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RESEARCH ARTICLES

Phytohormonal Regulation of In Vitro Micropropagation and Organogenesis in the Ethiopian Endemic Medicinal Shrub Weynageft (*Inula confertiflora* A. Rich.)

Desalegn Abebe Mekonen*[1,2], Hulubanch Tadele Kassa[1], Derejaw Demelash Eshete[1], Gashaw Belay Alemu[1], Leweye Getie Temesgen[5], Asmare Dejen Demeke[3], Haimanot Reta Terefe[5], Alemayehu Kefalew Shembo[5], Aragew Fetene Endalew[1], and Yikeber Getachew Dagnaw[4]

[1] Amhara Agricultural Research Institute (ARARI), Agricultural Biotechnology Research Division, Bahir Dar, Ethiopia
[2] Faculty of Agriculture, Department of Crop, Horticulture, and Soil, Egerton University, Kenya
[3] Amhara Agricultural Research Institute (ARARI), Bahir Dar, Ethiopia
[4] Amhara Agricultural Research Institute (ARARI), Crop Research, Bahir Dar, Ethiopia
[5] College of Natural and Computational Sciences, Debre Markos University, Ethiopia

*Corresponding author; email: desu.abebe33@gmail.com

Abstract

Weynageft (*Inula confertiflora* A. Rich.) is a medicinal shrub endemic to Ethiopia and is known for its antiviral, antibacterial, and anticancer properties. However, the species is threatened by overutilization and the lack of cultivation efforts. To address this challenge, we established and refined an in vitro micropropagation protocol to enable large-scale multiplication and preservation. We sterilized excised nodal explants with 1% sodium hypochlorite supplemented with five drops of Tween-20 for 10 min, then rinsed them with sterile distilled water. Sterile nodal explants were initiated on MS medium plus 1 mg/L gibberellic acid (GA_3), resulting in a 60% aseptic culture establishment and an 80% shoot response. We evaluated 10 cytokinin treatments and a hormone-free control. Cytokinin treatments included 6-benzylaminopurine (BAP; four concentrations), kinetin (Kin; three concentrations), and thidiazuron (TDZ; three concentrations) to promote shoot proliferation. For root induction, we evaluated various auxin hormones, including 10 treatments plus a hormone-free control. The auxins used for root induction were indole-3-butyric acid (IBA; four concentrations), indole-3-acetic acid (IAA; three concentrations), and naphthalene acetic acid (NAA; three concentrations). The highest shoot multiplication rate, averaging 12.55 ± 0.4 shoots per explant, was obtained with 1 mg/L kinetin, demonstrating a significant advantage over BAP and TDZ treatments. Application of 2 mg/L IAA resulted in a 100% rooting response, with an average of 6.2 ± 0.11 roots per plantlet and a mean root length of 4.38 ± 0.01 cm. Acclimatized plantlets survived completely under ex vitro conditions. These findings provide a potentially effective approach for the conservation and large-scale propagation of *I. confertiflora*, supporting future research on its pharmaceutical potential.

Keywords: in vitro propagation, *Inula confertiflora,* medicinal plant, proliferation, root induction, shoot tips

Introduction

Medicinal plants have been integral to traditional healthcare systems worldwide for

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thousands of years, with approximately 20% of pharmaceutical drugs formulated from plants (Fabricant & Farnsworth, 2001; Woldemariam et al., 2021). The genus *Inula* belongs to *the Asteraceae* family and comprises over 100 species, several of which possess significant anti-inflammatory, antimicrobial, and antiviral activities (Das et al., 2020).

Inula confertiflora A. Rich, commonly recognized as Weynageft, it is an endemic Ethiopian shrub with substantial ethnopharmacological importance. It is one of over 800 medicinal plant species that are crucial to Ethiopia's traditional healthcare system (Teklehaymanot, 2009; Woldemariam et al., 2021). This species is widely used to treat livestock disorders, human skin ailments and viral infections (Asfaw et al., 2022; Mekonen et al., 2026; Teshome & Yohannes, 2025). Studies have shown that methanolic leaf extracts of *I. confertiflora* possess in vitro antiviral activity against herpes simplex virus 1 (HSV 1) and coxsackievirus B3 (CVB3) (Gebre-Mariam et al., 2006; Woldemariam et al., 2021). Furthermore, *I. confertiflora* contains multiple bioactive compounds. Notably, the species contains two uncommon triterpenoids, epifriedelanol and dammara-20,24-dien-3-yl acetate, as well as sesquiterpenoid exhibit antibacterial, anticancer, anti-inflammatory properties, and antioxidant activities (Birhan et al., 2024; Gashu, 2022; Mekonen et al., 2026). These findings highlight the potential of this species as a source of novel pharmaceutical compounds.

Despite its promising therapeutic potential, *I. confertiflora* faces considerable threats from habitat loss, overharvesting, agricultural expansion, and climate change, driving the species toward regional extinction (Kalu & Seid, 2015; Yimam et al., 2022). Furthermore, this species shows limited seed germination and lacks established propagation techniques (Asfaw et al., 2022). Tissue culture offers a highly efficient method for the swift mass propagation and preservation of rare medicinal plants. Micropropagation protocols have been established for various *Inula* species, including *I. germanica* and *I. royleana* (Custódio et al., 2024; Kapoor, 2018; Kaur et al., 2010; Manchanda & Garg, 2011; Trejgell et al., 2018). A specific protocol for *I. confertiflora* is currently unavailable.

This study aimed to develop and refine the first micropropagation protocol for *I. confertiflora* using nodal-explant. We assessed the effects of three cytokinins (BAP, Kin, and TDZ) the impact of three cytokinins (BAP, kin, and TDZ) on shoot proliferation, as well as three auxins (IBA, IAA, and NAA) on root induction. This study assessed the potential of specific phytohormone treatments to enhance the efficiency of shoot and root regeneration for successful ex vitro acclimatization. Our goal was to establish an effective in vitro propagation technique to support dissemination and conservation initiatives, while also paving the way for future exploration of phytochemistry and pharmaceuticals.

Materials and Methods

Plant Material and Explant Preparation

Healthy and vigorous shoots of weynageft (*Inula confertiflora* A. Rich.) were selected as explant sources for initiating in vitro cultures. Segments of internodes and shoot tips, each ranging from 1.5 to 2.0 cm in length, were precisely excised from six-month-old mother plants that were cultivated under greenhouse conditions at the Amhara Agricultural Research Institute (ARARI) plant tissue culture laboratory in Bahir Dar, during the 2023-2024 growing period.

Due to the limited availability of the mother plants and the sensitivity of *I. confertiflora* to chemical sterilants. Explant sterilization involved rinsing the nodal and shoot tip explants under a stream of running tap water with a few drops of liquid detergent for 10 min. Based on preliminary trials, a surface-sterilization protocol was established. An initial treatment with 2% V/V NaClO for 10 min achieved 80% decontamination but resulted in 100% explant mortality. Consequently, the concentration was reduced to 1% (v/v) NaClO supplemented with five drops of Tween 20 for 10 min. Subsequently,

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the explants were rinsed five times in sterile distilled water under aseptic conditions.

Culture Medium Preparation and Establishment of Aseptic Culture

Sterilized explants were cultured on Murashige and Skoog (MS) basal medium Murashige and Skoog (1962) supplemented with 1 mg/L GA_3, 30 g/L sucrose, 100 mg/L inositol, and 1.2 g/L gelzan (gellan gum). The pH was adjusted to 5.6 before adding the gelling agent, followed by autoclaving at 121 °C for 15 min. Approximately 40 ml of the medium was dispensed into 7×10 cm culture vessels. The specific Gelzan concentration was selected based on institutional experience with sensitive in vitro cultures at the ARARI laboratory, where the standard concentration of 2.0 g/L was frequently observed to induce hyperhydricity. This lower concentration was adopted to ensure optimal physical properties of the medium and phytohormone diffusion, a practice supported by established protocols for other species (Magyar-Tábori et al., 2021).

Experimental Design

Shoot and root regeneration experiments were conducted using a completely randomized design (CRD). Each treatment consisted of three replications, with five independent culture vessels per replication, each containing a single explant, resulting in total of 15 vessels per treatment. Shoot tips (1.5-2.0 cm in length) harvested from the in vitro initiation stage were used as explants because of their high meristematic activity and cell-division capacity.

Shoot Regeneration

Shoot tips generated during the initiation stage were cultured on MS basal medium supplemented with different cytokinins: 6-benzylaminopurine (BAP, four levels) 1, 2, 3, and 4 mg/L; kinetin (kin, three levels) 1, 2, and 3 mg/L; thidiazuron (TDZ, three levels) 1, 2, and 3 mg/L; and hormone-free MS medium as a control. All media contained 30 g/L sucrose, 0.1 g/L inositol, and 1.2 g/L Gelzan. The pH was set to 5.6, the medium was boiled to dissolve the gelling agent, and it was sterilized at 121°C for 15 min. The cultures were maintained under the same environmental conditions outlined in the initiation stage.

Root Induction

To induce root formation, shoot tips regenerated from the initiation stage were cultured on MS medium formulated with various auxins: indole-3-butyric acid (IBA), four concentrations of 0.5, 1, 2, and 3 mg/L; indole-3-acetic acid (IAA), three concentrations of 1, 2, and 3 mg/L; and α-naphthaleneacetic acid (NAA), three concentrations of 1, 2, and 3 mg/L). Hormone-free Murashige and Skoog (MS) medium was used as the control. The medium formulation remained consistent throughout each experimental phase. The cultures were maintained under the same temperature and photoperiod conditions.

Ex Vitro Acclimatization

The acclimatization process was conducted in an acclimatization box (0.6 × 1.8 m) containing a sterilized sand-and-red-ash substrate at a 2:1 ratio. The substrate was autoclaved in a steam sterilizer at 121°C for 1.5 hr and pretreated with a systemic fungicide (Redomel) 24 hours before planting. One hundred well-rooted plantlets were carefully removed from the culture vessels and rinsed with a 0.1% (v/v) sodium hypochlorite solution to remove residual media, then rinsed with tap water. The plantlets were then transplanted into a sterile substrate and covered with moist cheesecloth for 20 days to maintain humidity that facilitates gradual hardening and adaptation to the external environment.

Data Collection

The data were recorded at 60 days for shoot multiplication, 40 days for rooting, and 30 days for ex vitro acclimatization. Each replication began

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with five independent vessels, each containing one explant, providing a sufficient buffer against potential in vitro losses such as contamination. To maintain a balanced experimental design and ensure equal representation across treatments, we randomly sampled three contamination-free plants from each replication for final data collection, totaling nine independent units per treatment. For statistical analysis, we used the mean of these independent experimental units within each replication as the experimental unit in the ANOVA (n = 3).

During the shoot multiplication stage, we recorded shoot number (SN), shoot length (SL, cm), node number (NN), and leaf number (LN). Additionally, we recorded callus response percentage (CR) and root response percentage (RR) as proportional data. The following rooting stage traits were evaluated: root number (RN), shoot length (SL), root length (RL), leaf number (LN), callus response percentage (CR), and root response percentage (RR). Finally, we evaluated the survival percentage during acclimatization.

Statistical Analysis

Statistical analyses were conducted using R software (version 4.4.1 (R Core Team, 2024). To satisfy the assumption of parametric analysis, percentage data for callus response and root response in shoot proliferation and rooting experiments were subjected to an arcsine square-root transformation before analysis. The remaining quantitative traits for shoot proliferation and rooting were analyzed directly. All experimental data were checked for normality using the Shapiro-Wilk test and for homogeneity of variances using Bartlett's test (Bartlett, 1937; Shapiro & Wilk, 1965). A one-way analysis of variance (ANOVA) was performed using the R agricola and dplyr packages (Wickham et al., 2023). Where a significant effect was detected, mean comparisons were carried out using Tukey's honestly significant difference (HSD) test at a significance level of $P < 0.05$. Data visualization was conducted using the Lattice, ggplot2, and cowplot packages (de Mendiburu, 2023; Sarkar, 2008; Wickham et al., 2023; Wilke, 2015).

Results and Discussion

Results

Establishment of Aseptic Cultures

Aseptic cultures were established by balancing decontamination efficiency with tissue survival. While the 2% NaClO initial trial failed due to total phytotoxicity. As a result, we tested a single treatment with 1% NaClO, which successfully yielded a 60% aseptic clean culture establishment rate, with 20% contamination, and 20% mortality rate, as illustrated in Figure 5a-c. By using both shoot tips and nodal segments during this critical phase, we ensured sufficient clean cultures were established despite the inherent risk of contamination and sterilant stress. These established cultures provided uniform shoot tip explants required for the multiplication and rooting experiments.

Shoot Multiplication Response to Cytokinins

Cytokinin application significantly influenced all growth parameters ($P < 0.001$), as shown in the ANOVA Table 1). Highly significant differences were observed across all assessed traits, including shoot number (SN), shoot length (SL), node number (NN), leaf number (LN), arcsine square root transformed callus response (CR-arc), and root response percentage (RR-arc) (Table 1).

Supplementary boxplots (Figure S1 and S2) illustrate the primary morphogenic responses and provide a comprehensive view of the underlying data distribution. In the box plots, red and white diamonds represent the mean values (Consistent with Table 2), and individual points denote the observed data for each experimental unit. The 1 mg/L kin treatment showed a relatively greater spread of observed values, reflecting a robust but naturally variable proliferative response. In contrast, the observed points for the BAP and TDZ treatments were tightly clustered around their lower mean values, indicating a highly uniform but restricted growth response.

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Table 1

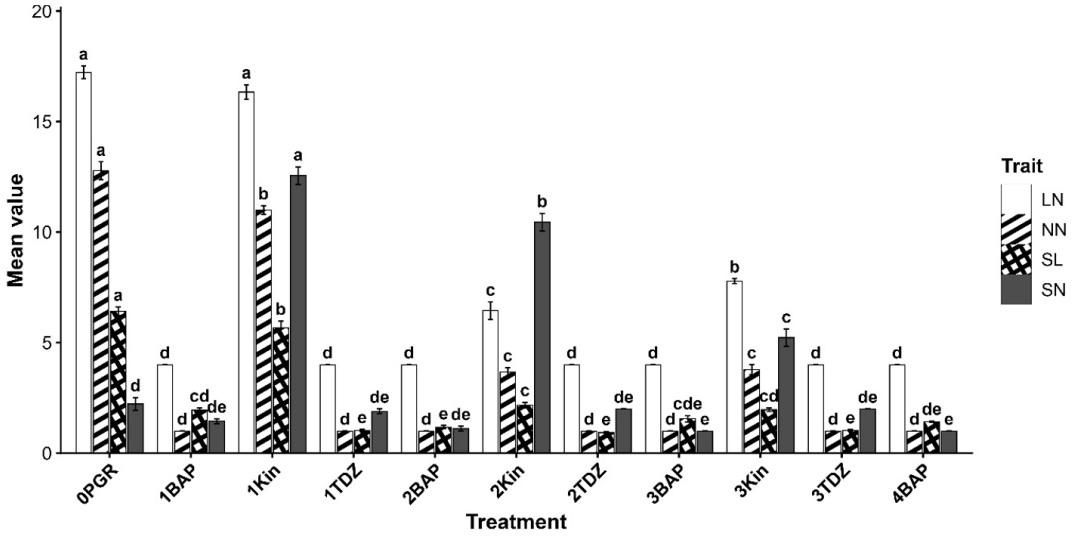
Analysis of Variance (Mean Squares) for Shoot Multiplication of Inula confertiflora Cultured In Vitro

		Mean square					
Source of variation	df	SN	SL	NN	LN	CR_arc	RR_arc
Treatments	10	49.15***	10.87***	55.85***	76.62***	0.70***	0.52***
Residuals	22	0.16	0.05	0.08	0.10	0.04	0.02
F value	-	298.94	201.09	722.37	760.83	18.77	23.24
P value	-	$P \leq 0.001$	$P \leq 0.001$	$P \leq 0.001$	$P \leq 0.001$	$P \leq 0.001$	$P \leq 0.001$

Notes. SN = shoot number; SL = shoot length; NN = node number; LN = leaf number; CR_arc = arcsine square-root-transformed callus response; and RR_arc = arcsine square-root-transformed root response. Values are mean squares obtained from one-way ANOVA. *** significant at $P \leq 0.001$.

Figure 1

Influence of Cytokinin Treatments on Shoot Multiplication and Growth Response of Inula confertiflora Cultured In Vitro



Notes. Bar plots represent mean ± standard error (SE) for SN = shoot number; SL = shoot length; NN = node number; and LN = leaf number. Means followed by different letters within each trait are significantly different according to Tukey's honestly significant difference (HSD) test at $P \leq 0.05$.

Comparative Effectiveness of Cytokinins on Shoot Proliferation

Among the evaluated cytokinins, 1 mg/L Kin demonstrated the highest efficacy for shoot proliferation, yielding a maximum mean of 12.55 ± 0.4 shoots per explant (Figure 5f). According to Tukey's honestly significance difference (HSD) test (Figures 1 and Figure 2), this treatment produced a significantly higher mean for SN value (12.55 ± 0.4) (labelled 'a' in Table 1) and for other traits, demonstrating the highest mean values observed in cytokinin concentration and type. This represents a 5.6-fold increase in shoot number compared to the hormone-free control (2.22 ± 0.29 shoots and 11.3-fold increase over 2 mg/L BAP (1.11 ± 0.11 shoots). This difference is clearly illustrated in Figure 5d-e. The 1 mg/L kin treatment resulted in a 6.3-fold increase in shoot number compared with the 2 and 3 mg/L TDZ treatments (1.89 ± 0.11 shoots).

While the hormone-free medium (0PGR)

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produced the highest mean values for shoot elongation (6.41 ± 0.2a cm) and node number (12.78 ± 0.4a), the application of 1 mg/L Kin followed closely as the second-best treatment for this trait (labelled 'b'). Notably, both 0PGR and 1 mg/L Kin were statistically comparable in terms of leaf number (LN), sharing the 'a' significance group (Figure 1; Table 2). In contrast, all concentrations of BAP and TDZ resulted in significantly lower growth across all four parameters, with means consistently falling into the 'd' and 'e' statistical groups (Figure 1; Table 2).

Morphologically, the 1 mg/L Kin treatment promoted direct organogenesis. Although the primary explants showed a basal callus response of 55.5% (CR-arc = 0.84), regenerated shoots emerged directly from the nodal shoot tips. These explants proliferated multiple shoots and developed callus at the base of the plantlet attachment points. In contrast, no callus formation was observed in the hormone-free treatment (Figures 5f and g). Conversely, treatments with BAP and TDZ produced an undifferentiated, compact green mass of cells (CR-arc = 1.57) that failed to differentiate into shoots during the culture period, as clearly shown by 3 mg/L TDZ (Figures 2 & 5d) and 3 mg/L BAP (Figure 5e). This distinction highlights the effectiveness of 1 mg/L Kin in achieving clean, direct shoot regeneration with minimal callus formation.

Effect of Auxin on Root Induction

Both auxin type and concentration significantly influenced root induction and subsequent plantlet development ($P < 0.001$). These effects were observed for root number (RN), root length (RL), leaf number (LN), and shoot length. The root response (RR-arc) also differed significantly across treatments ($P < 0.05$) (Table 3).

Root morphogenic responses are depicted in Tables 3 and 4. Boxplots (Figure S3 and Figure 4) illustrate data distribution. The white diamond in the box plot indicates the mean value, and individual points denote the observed data points of the experimental unit. This visualization showed that the 2 mg/L IAA treatment exhibited the greatest spread of observed values, while responses were relatively consistent across the other treatments.

Table 2

Effect of Cytokinin on Shoot Proliferation

Treatment	Mean ± SE					
	SN	SL	NN	LN	CR_arc	RR_arc
0PGR	2.22 ± 0.29 d	6.41 ± 0.20 a	12.78 ± 0.40 a	17.22 ± 0.29 a	0.00 ± 0.00 c	1.37 ± 0.20 a
1BAP	1.44 ± 0.10 de	1.94 ± 0.10 cd	1.00 ± 0.00 d	4.00 ± 0.00 d	1.57 ± 0.00 a	0.00 ± 0.00 b
1Kin	12.55 ± 0.40 a	5.68 ± 0.30 b	11.00 ± 0.19 b	16.33 ± 0.33 a	0.84 ± 0.12 b	0.41 ± 0.20 b
1TDZ	1.89 ± 0.11 de	1.02 ± 0.05 e	1.00 ± 0.00 d	4.00 ± 0.00 d	1.57 ± 0.00 a	0.00 ± 0.00 b
2BAP	1.11 ± 0.11 de	1.18 ± 0.07 e	1.00 ± 0.00 d	4.00 ± 0.00 d	1.57 ± 0.00 a	0.00 ± 0.00 b
2Kin	10.45 ± 0.40 b	2.17 ± 0.14 c	3.67 ± 0.19 c	6.45 ± 0.40 c	1.37 ± 0.20 ab	0.00 ± 0.00 b
2TDZ	2.00 ± 0.00 de	0.93 ± 0.05 e	1.00 ± 0.00 d	4.00 ± 0.00 d	1.57 ± 0.00 a	0.00 ± 0.00 b
3BAP	1.00 ± 0.00 e	1.56 ± 0.14 cde	1.00 ± 0.00 d	4.00 ± 0.00 d	1.57 ± 0.00 a	0.00 ± 0.00 b
3Kin	5.22 ± 0.40 c	1.97 ± 0.08 cd	3.78 ± 0.22 c	7.78 ± 0.11 b	1.37 ± 0.20 ab	0.00 ± 0.00 b
3TDZ	2.00 ± 0.00 de	1.03 ± 0.03 e	1.00 ± 0.00 d	4.00 ± 0.00 d	1.57 ± 0.00 a	0.00 ± 0.00 b
4BAP	1.00 ± 0.00 e	1.42 ± 0.03 de	1.00 ± 0.00 d	4.00 ± 0.00 d	1.37 ± 0.20 ab	0.00 ± 0.00 b

Notes: SN = shoot number; SL = shoot length; NN = node number; LN = leaf number; CR_arc = arcsine square root transformed callus response; and RR_arc = arcsine square-root transformed root response. Values are presented as mean ± SE = means followed by different superscript letters within the same column differ significantly according to Tukey's honestly significant difference (HSD) test at $P \leq 0.05$.

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Table 3

Analysis of Variance (ANOVA) for Rooting Traits of Inula confertiflora Cultured In Vitro

Source of variation	Mean square						
	df	RN	RL	SL	LN	CR_arc	RR_arc
Treatments	10	5.11***	3.10***	2.99***	12.07***	1.72***	0.080*
Residuals	22	0.16	0.028	0.056	0.74	0.033	0.033
F value	-	31.64	109.10	53.70	16.27	52.33	2.45
P value	-	$P \leq 0.001$	$P \leq 0.001$	$P \leq 0.001$	$P \leq 0.001$	$P \leq 0.001$	$P \leq 0.05$

Notes. RN = root number; SL = shoot length; RL = root length; LN = leaf number; RR_arc = arcsine square root transformed root response, and CR_arc = arcsine square-root transformed callus response. Values are mean squares from one way ANOVA. * and *** indicate significance at $P \leq 0.05$ and $P \leq 0.001$, respectively.

Table 4

Influence of Auxin Treatments on Root Induction and Rooting Performance of Inula confertiflora Cultured In Vitro

Treat-ment	Mean ± SE					
	RN	RL	SL	LN	CR_arc	RR_arc
0.5IBA	2.22 ± 0.11gh	1.52 ± 0.12de	3.89 ± 0.03e	14.11 ± 0.49ab	1.57 ± 0.00a	1.57 ± 0.00a
0PGR	4.00 ± 0.19bcd	3.91 ± 0.11a	5.88 ± 0.17ab	15.22 ± 0.62a	0.00 ± 0.00b	1.57 ± 0.00a
1IAA	3.11 ± 0.29defg	1.67 ± 0.07cd	5.30 ± 0.20bc	14.44 ± 0.11ab	0.00 ± 0.00b	1.57 ± 0.00a
1IBA	2.44 ± 0.29fgh	1.85 ± 0.10cd	4.58 ± 0.08d	13.67 ± 0.38abc	1.37 ± 0.20a	1.37 ± 0.20a
1NAA	3.78 ± 0.29be	2.10 ± 0.02bc	4.91 ± 0.17cd	11.33 ± 0.38cd	1.57 ± 0.00a	1.57 ± 0.00a
2IAA	6.22 ± 0.11a	4.38 ± 0.01a	6.24 ± 0.09a	15.22 ± 0.11a	0.00 ± 0.00b	1.57 ± 0.00a
2IBA	2.78 ± 0.22efgh	1.71 ± 0.11cd	4.64 ± 0.11cd	13.56 ± 0.56abcd	1.57 ± 0.00a	1.57 ± 0.00a
2NAA	4.44 ± 0.29bc	2.36 ± 0.10b	5.27 ± 0.18bc	12.00 ± 0.19 bcd	1.57 ± 0.00a	1.57 ± 0.00a
3IAA	4.78 ± 0.11b	1.72 ± 0.04cd	5.11 ± 0.15cd	12.44 ± 0.59bcd	0.00 ± 0.00b	1.57 ± 0.00a
3IBA	1.66 ± 0.33h	1.08 ± 0.05e	4.82 ± 0.12cd	11.11 ± 0.91de	1.05 ± 0.28a	1.05 ± 0.28a
3NAA	3.56 ± 0.11cdef	2.03 ± 0.18bc	2.51 ± 0.12f	8.67 ± 0.51e	1.57 ± 0.00a	1.57 ± 0.00a

Notes. RN = root number; SL = shoot length; RL = root length; LN = leaf number; RR_arc = arcsine-transformed root response; CR_arc = arcsine-transformed callus response. Values are expressed as mean ± SE (n = 3). Means followed by different superscript letters within a column differ significantly according to Tukey's HSD test ($P \leq 0.05$).

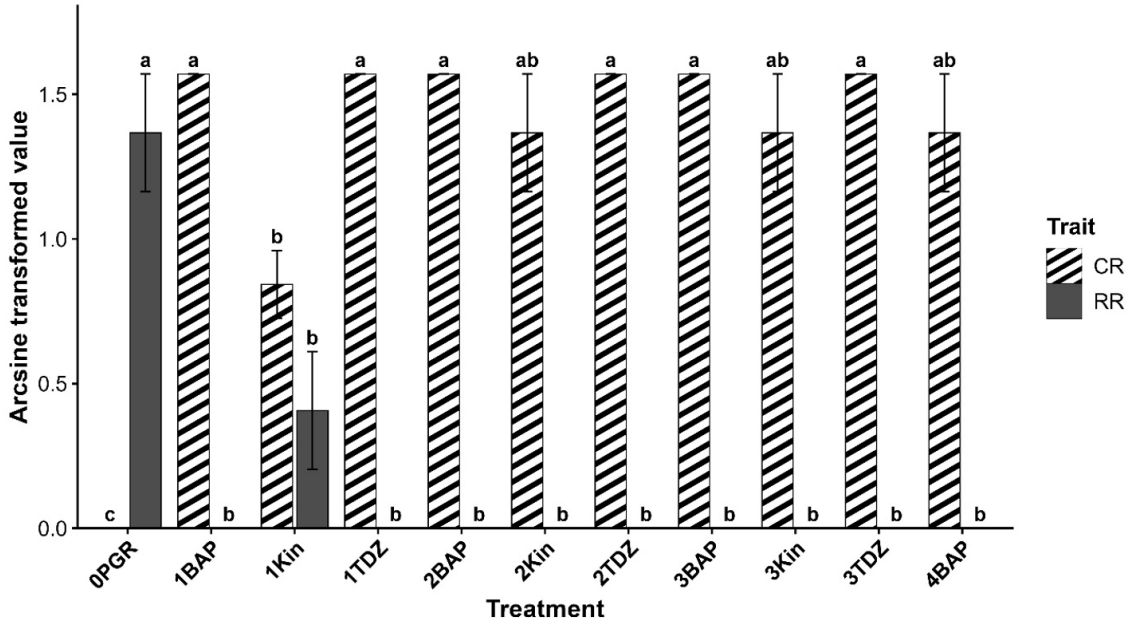
This concentration showed the highest average root number (6.22 ± 0.11), the greatest root length (4.38 ± 0.01 cm), and significant shoot elongation (6.24 ± 0.09 cm), all achieved without callus development (Figure 3 and 4; Table 4). This difference is also clearly illustrated in figure 5j. According to the HSD test (Figures 3 and 4), this treatment was significantly superior for all rooting parameters except for the callus response.

In contrast, all NAA concentrations led to 100% basal callus formation while concurrently inhibiting root and shoot development, as observed with 3 mg/L NAA (Figure 5h). Similarly, 0.5 and 2mg/L IBA resulted in significant basal callusing and growth inhibition. These results underscore the effectiveness of IAA in facilitating direct root organogenesis and producing high-quality plantlets with robust, dense root systems (Figures 5i & j).

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Figure 2

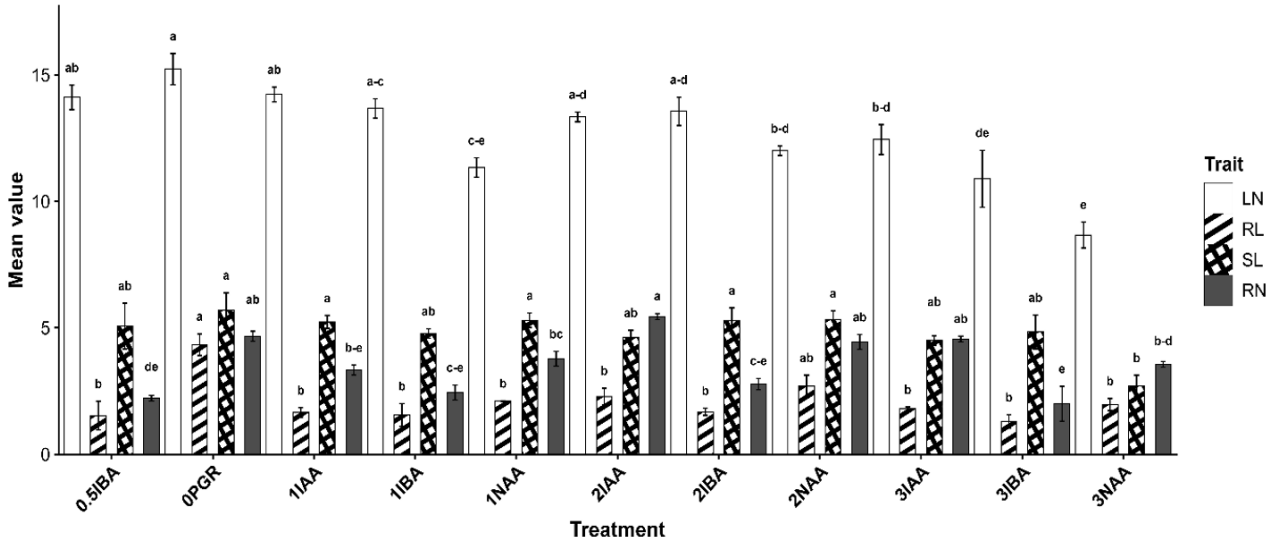
Influence of Cytokinin Treatments on Callus and Root Response of Inula confertiflora Cultured In Vitro



Notes. Bar plots represent mean ± standard error (SE) for CR_arc = arcsine square root transformed callus response and RR_arc = arcsine square root transformed root response. Means followed by different letters within each trait are significantly different according to Tukey's honestly significant difference (HSD) test at $P \leq 0.05$.

Figure 3

Influence of Auxin Treatments on In Vitro Rooting Performance and Growth of Inula confertiflora

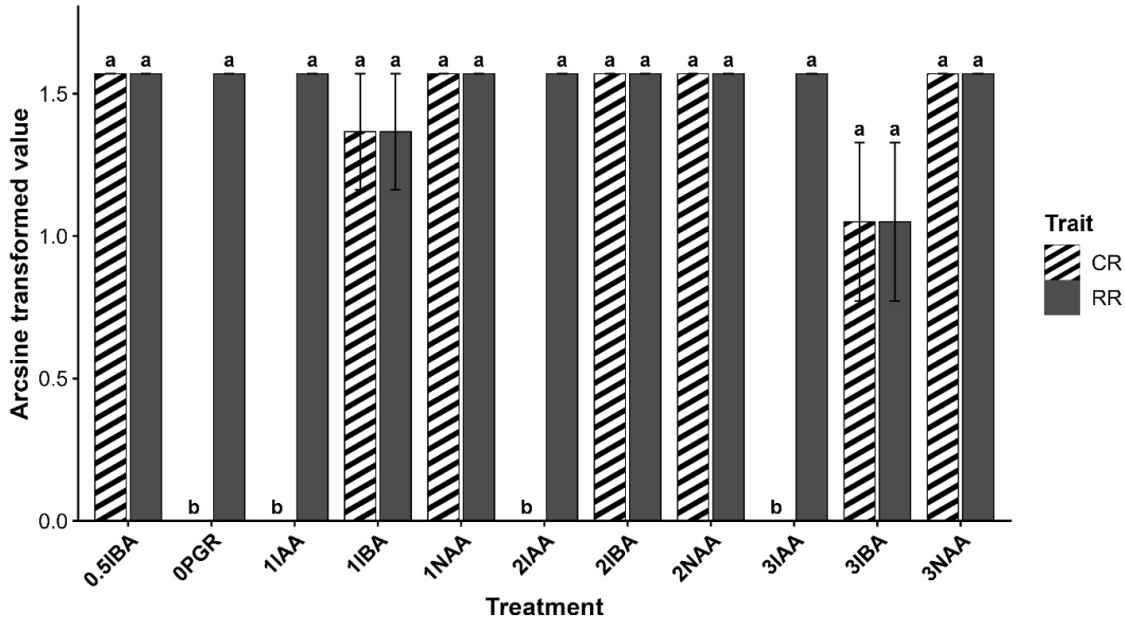


Notes. Mean values ± (SE) standard error are expressed for LN = leaf number; RL = root length; RN = root number; and SL = shoot length. Different letters above bars indicate significant difference among treatments within each trait according to Tukey's HSD test ($P \leq 0.05$).

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Figure 4

Influence of Auxin Type and Concentration on Callus Induction and Rooting Response of Inula confertiflora In Vitro



Notes. Mean values ± standard error (SE) of arcsine square-root transformed callus response (CR) and root response (RR) are shown. Different letters above bars indicate significant differences among treatments within each trait according to Tukey's HSD test ($P \leq 0.05$).

Figure 5

Micropropagation Stages: (a) Mother Plant; (b) Explant Sterilization; (c) Clean Culture; (d-g) Shoot Multiplication; (h-j) Root Induction; (k) Primary Acclimatization; (l) Seedling Ready for Dissemination



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Ex Vitro Survival and Adaptation

Acclimatization of the in vitro regenerated plantlets was highly successful. Among the plantlets subjected to hardening, survival was 100% after 30 days of acclimatization. The plantlets adapted successfully to greenhouse conditions. This demonstrates the effectiveness of the sand and red ash (2:1) substrate in supporting the recovery of in vitro-regenerated plantlets, which eventually developed into healthy, established plants (Figures 5k and l).

Discussion

In Ethiopia, approximately 80% of the human population and 90% of livestock rely on traditional medicinal systems to meet their primary healthcare needs (Woldearegay & Regassa, 2023). Notably, over 95% of these traditional remedies are plant-derived, demonstrating the significant pharmacological potential of Ethiopia's native flora (Birhan et al., 2024; Woldemariam et al., 2021). Worldwide, about 20% of pharmaceutical drugs originate from plants, indicating the critical need to safeguard ethnomedicinal knowledge and the biodiversity that underpins it.

The results of this study demonstrate an efficient in vitro propagation protocol for *Inula confertiflora* A. Rich., which has recently been taxonomically transferred to *Pentanema confertiflorum* (A. Rich.) (Englund et al., 2009; Gutiérrez-Larruscain et al., 2018). This reclassification is particularly relevant because the species is occasionally cited under different names in literature, potentially causing nomenclatural confusion. Phylogenetic evidence indicates that the traditional genus *Inula* is paraphyletic, supporting the transfer of several species, including *I. confertiflora*, to the genus *Pentanema* (Englund et al., 2009; Gutiérrez-Larruscain et al., 2018). The use of *Pentanema confertiflorum* as the revised name for *I. confertiflora* promotes nomenclatural consistency and facilitates accurate interpretation of ethnopharmacological and biotechnological research (Birhan et al., 2024; Gashu, 2022).

*Inula confertiflora (*syn*. Pentanema confertiflorum* A. Rich.*)*, commonly referred to as weynageft, is an endemic medicinal plant facing significant conservation challenges. Ethnobotanical surveys have documented its use in treating various human and livestock ailments, while its diverse repertoire of bioactive compounds, including triterpenoids and sesquiterpene lactones, which exhibit antiviral, antibacterial, and anti-inflammatory properties (Ahmed et al., 2024; Asfaw et al., 2022; Gashu, 2022). However, recent ethnobotanical assessments and field observations indicate that the species is facing increasing scarcity, often being characterized as "locally endangered" by indigenous communities and researchers (Kalu & Seid, 2015; Yimam et al., 2022). Although the species is not yet officially listed on national conservation red lists, the cumulative pressure of unsustainable harvesting, habitat degradation, and climate change underscores the urgent need for proactive measures. Establishing an effective in vitro propagation protocol is both timely and critical for conservation, and increased availability may enhance its future potential for pharmaceutical application (Birhan et al., 2024).

Clean Culture Establishment

Establishing aseptic cultures is an essential first step in micropropagation, as it introduces plant material into laboratory culture. In this study, after preliminary trials to determine the tolerance of shoot tip and nodal tissues, we adopted a single, uniform sterilization protocol using 1% sodium hypochlorite (NaClO) for the main experiment. This treatment yielded a 60% clean culture establishment, which is comparable to the results reported for *Inula crithmoides* (67% success) using more rigorous mercuric chloride treatments (Rodrigues et al., 2023). By selecting a single treatment based on our preliminary trial of 2% NaClO, we aimed to balance microbial suppression with explant viability. This specific treatment was effective under the conditions of the present study. The observed survival rates highlight the inherent challenges of surface sterilization in wild-sourced material

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and suggest that future large-scale applications should explore further refinements. This focused approach provided consistent and viable starting material for subsequent multiplication and rooting phases.

Shoot Multiplication and Cytokinin Efficacy

Shoot tips were used as the primary explant source because of their high regenerative capacity and lower risk of somaclonal variation than callus-derived tissues. Our results identified 1 mg/L kinetin (Kin) as the most effective treatment for shoot proliferation, yielding a significantly higher multiplication rate than the hormone-free control treatment. This finding aligns with previous studies on *Inula germanica* and *Inula royleana*, indicating that the combination of BAP with a low concentration of NAA/Kin effectively promotes shoot regeneration (Stojakowska & Malarz, 2004; Trejgell et al., 2018)hypocotyls and roots were isolated from ten-day-old seedlings. Explants were put on MS medium containing 1.0 mg l-1 benzylaminopurine and 0.1 mg l-1 naphthaleneacetic acid and cultured under continuous white fluorescent light (45 µmol.m-2.s-1. Remarkably, our findings with Kin contrast with those reported for *Inula crithmoides*, where BAP was identified as the most effective cytokinin for shoot multiplication, yielding up to 7.8 shoots per explant (Rodrigues et al., 2023).

The differential responses among Kin, BAP, and TDZ underscore the critical importance of cytokinins in establishing shoot proliferation in *Inula confertiflora*. Kinetin significantly influenced shoot proliferation, as shown by its effects on key morphogenetic traits: shoot number (SN) and node number (NN). Notably, increasing concentrations of Kin significantly reduced both shoot and node numbers.

In contrast, BAP and TDZ concentrations did not significantly affect shoot proliferation. Although not statistically significant, the numerical trends for BAP generally mirrored those for Kin, showing decreased shoot proliferation with increasing concentration. Conversely, increasing the TDZ concentration increased the shoot number.

Cytokinin responses among various species and genotypes are thoroughly documented and probably indicate variations in endogenous hormone levels, receptor sensitivity, and the efficiency of signal transduction (Cui et al., 2019). Various types of cytokinins, either alone or in combination, have been used in related species and have produced differential responses (Custódio et al., 2024; Kapoor, 2018; Perica et al., 2008; Rodrigues et al., 2023; Stojakowska & Malarz, 2004). These findings suggest that cytokinin types and concentration significantly affect in vitro propagation.

Kinetin application promoted vigorous shoot elongation and multiplication, whereas higher concentrations of BAP and TDZ substantially increased basal callus formation and reduced shoot number and growth. Our finding that kinetin was more effective than the combined application of kinetin and NAA in *I. royleana* produced 5.2 shoots per explant (Stojakowska & Malarz, 2004). Additionally, in *I. royleana,* the largest number of shoots were produced from callus using BAP and IAA in combination (Amin et al., 2018). Rodrigues et al. (2023) BAP enhanced multiplication in *I. crithmoides*; however, it simultaneously decreased shoot height as concentration increased. This is similar to previous findings in other plants, where high levels of cytokinins may suppress apical dominance and elongation (Sultana et al., 2022).

Root Induction and Auxin Selection

Root formation is an intricate physiological process governed by the interplay between internal and external auxin levels. Different plant species and various genotypes within a single species exhibit unique root characteristics (Pandey et al., 2010). In our study, IAA produced a denser root system than other auxin types. This study shows that IAA at 2 mg/L effectively enhances root number, root length, and shoot elongation without causing callus formation. This is consistent with the observations in *Inula verbascifolia*, where IAA resulted in more effective root systems than IBA or NAA (Perica et al., 2008).

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In contrast, IBA and NAA treatments, particularly at higher concentrations, induced significant callogenesis at the shoot base, which appeared to physically obstruct root emergence and led to stunted shoot and root growth. Although callus formation at the root base could potentially be repurposed in future studies for secondary metabolite production, elevated concentrations resulted in substantial callus formation accompanied by stunted shoot and root growth. Callogenesis is often regarded as an unwanted consequence of root morphogenesis. This may still benefit phytochemical extraction because secondary metabolites accumulate (Custódio et al., 2024). For optimal plantlet regeneration, it is advantageous to minimize callus formation, as this is associated with enhanced root and shoot morphologies and a greater likelihood of survival during ex vitro acclimatization.

Implications for Conservation and Propagation

The successful ex vitro acclimatization of regenerated plantlets demonstrated the protocol's feasibility. While the initial batch achieved 100% survival, this high rate demonstrates technical feasibility but does not necessarily show protocol robustness under variable environmental conditions. The use of a sterilized sand and red ash substrate (2:1), together with wet cheesecloth covering, provided a suitable microenvironment for the plantlets to develop functional stomata and cuticular waxes (Donnelly & Tisdall, 1993; Timofeeva et al., 2014).

This study presents the first comprehensive in vitro propagation protocol for *I. confertiflora.* The results not only offer a sustainable pathway for mass propagation of this endangered endemic species but also establish a foundation for future biotechnological applications. Future research may focus on phytochemical profiling of in vitro-derived tissues to confirm whether they retain their medicinal properties and to explore the potential of callus-forming treatments for industrial production of bioactive sesquiterpene lactones.

Conclusions

This study successfully established a comprehensive and reproducible in vitro propagation protocol for weynageft (*Inula confertiflora*), an endemic and locally endangered Ethiopian medicinal plant of notable ethnopharmacological significance. Using shoot tip meristems as explants, we established a reliable system that supports extensive clonal propagation and conservation. The optimal shoot proliferation rate was observed on MS medium enriched with 1 mg/L Kin, which notably increased shoot number, node number, and leaf count while effectively reducing unintended callus formation. The most effective medium for root induction was MS medium enriched with 2 mg/L IAA, which produced well-developed adventitious roots, increased shoot and root elongation, and a higher leaf count while reducing callus formation. Moreover, plantlets rooted under this treatment showed a 100% survival rate during ex vitro acclimatization, demonstrating a successful transition to greenhouse conditions. This protocol presents a practical approach for the conservation and commercial propagation of *I. confertiflora*. These findings pave the way for future exploration of the phytochemical composition and therapeutic potential of this species, reinforcing its role in future pharmacological research and sustainable use of medicinal plants.

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