of 3 calli, resulting in a total of 3 calli per treatment (n = 3). All data were expressed as mean ± standard deviation (SD). Percentage data (including callus induction frequency, regeneration frequency, and survival rate) were arcsine-transformed prior to statistical analysis to meet the assumptions of normality and homogeneity of variance, as recommended for proportional data in plant tissue culture studies (Zare et al. 2020; Roozbeh et al. 2021). Normality of the data was assessed using the Shapiro-Wilk test ($p > 0.05$), which confirmed a normal distribution for all measured parameters of callus length, width, height, and fresh weight (the results along with raw data are provided in Online Resource 1 and 2). One-way analysis of variance (ANOVA) was performed to compare treatment means for callus growth parameters. This analysis revealed significant differences among treatments for callus length ($p = 0.003$), fresh weight ($p = 0.002$), and callus width ($p = 0.07$), while no significant difference was detected for callus height ($p > 0.05$). To evaluate the independent effects of each cytokinin, a two-way ANOVA was conducted without analyzing the interaction effect between BAP and TDZ. This analysis showed that both hormones significantly influenced callus length and fresh weight, while neither hormone had a significant effect on callus width or height (Details are presented in Online Resource 3). Mean comparisons among treatments and among different concentrations of each hormone were performed using Duncan's multiple range test (DMRT) at a significance level of $p < 0.05$ (Ossai et al. 2025). All statistical analyses were conducted using SAS software (version 9.4, SAS Institute Inc., Cary, NC, USA). Graphical representations were prepared using GraphPad Prism (version 9.0, GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Embryo Culture and Plantlet Establishment

Isolated mature embryos of *F. assa-foetida* cultured on half-strength Murashige and Skoog (MS/2) medium without plant growth regulators showed rapid and uniform germination. Within 5–7 days of culture, radicle emergence was observed in more than 95% of the embryos. After 14 days, all germinated embryos had developed into healthy, morphologically uniform plantlets with well-developed roots (approximately 3–4 cm in length) and shoots bearing 2–3 expanded leaves (Fig. 5). These results

demonstrate that embryo culture is an efficient strategy to bypass seed dormancy and rapidly produce sterile, juvenile plantlets suitable as a source of explants for subsequent tissue culture experiments.

3.2. Callus Induction from Root, Leaf, and Stem Explants

Root segments (1 cm) excised from 21-day-old plantlets were cultured on MS medium supplemented with various combinations and concentrations of BAP and TDZ. Callus formation was observed only in four specific hormone combinations. No callus was induced on control medium (without PGRs) or on media containing BAP or TDZ alone at any concentration tested (0, 0.5, 1.0, or 1.5 mg/L). The four successful hormone treatments were: T1 (0.5 mg/L BAP + 0.5 mg/L TDZ), T2 (1.5 mg/L BAP + 0.5 mg/L TDZ), T3 (0.5 mg/L BAP + 1.0 mg/L TDZ), and T4 (1.5 mg/L BAP + 1.5 mg/L TDZ) (Fig. 3). Callus initiation began 10–14 days after culture. The induced calli were friable, yellowish-white, and proliferated actively on the surface of the root explants.

Leaf explants showed very limited callogenic potential. Among all PGR treatments tested, only the combination of 0.5 mg/L BAP + 1.0 mg/L TDZ induced a small amount of callus. This indicates that leaf tissues require a precise and narrow hormonal balance for callogenesis. In contrast, stem explants did not produce callus under any of the hormone combinations tested. Stem tissues were significantly less responsive to BAP and TDZ compared to root tissues, suggesting that alternative PGR protocols or pre-treatments are required for callus induction from stem explants in this species. Consequently, all subsequent experiments focused on root-derived callus (Fig. 4).

3.3. Morphological Evaluation of Root-Derived Calli

After four weeks of culture, callus growth parameters—including length (cm), width (cm), height (cm), and fresh weight (g)—were measured for the four hormone treatments (T1–T4) that successfully induced callus from root explants. Analysis revealed highly significant differences among treatments for callus length and fresh weight ($p < 0.01$), a marginally significant difference for callus width ($p < 0.10$), and no significant difference for callus height. Treatment T1 (0.5 mg/L BAP + 0.5 mg/L TDZ) produced the highest callus length (4.73 cm) and fresh weight (5.94 g), both of which were statistically superior to all other treatments ($p \leq 0.05$). This treatment also yielded relatively high callus width (2.53 cm), although it was not significantly different from T2 or T4 at the conventional $p \leq 0.05$ level. Regarding callus width, T2 (1.5 mg/L BAP + 0.5 mg/L TDZ) showed the greatest value (2.87 cm), which was significantly larger than T3 (1.73 cm) at $p \leq 0.10$, but statistically comparable to T1 and T4. Callus height remained relatively stable across all treatments (ranging from 1.33 cm in T3 to 1.63 cm in T4), with no statistically significant differences detected. Fresh weight followed a similar pattern to callus length: T1 significantly outperformed all other treatments ($p \leq 0.05$), while T3 (1.14 g) produced the lowest biomass. These results collectively indicate that the combination of lower concentrations of both BAP and TDZ (0.5 mg/L each) is optimal for promoting vigorous callus growth in *F. assa-foetida* root explants, while increasing either cytokinin concentration adversely affects callus proliferation (Fig. 4).

3.4. Individual Effects of BAP and TDZ

The results revealed that both BAP and TDZ significantly influenced callus length and fresh weight, while neither hormone had a significant effect on callus width or height. Both BAP and TDZ significantly influenced callus length and fresh weight, albeit with different levels of statistical confidence. For callus length, TDZ showed a highly significant effect ($p < 0.01$), while BAP exhibited a significant but weaker effect ($p < 0.05$). For fresh weight, both hormones demonstrated highly significant effects ($p < 0.01$). Notably, the mean square values—which reflect the magnitude of variance explained by each factor—were substantially larger for TDZ (4.08 for length; 17.44 for fresh weight) compared to BAP (1.31 for length; 10.65 for fresh weight). This indicates that TDZ had a stronger influence than BAP on both callus length and fresh weight, suggesting that TDZ is the primary determinant of callus growth vigor in this species under the tested conditions.

In contrast, neither BAP nor TDZ had a significant effect on callus width ($p > 0.05$ for both) or callus height ($p > 0.05$ for both). The variation observed in these two parameters among treatments was not statistically attributable to the concentrations of either cytokinin, indicating that other factors—potentially including genotype, explant physiology, or uncontrolled environmental variation—may have played a more dominant role in determining these dimensions (Table 2).

Table 2
Two-way ANOVA summary for the effects of TDZ and BAP on callus growth parameters.

Parameter	Source	df	MS	p-value
Callus Length	TDZ	2	4.08	0.001^{**}
	BAP	1	1.31	0.048^{*}
Fresh Weight	TDZ	2	17.44	0.001^{**}
	BAP	1	10.65	0.010^{**}
Callus Width	TDZ	2	0.79	0.064^{*}
	BAP	1	0.13	0.436^{ns}
Callus Height	TDZ	2	0.05	0.532^{ns}
	BAP	1	0.01	0.784^{ns}

Significance levels: **p < 0.01; *p < 0.05; ns = non-significant (p > 0.05).MS: Mean Square, TDZ had a stronger effect than BAP on both callus length (F = 16.90 vs. 5.41) and fresh weight (F = 18.67 vs. 11.41), indicating that TDZ is the primary determinant of callus growth vigor in this species under the tested conditions

3.5. Dose-Response Analysis: Optimal Concentrations of TDZ and BAP

The dose-response analysis revealed distinct patterns for TDZ and BAP. For TDZ, the concentration of 0.5 mg/L consistently produced the highest mean values across three out of four measured parameters: callus length (4.27 cm), callus width (2.72 cm), and fresh weight (4.61 g). Increasing the TDZ concentration to 1.0 mg/L resulted in a sharp and statistically significant decline in all these parameters, with fresh weight dropping to its lowest level (1.14 g). Interestingly, when TDZ was further increased to 1.5 mg/L, a partial recovery was observed: callus length (3.83 cm) and fresh weight (2.82 g) returned to levels statistically comparable to those achieved at 0.5 mg/L, while callus width (2.27 cm) showed an intermediate response not significantly different from either 0.5 or 1.0 mg/L. This non-linear (U-shaped or J-shaped) dose-response suggests a complex regulatory mechanism of TDZ on callogenesis in *F. assa-foetida*. For BAP, both concentrations tested (0.5 and 1.5 mg/L) produced comparable callus length (3.57 vs. 3.82 cm, respectively) and callus width (2.15 vs. 2.57 cm, respectively), with no statistically significant differences between them. However, a significant difference was observed for fresh weight: the lower BAP concentration (0.5 mg/L) yielded a significantly higher fresh weight (3.54 g) compared to the higher concentration (3.05 g). This indicates that while BAP concentration does not strongly influence the dimensional expansion of callus, it plays a critical role in determining callus biomass accumulation, with lower concentrations being more favorable. Regarding callus height, neither TDZ nor BAP concentration had a statistically significant effect ($p > 0.05$). Mean callus height values ranged narrowly from 1.33 cm (TDZ at 1.0 mg/L) to 1.63 cm (TDZ at 1.5 mg/L), confirming the earlier ANOVA results that callus height is the least responsive parameter to cytokinin manipulation in this species. Overall, these results indicate that 0.5 mg/L TDZ and 0.5 mg/L BAP represent the optimal concentrations for maximizing callus growth, particularly in terms of fresh weight and linear dimensions. Higher concentrations of either cytokinin either reduce (BAP) or non-linearly modulate (TDZ) callus proliferation (Fig. 5).

3.6. Plantlet Regeneration from Root-Derived Callus

Calli from all four treatments were transferred to regeneration medium. Only calli derived from T1 (0.5 mg/L BAP + 0.5 mg/L TDZ) successfully regenerated whole plantlets (Fig. 6A). Calli from T2, T3, and T4 produced no shoots or abnormal structures even after 8 weeks of culture. The regenerated plantlets initially cultured on ½ MS medium showed severe vitrification (hyperhydricity), characterized by translucent, brittle leaves and stems due to high nitrogen content in the medium (Fig. 6B). To overcome this, vitrified plantlets were transferred to ½ QL (Quoirin and Lepoivre) medium, which has a lower nitrogen concentration. Within 2–3 weeks, plantlets recovered completely, exhibiting normal green morphology, vigorous growth, and healthy leaf expansion (Fig. 6C). This confirmed that reduced nitrogen levels are critical for producing morphologically normal plantlets in *F. assa-foetida*. Plantlets transferred to ½ QL medium developed a robust root system within 4 weeks (Fig. 6D). In contrast, plantlets maintained on ½ MS medium showed poor rooting and continued vitrification. After rooting, plantlets were transferred to a sterile substrate (peat moss: perlite: vermiculite, 2:1:1) and acclimatized under high humidity. After 4 weeks, more than 85% of plantlets survived and were successfully established under greenhouse conditions, showing no morphological abnormalities (Fig. 6E).

4. Discussion

4.1. Embryo culture, explant selection, and the role of juvenility in callogenesis

F. assa-foetida is a monocarpic, endangered medicinal plant native to the cold steppes of Iran and Afghanistan. Its natural regeneration is severely limited by complex seed dormancy and the fact that each plant flowers and produces seeds only once before dying. Overexploitation for its valuable oleo-gum-resin has further endangered wild populations. The present study addressed these challenges by developing an efficient micropropagation protocol using root-derived callus from seed-embryo plantlets. The present study demonstrated that culture of isolated mature embryos on half-strength MS medium without plant growth regulators resulted in rapid (5–7 days) and uniform germination with over 95% success. This finding is particularly significant given that intact seeds of *F. assa-foetida* exhibit complex morphophysiological dormancy under natural conditions. Tavili and Saberi (2020) reported that asafoetida seeds require 6–8 weeks of cold stratification at 4°C to achieve only 40–50% germination. Similarly, Karimzadeh and Mahmoudi (2022) confirmed that seed dormancy in this species is a major bottleneck for conventional propagation. The dramatic acceleration of germination achieved through embryo culture can be attributed to the removal of physical barriers imposed by the thick seed coat and endosperm, as well as the elimination of chemical inhibitors such as phenols and coumarins that accumulate in the seed coat, as previously explained by Booth and Sowa (2001). These findings support the notion that asafoetida seed dormancy is a combination of both physiological and morphological factors. Jonoubi et al. (2025) reached similar conclusions while working on the closely related species *F. gummosa*, demonstrating that embryo culture not only shortened the germination period but also provided a clean source of sterile explants for subsequent micropropagation. The use of hormone-free ½ MS medium was adequate for embryo development into whole plantlets within two weeks, confirming that exogenous plant growth regulators are not required at this initial developmental stage. This aligns with the proteomic findings of Punia et al. (2024), who demonstrated that cold stratification activates endogenous signaling cascades, energy metabolism, and ROS scavenging systems sufficient to drive germination without external hormonal supplementation. The resulting plantlets provided a clean, sterile, and juvenile source of root explants for subsequent callus induction and regeneration. A striking finding of this study was the marked differential responsiveness of various explant types to callus induction. Root explants showed robust callus formation under specific BAP and TDZ combinations, while leaf explants exhibited only very limited callogenic potential (only at 0.5 mg/L BAP + 1.0 mg/L TDZ), and stem explants failed to produce any callus across all treatments tested. This pattern of explant-dependent regeneration capacity is consistent with previous reports on Ferula species. Zare et al. (2020) and Bahramali et al. (2023) also reported that root explants from *F. assa-foetida* seedlings showed superior callus induction compared to leaf and stem explants. However, the poor responsiveness of stem and leaf explants observed in our study contrasts with the findings of Kumari et al. (2025), who successfully achieved somatic embryogenesis and shoot organogenesis from leaf explants of two-year-old greenhouse-grown plants using TDZ alone. This discrepancy can be explained by differences in

physiological age and explant origin. Our study utilized roots from juvenile (21-day-old) in vitro-derived plantlets, which possess highly meristematic and less differentiated cells with greater reprogramming capacity. In contrast, Kumari et al. (2025) used leaves from mature greenhouse plants with a different physiological status. The greater responsiveness of juvenile root tissues to cytokinin-induced dedifferentiation aligns with the well-established principle that juvenility enhances morphogenic competence in plant tissue culture, as reviewed by Hashemi et al. (2023). Furthermore, our observation that no callus formed on media containing BAP or TDZ alone—only in specific combinations—emphasizes the synergistic requirement of these two cytokinins for callogenesis in this species, a finding that differs from Kumari et al. (2025) where TDZ alone was sufficient for leaf explants.

4.2. Optimal hormonal regime, the pivotal role of TDZ, and regeneration competence

Among the four successful hormone combinations tested, treatment T1 (0.5 mg/L BAP + 0.5 mg/L TDZ) consistently produced the highest callus length (4.73 cm) and fresh weight (5.94 g), significantly outperforming all other treatments. Two-way ANOVA revealed that both BAP and TDZ significantly influenced callus length and fresh weight, but TDZ exhibited a substantially stronger effect compared to BAP, indicating that TDZ is the primary determinant of callus growth vigor in *F. assa-foetida* under the tested conditions. The superior efficacy of TDZ is consistent with its known properties as a non-classical phenylurea-type cytokinin. Mok et al. (1982) first demonstrated that TDZ exhibits much stronger cytokinin-like activity compared to adenine-type cytokinins such as BAP. Recent groundbreaking work by Kumari et al. (2025) using deep learning and machine learning modeling (convolutional neural networks with 87% accuracy) identified TDZ as a "key modulator" of somatic embryogenesis and shoot organogenesis in *F. assa-foetida*. Mechanistically, Wang et al. (2025) and Kumari et al. (2025) explained that TDZ promotes cell division and meristematic reprogramming by activating strong cytokinin signaling pathways through Arabidopsis Response Regulators (ARR-A and ARR-B). The dose-response analysis revealed a non-linear (U-shaped or J-shaped) relationship for TDZ: 0.5 mg/L produced the highest values across most parameters, 1.0 mg/L caused a sharp decline (fresh weight dropping to 1.14 g), and 1.5 mg/L resulted in partial recovery. This non-linear pattern suggests a complex regulatory mechanism where high concentrations of TDZ may induce cytotoxic effects or trigger feedback inhibition of cytokinin signaling pathways. For BAP, both concentrations (0.5 and 1.5 mg/L) produced comparable dimensional growth, but the lower concentration yielded significantly higher fresh weight (3.54 g vs. 3.05 g), indicating that BAP primarily influences biomass accumulation rather than dimensional expansion. A critical finding of this study was that only calli derived from T1 (0.5 mg/L BAP + 0.5 mg/L TDZ) successfully regenerated whole plantlets. Calli from T2, T3, and T4 produced no shoots or abnormal structures even after 8 weeks of culture. This demonstrates that the combination of lower concentrations of both cytokinins is not only optimal for callus proliferation but is also essential for maintaining organogenic competence. The inability of calli from higher cytokinin treatments to regenerate suggests that supraoptimal cytokinin concentrations may induce sustained undifferentiated proliferation at the expense of shoot differentiation, a phenomenon previously described by Su et al. (2011) and Xu et al. (2024) in other plant species.

4.3. Vitrification recovery, acclimatization, and conservation-commercial implications

Regenerated plantlets initially cultured on ½ MS medium exhibited severe vitrification (hyperhydricity), characterized by translucent, brittle leaves and stems. This physiological disorder is commonly associated with high ammonium and total nitrogen concentrations in MS medium, which can cause oxidative stress, abnormal water accumulation, and reduced lignin deposition, as reviewed by Hashemi et al. (2023). Transferring vitrified plantlets to ½ QL (Quoirin and Lepoivre) medium, which has a substantially lower nitrogen content (approximately 60% less than MS), resulted in complete recovery within 2–3 weeks. This finding is particularly important for Ferula species, as it demonstrates that nitrogen reduction is critical for producing morphologically normal plantlets. Subsequent rooting on ½ QL medium produced a robust root system, and more than 85% of plantlets survived acclimatization and were successfully established under greenhouse conditions. This survival rate is comparable to recent reports on asafoetida micropropagation. Roozbeh et al. (2021) reported 80–90% acclimatization success for F. assa-foetida plantlets derived from stem explants. Similarly, Zare et al. (2020) achieved 75–85% survival for tissue-cultured plantlets of the same species. The findings of the present study offer a practical and efficient solution for large-scale micropropagation of this endangered medicinal plant. Using only one centimeter of root derived from a seed-embryo plantlet, approximately 30 healthy plantlets can be produced through the protocol developed here (0.5 mg/L BAP + 0.5 mg/L TDZ for callus induction and regeneration, followed by recovery on ½ QL medium). This multiplication rate is highly favorable compared to conventional propagation methods, which are severely limited by seed dormancy and slow seedling growth, as emphasized by Kumar et al. (2025) and Punia et al. (2024). The successful acclimatization of regenerated plantlets with over 85% survival under greenhouse conditions confirms the practical applicability of this protocol for restoration programs. Hemavathi et al. (2025) recently highlighted that plant tissue culture methods have gained industrial importance in rapid propagation, plant resource conservation, and secondary metabolite production. Several recent studies have confirmed that plant tissue culture is an emerging viable technology to protect biodiversity in the face of biodiversity loss (Hashemi et al., 2023). Future research should focus on: (1) evaluating the genetic fidelity of micropropagated plants using molecular markers such as ISSR or SCoT to ensure clonal uniformity, as recommended by Roozbeh et al. (2021); (2) optimizing TDZ and BAP combinations for direct shoot organogenesis without an intervening callus phase; (3) exploring the integration of machine learning approaches, as demonstrated by Kumari et al. (2025), to further refine PGR predictions; and (4) assessing the field performance and oleo-gum-resin yield of tissue-culture-derived plants compared to wild populations. In conclusion, the protocol established in this study provides a foundation not only for the conservation of *F. assa-foetida* but also for its sustainable commercial cultivation, reducing pressure on remaining wild populations. The use of root explants derived from seed-embryo plantlets represents a novel and efficient approach that overcomes the reproductive limitations imposed by the species' monocarpic nature and seed dormancy, offering a practical pathway for the preservation of this valuable medicinal plant for future generations.

5. Conclusion

This study developed the first efficient micropropagation protocol for *Ferula assa-foetida* using root-derived callus from seed-embryo plantlets. Embryo culture on ½ MS medium achieved > 95% germination within 5–7 days, bypassing natural seed dormancy, which under natural conditions requires 6–8 weeks of cold stratification for only 40–50% germination. Treatment T1 (0.5 mg/L BAP + 0.5 mg/L TDZ) produced the highest callus growth (4.73 cm length; 5.94 g fresh weight) and was the only treatment supporting whole plantlet regeneration, confirming that TDZ acts as the primary determinant of callus growth vigor and that lower balanced cytokinin concentrations are essential for maintaining organogenic competence. Vitrified plantlets recovered completely on ½ QL medium (lower nitrogen content, approximately 60% less than MS), with > 85% greenhouse survival, demonstrating that nitrogen reduction is critical for producing morphologically normal plantlets. Using only one centimeter of root derived from a seed-embryo plantlet, approximately 30 healthy plantlets can be produced. This protocol offers a practical solution for large-scale asexual propagation, conservation, and sustainable cultivation of this endangered monocarpic medicinal species, addressing critical limitations imposed by seed dormancy and overexploitation. The findings also highlight the synergistic requirement of BAP and TDZ for callogenesis in this species, the superiority of juvenile root explants over leaf and stem explants, and the importance of nitrogen reduction for successful acclimatization, providing a foundation for future research on genetic fidelity assessment, direct shoot organogenesis, and field performance evaluation of tissue-culture-derived plants.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Ethics Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent

For this type of study, informed consent is not required.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contribution

Conceptualization and formal analysis were performed by N. Meskin. Methodology was developed by M. Rasouli Alamouti. The first draft of the manuscript was written by A. Tavili. Stattistical analyses was done by M.A. Zare Chahouki. Supervision of the project was carried out by A. Tavili and V. Karimian. All authors contributed to the review, editing, and revision of the manuscript. All authors read and approved the final manuscript.

Data Availability:

The datasets generated and/or analyzed during the current study are included in this published article and its supplementary information files.

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Figures

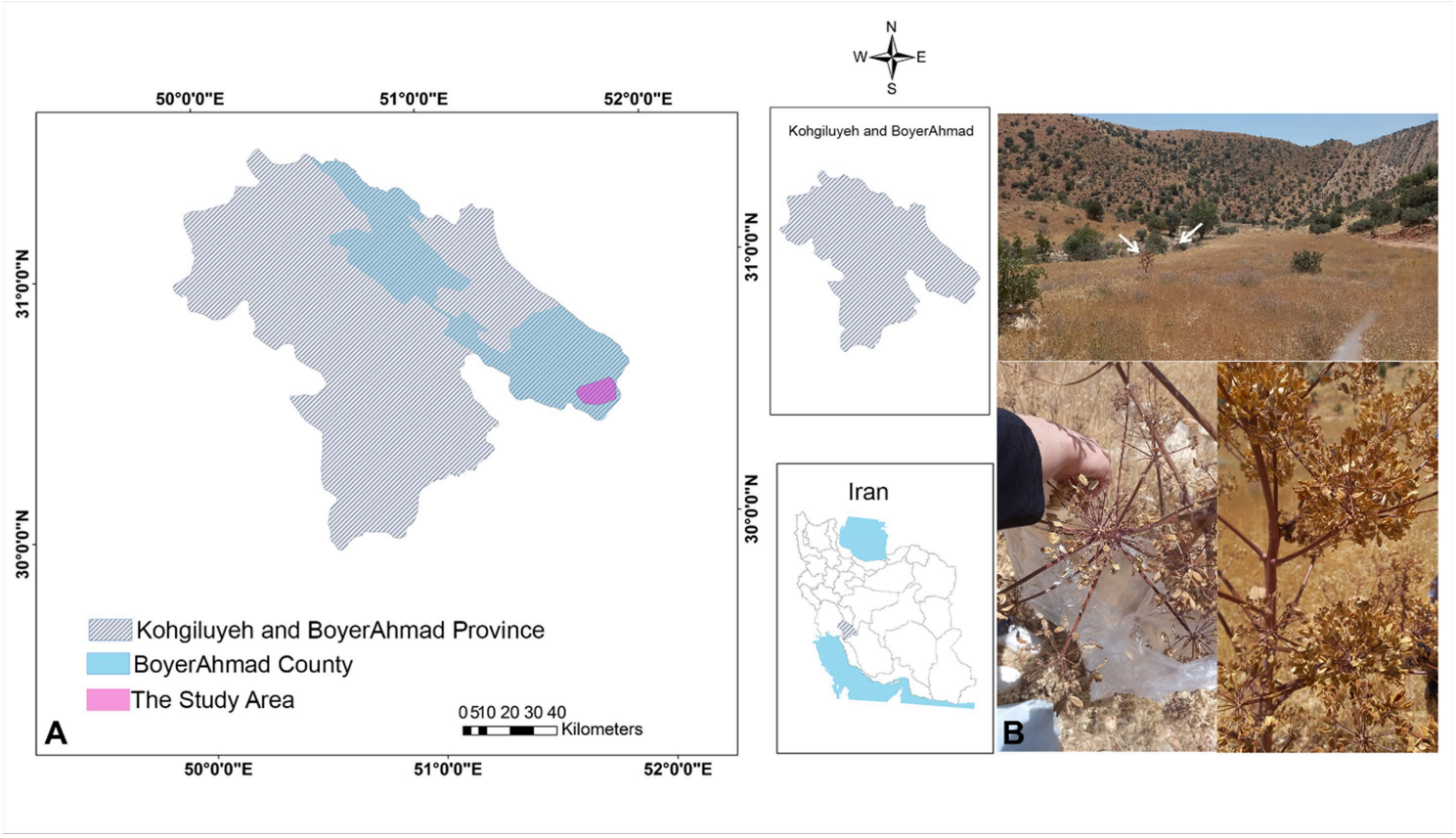

Figure 1

Geographic location of the study area of Tang-e-Sorkh in Boyer-Ahmad county, Kohgiluyeh and Boyer-Ahmad Province, Iran A and field observation and sampling of F. assa-foetida in natural habitat B

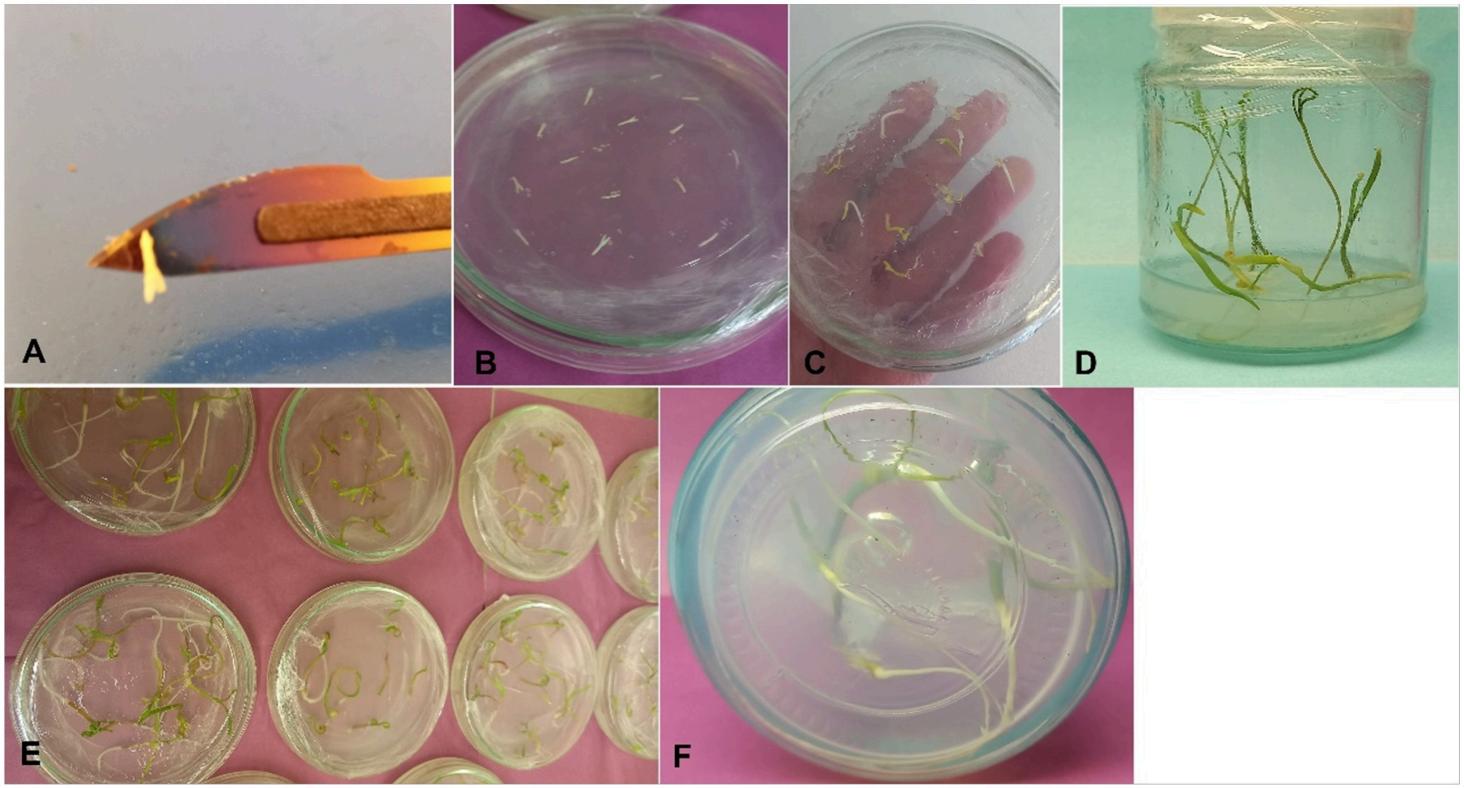

Figure 2

Different stages of plantlet and explant production from seed embryos of F. assa-foetida A Excised embryo before culture, B Embryo culture on hormone-free ½ MS medium, C Greening of seed embryos after a few days D, E Production of leaf and stem explants, F Root explant. All explants were derived from 14-day-old in vitro plantlets and used for subsequent callus induction experiments

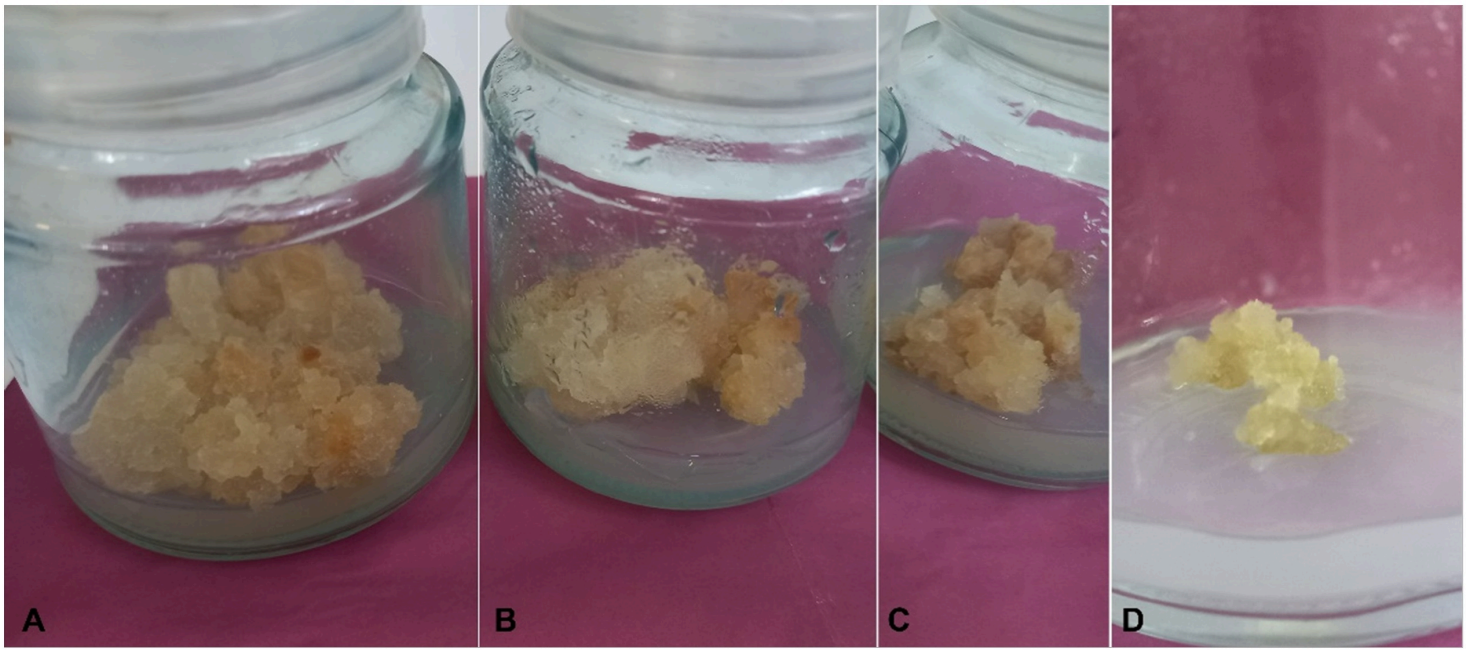

Figure 3

Callus induction from embryo-derived root explants of F. assa-foetida on MS medium supplemented with different concentrations of BAP and TDZ A 0.5 mg/L BAP + 0.5 mg/L TDZ, B 1.5 mg/L BAP + 0.5 mg/L TDZ, C 0.5 mg/L BAP + 1 mg/L TDZ, D 1.5 mg/L BAP + 1.5 mg/L TDZ

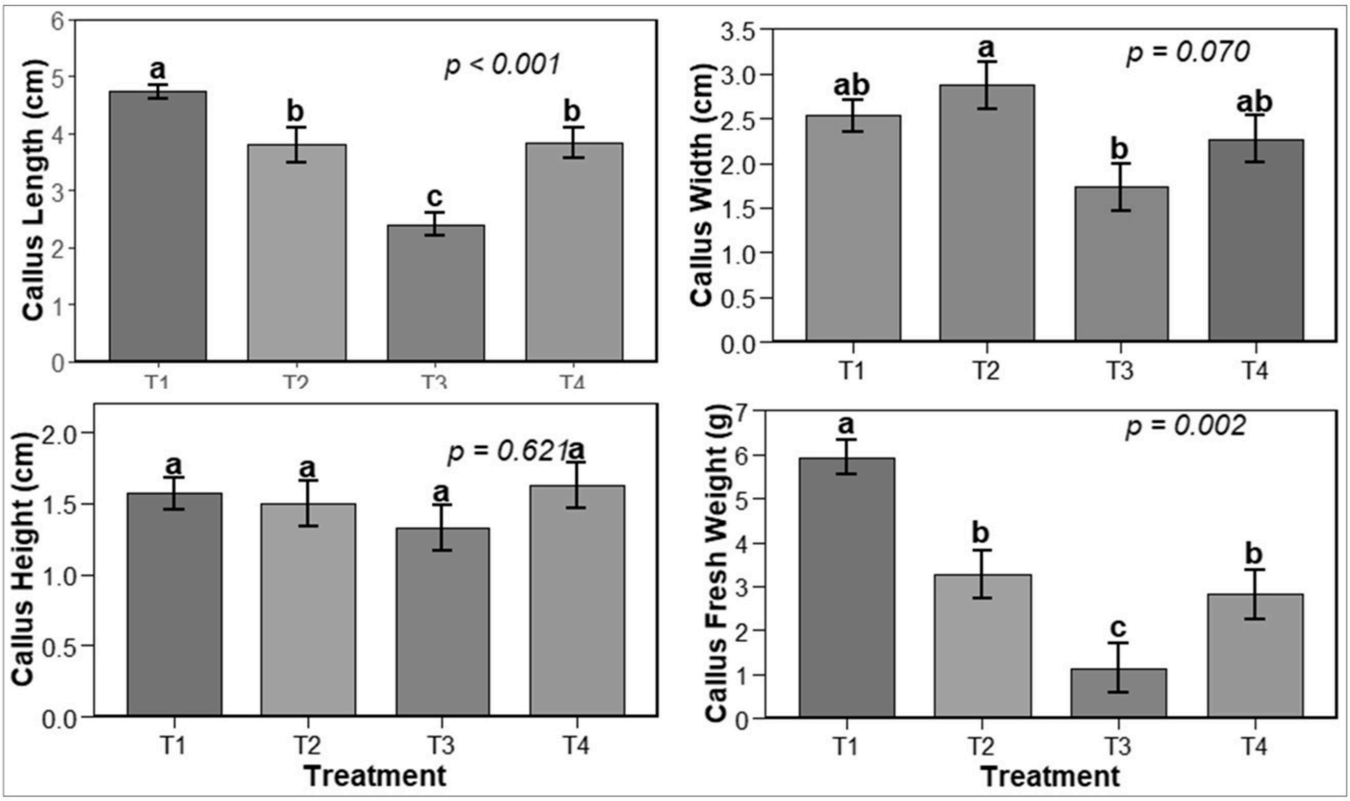

Figure 4

Growth parameters of callus derived from root explants of *F. assa-foetida* cultured on MS medium supplemented with different combinations of BAP (mg/L) and TDZ (mg/L) after four weeks of culture. T1: BAP 0.5 + TDZ 0.5 mg/L, T2: BAP 1.5 + TDZ 0.5 mg/L, T3: BAP 0.5 + TDZ 1.0 mg/L, T4: BAP 1.5 + TDZ 1.5 mg/L

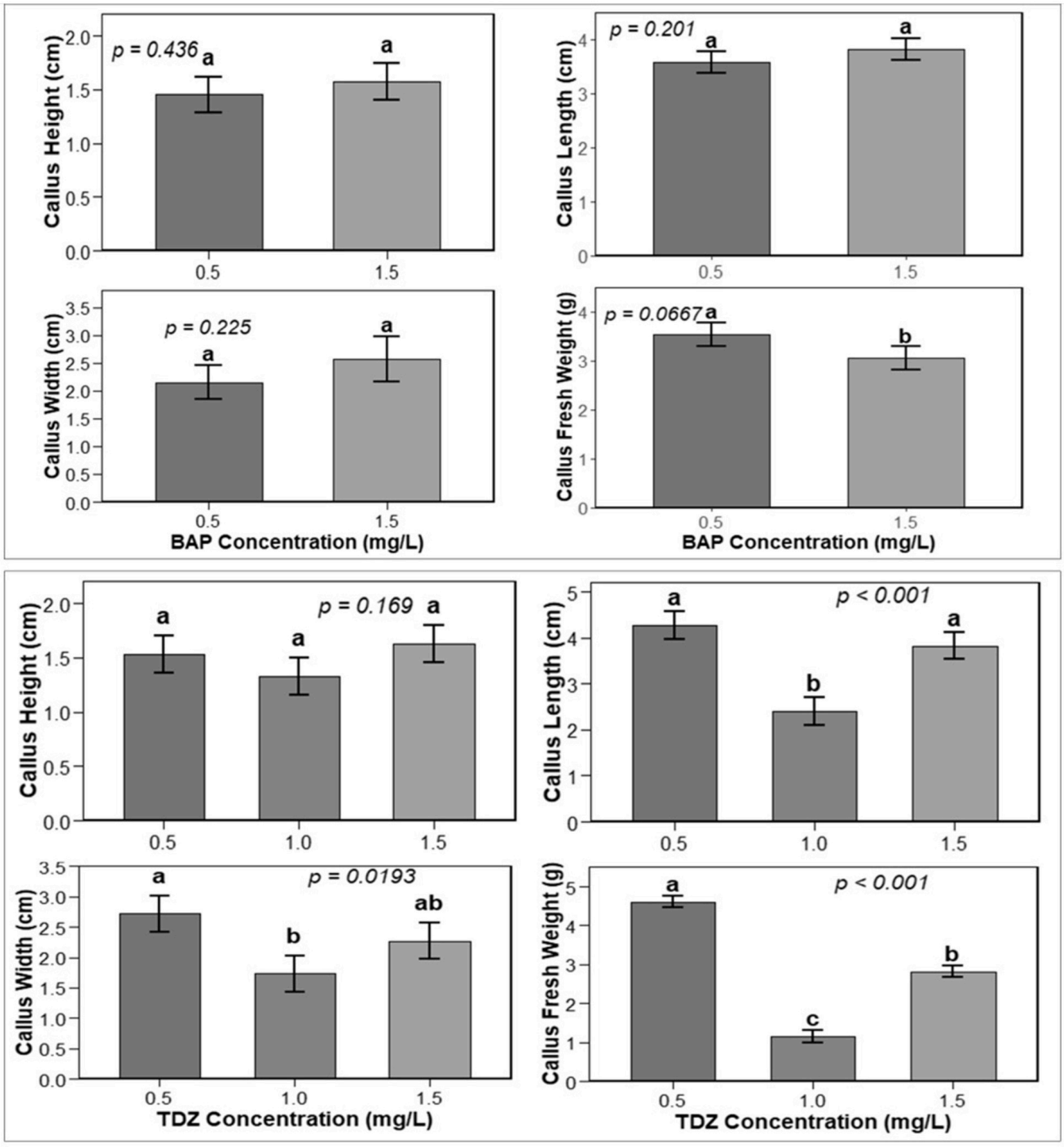

Figure 5

Callus characteristics (length, width, height and fresh weight) under different concentrations of TDZ and BAP in *F. assa-foetida*

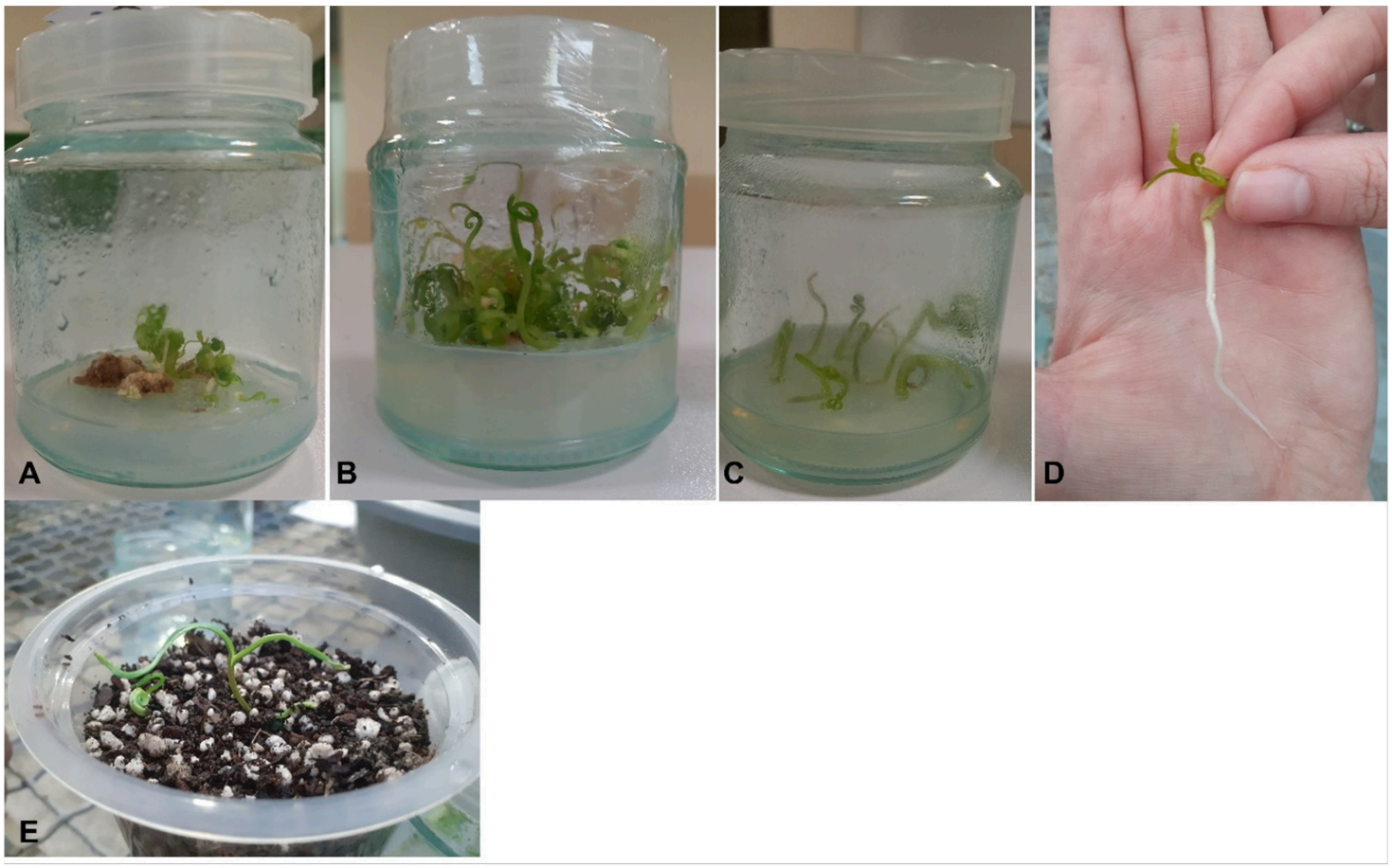

Figure 6

Regeneration of *F. assa-foetida* from root-derived callus obtained from seed embryos: Plant regeneration through callus derived from seed embryo roots (0.5 mg/L BAP and 0.5 mg/L TDZ) A Vitrification of regenerated plantlets on ½ MS medium caused by high nitrogen concentration B Healthy growth of plantlets following transfer to ½ QL medium with D Acclimatized plantlets were established in sterilized soil E Acclimatized plantlets successfully transferred to greenhouse conditions