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An efficient and scalable in vitro propagation protocol of Atractylodes macrocephala Koidz.: A key traditional medicinal plant within Food-Medicine Continuum

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Research Article

Keywords: Atractylodes macrocephala Koidz., Food-Medicine continuum, In vitro propagation, Plant growth regulators, Tissue culture

Posted Date: December 20th, 2025

DOI: https://doi.org/10.21203/rs.3.rs-8262830/v1

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Version of Record: A version of this preprint was published at Plant Cell, Tissue and Organ Culture (PCTOC) on April 22nd, 2026. See the published version at https://doi.org/10.1007/s11240-026-03432-z.

Abstract

Atractylodes macrocephala Koidz., an important medicinal species within the food–medicine continuum, is widely utilized across Korea, China, and Japan. However, its traditional seed-based cultivation is increasingly unsustainable due to low germination rates, slow multiplication, and short seed viability, necessitating alternative propagation strategies to meet growing demand. This study developed an efficient and scalable in vitro propagation protocol using shoot tip explants. The effects of culture media, cytokinins for shoot proliferation, and auxins for root induction were assessed, alongside physiological, biochemical, and genetic comparisons between in vitro-regenerated and maternal plants. Shoot tips cultured on Murashige and Skoog (MS) medium supplemented with 1.5 mg/L benzyl aminopurine (BAP) yielded the highest shoot proliferation, producing 17.5 ± 1.21 vigorous shoots per explant. Optimal rooting was achieved on MS medium with 1.5 mg/L indole-3-acetic acid (IAA), generating 21.2 ± 1.32 roots with an average root length of 8.76 ± 0.85 cm and a surface area of 252.20 ± 4.92. Acclimatization produced a 93.33% survival rate, indicating strong adaptation of regenerated plantlets to ex vitro conditions. Comparative assessments showed that regenerated plants displayed similar chlorophyll content, photosynthetic efficiency, genetic stability (flow cytometry), and biochemical characteristics (FT-NIR) to maternal plants, confirming the fidelity and quality of in vitro-derived plants. Overall, this cost-effective and reproducible micropropagation protocol enables rapid mass production of uniform, disease-free *A. macrocephala* plantlets. The platform offers significant potential for conservation, commercial production, and long-term germplasm preservation of this valuable food-medicinal plant.

Introduction

Atractylodes macrocephala Koidz.(*A. macrocephala*), is a wild perennial medicinal plant found in the cool climate regions of Southeast Asia, including Korea, China and Japan. It has been cultivated for over 700 years for its medicinal properties (Zhu et al., 2018). Known by various names “Baekchul in Korea, “Bai-zhu” in Chinese, and “Baikujustu” in Japanese, *A. macrocephala* belongs to the Atractylodes genus and Asteraceae family. In China, it is classified as a homologous resource for medicine and food, and has been widely applied within food-medicine continuum (Wu et al., 2020; Liu et al., 2022; Luo et al., 2025a). *A. macrocephala* is incorporated into a variety of products as a functional food ingredient. Currently, several health care products on the market contain *A. macrocephala* rhizome (AMR), tangerine peel, and villous amomum fruit. The plant contains a variety of bioactive components, including hydrophobic lactones, volatile oils, and hydrophilic carbohydrates that supports its application in functional foods (Luo et al., 2025a). To date, over 180 compounds have been isolated from *A. macrocephala*, spinning diverse classes such as sesquiterpenoids, triterpenoids, polyacetylenes, coumarins, phenylpropanoids, flavonoids, flavonoid glycosides, benzoquinones, polysaccharides, among other compounds. These compounds are responsible for *A. macrocephala*’s wide range of pharmacological activities, including anti-tumor, immunomodulatory, anti-inflammatory, anti-oxidant, and neuroprotective effects, as well as support for gastrointestinal functions. As such, *A. macrocephala* is widely recognized for its significant medicinal, nutritional, economic, and ecological values (Zhu et al., 2018; Luo et al., 2025a). The plant has traditionally been used to manage several conditions including diabetes mellitus, digestive system

diseases that include spleen disorder, gastrointestinal function, food intake reduction, abdominal distention, bloating, diarrhoea, boosting immune system and repairing gastrointestinal mucosal damage. The plant has also been reported to support the treatment and management of bone hyperplasia, headache, and obesity (Liu et al., 2022; Yan and Li, 2023; Singh et al., 2024; Yang et al., 2025). Several reports have indicated the use of *A. macrocephala* in managing cancer, edema, dizziness, treating tumor, and relieving inflammation (Jain et al., 2015; Luo et al., 2025b). Initially harvested from the wild, the plant has long been incorporated into traditional Chinese medicine, and its domestication has been practiced for many years. To preserve its genetic stability, *A. macrocephala* was traditionally propagated from seeds and cultivated in the open field (Chen et al., 2019). The seeds were collected from mature *A. macrocephala* plants consisting multiple capitula, forming a clear distinguished “banquet” (a cluster of flower heads) arranged in a regular pattern. These plants, typically harvested from the naturally growing ecosystem, have traditionally provided the seeds for propagation (Ye et al., 2022).

However, conventional propagation of *A. macrocephala* through seeds faces various challenges including, low seed germination rates, delayed multiplication, limited seed sets even with manual pollination, environmental stress during seed germination due to climatic variability, and a short seed viability period (Yang et al., 2023), (Mao et al., 2009), (Lee et al., 2025). Given these challenges, *In vitro* propagation emerges as an urgent and viable alternative for the effective cultivation of *A. macrocephala*. This technique has already been successfully used to rapidly and sustainably propagate several other traditional medicinal plants including *Polygonum multiflorum* (Kang et al., 2018), *Codonopsis pilosula* (Gang et al., 2023), *Asparagus racemosus* Wild (Bopana and Saxena, 2008), *Rhodiolo dumulosa* (Lu et al., 2023), among others. The application of *in vitro* propagation to *A. macrocephala* would ensure mass production of the plant’s raw materials, and steady supply for herbal medicine production. However, Pence (2011) emphasize that the success of *in vitro* propagation relies on the efficiency and scalability of the protocol. This paper presents a highly efficient and scalable *in vitro* propagation protocol of *A. macrocephala*, offering a potential strategy for the mass production and conservation of the plant. Although a few studies have explored the micropropagation of *A. macrocephala (*Mao et al., 2009; Tai et al., 2023), these studies have primarily focused on propagation using a narrow range of media and a limited evaluation of alternative plant growth regulators (PGRs). Additionally, no studies to date have compared *in vitro*-regenerated plants with their maternal counterparts in terms of genetic stability, physiological performance, FT-NIR spectra, and related traits. Such comparisons are important in validating the reliability of *in vitro* propagation, maintaining the genetic fidelity, and assessing potential physiological or metabolic variations that may affect large-scale cultivation or conservation programs. This gap is particularly notable following the observations of Duta-Cornescu et al. (2023), who highlighted the potential of micropropagation to induce somaclonal variations between maternal plants and tissue culture-regenerated plants. Additionally, Polivanova and Bedarev (2022) and Hazarika (2006) reported that tissue culture techniques can induce physiological disorder in *in vitro*-regenerated plants. Their observation underscore the need for more comprehensive research on *in vitro*-regenerated plants, as an important tissue culture technique. Therefore, The limited scope of the previous studies has hindered a comprehensive understanding of the plant's full *in vitro* propagation potential.

To fill these gaps, the current study provides a thorough and comprehensive analysis of the effects of various media and a range of PGRs at varying concentrations on shoot and root proliferation, aiming at optimizing shoot and root number and length in the *in vitro* propagation protocol for *A. macrocephala*. Additionally, a comparative analysis was conducted between *in vitro*-regenerated plants and maternal plants to evaluate their physiological parameters using Soil Plant Analysis Development (SPAD) readings, FluorPen FP110 measurements, and FT-NIR spectrometry to asses chlorophyll pigment content, photosynthetic efficiency, and chemical composition respectively. To examine potential somaclonal variations, genomic stability of the *in vitro*-regenerated *A. macrocephala* was assed using flow cytometry.

This research will play a critical role in optimizing *A. macrocephala* propagation techniques, ensuring genetic consistency, and providing insights that can support the sustainable cultivation and conservation of this homologus resource of medicine and food. Additionally, the findings of this study contribute significantly to the efficient mass propagation of *A. macrocephala* for commercial production.

Materials and Methods

Seed germination and Explant Preparation

A. macrocephala seeds used in this study were donated by Chungbuk Agricultural Research and Extension Services in Chungju, Korea. Seeds coats were gently and carefully removed, and the seeds were treated with detergent Tween 20 (0.1%) for 15 minutes and again washed with tap water until the detergents washed clearly. After that, the seeds were surface sterilized by dipping them in 70% alcohol for 2 minutes followed by 1% sodium hypochlorite solution for 15 minutes and were subsequently rinsed in sterilized water at least three times. The sterilized seeds were spread over a few sterile papers under a laminar flow hood to be dried for 5 minutes. For germination, Murashige and Skoogs (MS) basal medium (Murashige and Skoog, 1962), plus 30.0 gL^{-1} sucrose was used without any growth regulator. The pH of the medium was adjusted to 5.7–5.8 before adding 3.0 g/L gelrite prior to autoclaving at 121°C for 15 minutes Afterwards, the medium was poured into 90 x 15 mm disposable plastic petri dishes (25 mL into each of them). For efficient germination, the parafilm-sealed plates were kept in a growth chamber at constant temperature of 22 ± 2°C (16/8h photo period) under radiation of 35 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent tubes (Master TL-D 840, Philips, Pila, Poland) at 70 ± 10% relative humidity. The seeds were thinly spread over the surface of MS basal medium under aseptic condition as seed in (Fig. 1A). Seedlings started to emerge after 2 weeks of culture and subsequently developed into a plantlet (Fig. 1B). After six weeks of growth, the plantlet derived from the seeds was selected as a primary explants, serving as the source of the shoot tip used for *in vitro* propagation.

Effect of growth media on the growth of A. macrocephala.

To evaluate the effect of The effects of six basal media on shoot and root development of *A. macrocephala*, excised shoot tip (0.8–1.3 cm) were cultured in 100 ml of gelled media (MS, DJ, LS, QL,

WPM, and CHU) contained in polystyrene culture vessels (125 × 110 mm, Gaooze 1011C, Gyeonggi-do, South Korea). At inoculation, the initial shoot length was recorded. Each treatment consisted of 10 replicates with two explants per vessel as shown in (Fig. 1C1). The experiment was repeated three times. Cultures were maintained at 24–25°C under 500–1000 lux illumination with a 16/18 h light/dark photoperiod, using cool white fluorescent lamps providing a light intensity of 33.73 µmol m^2 s^{-1} at 80% relative humidity. All media were supplemented with 30 g/L sucrose and vitamins, adjusted to pH 5.7–5.8 with 0.1 N NaOH or HCl, and solidified with 3 g/L gelrite before autoclaving at 121°C for ~ 20 minutes. Explants were inoculated in the solidified media without roots (Fig. 1C1). After six weeks of shoot tip culture (Fig. 1C2), data were collected from the plants focusing on mean shoot length and root number. Based on the collected data, MS produced the highest mean shoot length and root number and was therefore selected for subsequent experiments.

Effect of cytokinin on shoot multiplication

MS medium supplemented with vitamins was independently enriched with four different cytokinins; Thidiazuron (TDZ), 6-Benzylaminopurine (BAP), Kinetin (KIN), and isopentenyl adenine (2iP) at concentrations of 0.1, 0.5, 1.0, 1.5, and 2.0 mg/L. Each medium-cytokinin combination was also supplemented with 30.0 g/L sucrose, and the pH was adjusted to 5.7 ± 5.8 with 0.1 NaOH or HCl before adding 3.0 g/L gelrite. The medium-cytokinin combination of BAP and KIN were autoclaved at 121°C for 20 minutes, while TDZ and 2iP were filter sterilized. After sterilization, 100 mL of the prepared medium was dispensed into polystyrene culture vessels (125 × 110 mm; Gaooze 1011C, Gyeonggi-do, South Korea). Shoot tips (measuring 0.8–1.3 cm long) were carefully cultured into the cooled, solidified MS basal medium containing different cytokinins at varying concentrations. Each vessel contained two shoot tips as shown in (Fig. 1D1), and each treatment consisted of 10 replicates. After six weeks of induction, the number of shoots that developed per explant were recorded. (Fig. 1D2) shows the shoots that proliferated after six weeks of induction in BAP 1.5 mg/L.

Effect of Auxin on root proliferation

MS medium supplemented with vitamins was independently enriched with four different auxins naphthaleneacetic acid (NAA), Indole-3-butyric acid (IBA), Indole-3-Acetic Acid (IAA), and 2,4-Dichlorophenoxyacetic acid (2-4D) at concentrations of 0.1, 0.5, 1.0, 1.5, and 2.0 mg/L. Each medium was also supplemented with 30 g/L sucrose, and the pH was adjusted to 5.7 ± 5.8 with 0.1 NaOH or HCl before adding 3 g/L gelrite. All medium hormone combinations were autoclaved at 121°C for 20 minutes. After sterilization, 100 mL of the prepared medium was dispensed into polystyrene culture vessels (125 × 110 mm; Gaooze 1011C, Gyeonggi-do, South Korea). Shoot tips measuring 20–40 mm were excised from previously proliferated plants. Shoot tips were placed vertically into the cooled, solidified MS media containing different auxin at varying concentrations. Each test tube contained one shoot apex (Fig. 1E1), with 10 replicates per treatment. After six weeks of inoculation (Fig. 1E2), root number, root length, and root surface were recorded for all treatments. The root surface area was

obtained using the WinRHIZO Pro software (Reagent Instruments, Queben, Canada) after acquiring 2D root images (Fig. 1E3) by scanning the roots using an expression 12000XL scanner (Epson, Sueas, Nagano, Japan) developed per explant was recorded.

Experimental Design

The experiment was conducted in a completely randomized design (CRB) with 10 replications. Data were recorded as centimeters for shoot length and root length, number of components for each plant, square centimeter for roots surface area, grams for fresh, dry weight of leaves, leaf powder and root powder. The cultures were periodically monitored to record the percentage response for shoot bud regeneration, multiple shoots development and rooting. The total number of shoots and roots were determined by visual observation, root surface area was assed using WinRHIZOR, weight of leaves was measured using weighing scale.

Acclimatization

After six weeks of culturing, well-rooted *A. macrocephala* plantlets were taken out of test tubes and carefully washed with sterile water to remove any remaining medium adhering to the roots (Fig. 1F). The cleaned plantlets were transplanted into plastic pots (7 cm in diameter, 8 cm in height) containing a sterile horticultural soil and perlite mixture (2:1, pH 6.5). Each pot was covered with a transparent plastic bag to maintain adequate humidity during the hardening process (Fig. 1H). Watering was carried out twice weekly for the first three weeks and then reduced to once weekly in the following weeks. After 15 days, the plastic covers were gradually removed, allowing the plantlets to adapt to natural conditions. The survival rate of plantlets was evaluated after eight weeks of acclimatization (Fig. 1H), the survival rate of the plants was determined.

Figure 1

Assessment of leaf Chlorophyll Content

After nine weeks of acclimatization, the leaf chlorophyll content of *in vitro*-regenerated and maternal *A. macrocephala* plants was assessed using a calibrated Soil Plant Analysis Development (SPAD) chlorophyll meter (SPAD-502 Plus, Konica Minolta, Inc., Japan) as shown in (Fig. 2A). For each plant, eight leaves were randomly selected: One pair from the apical region, two pairs from the mid-stem, and one pair from the lower stem. SPAD measurements were taken at four different points per leaf as shown in (Fig. 2B), and the average value was calculated for each leaf. The mean of the eight leaf averages was considered the overall chlorophyll content for that plant. Measurements were recorded weekly over eight weeks for a total of 20 plants (10 *in vitro*-regenerated and 10 maternal plants) and the results are as shown in (Fig. 2C).

Figure 2

Chlorophyll florescence measurements

Chlorophyll fluorescence measurements were carried out using the FluorPen FP110 (Drásov 470, 664 24 Drásov, Czechia) following dark adaptation of the leaves for approximately one hour (from 9:00 to 10:00) using leaf clip gaskets. Ten fully expanded leaves were randomly selected from different sections of ten replicates of both *in vitro*-regenerated and maternal plants, and chlorophyll fluorescence was measured accordingly as shown in (Fig. 3A). Data collection was conducted weekly over an 8-week period as presented in (Fig. 3B). A saturating light pulse exceeding 4,000 μmol m^{-2} s^{-1} was applied to determine the Fv/Fm values, following the FluorPen FP110 OJIP protocol. In the dark-adapted state, the minimal chlorophyll fluorescence (Fo), the maximum fluorescence (Fm), and the variable fluorescence (Fv) were recorded. These values were measured under conditions where photosystem II (PSII) reaction centers were fully open, a saturation pulse was applied, and non-photochemical quenching processes were minimized. Fv/Fm represents the maximum efficiency of Photosystem II (PSII) under optimal conditions (Bahmanbiglo and Eshghi, 2021).

Figure 3

Measuring relative water content

To assess the relative water content (RWC) of the leaves from *in vitro* regenerated and *A. macrocephala* maternal plants, the technique was modified from (Hewlett and Kramer, 1963). The measurement was conducted after 10 weeks of acclimatization. Fully expanded leaves that had received light were selected for the experiment. During preparation, empty sample tubes were numbered and weighed. Five fully expanded leaves were selected and cut from randomly chosen plants in each plant together with their leaf stalk as shown in (Fig. 4A). The top and bottom parts of each leaf were removed, and any dead or dying tissue was discarded as illustrated in (Fig. 4B). The prepared leaves were immediately placed into the centrifuge tube and sealed with lid to prevent moisture loss or gain. The sample tubes were weighed (tubeW + FW) as illustrated in (Fig. 4C). Next, 1 cm of distilled water was added to each tube as shown in (Fig. 4D), and the tubes were placed in a refrigerator at 4°C in darkness for 24 hours, allowing the leaves to reach full turgidity. After 24 hours, the leaves were carefully removed from the tubes, blotted dried with a paper towel as illustrated in (Fig. 4E), and weighed (TW; turgid weight). The leaf samples were then placed in labeled envelopes and dried at 60°C for 24 hours or until they reached a constant mass as shown in (Fig. 4F), after which the dry weight (DW) was recorded.

To calculate the fresh weight (FW) of the leaf samples, the formula used was:

FW = tubeW + FW - tubeW

Finally, the leaf RWC was calculated using the formula:

$$Leaf\ RWC\ (\%) = \frac{FW - DW}{TW - DW} X\ 100$$

Where: FW = Fresh weight, TW = Turgid weight, DW = Dry weight., tube W = tube weight

Figure 4

Flow cytometry analysis

The genomic stability and ploidy level of *in vitro* regenerated *A. macrocephala* plants were assessed using flow cytometry, following the protocol described by Abdolinejad et al. (2020) with slight modifications. In brief, leaf samples were randomly collected from both *in vitro* regenerated and maternal plants. *Secale cereale*, with a known 2C DNA content of 16.19 pg, (Zwyrtková et al., 2020) was used as an internal standard. Approximately 0.5 cm² of leaf tissue from each sample and the internal standard were placed in a plastic petri dish. To each dish, 500 µL of nuclei extraction buffer (CyStain UV Precise P Nuclei Extraction Buffer; Sysmex Partec, Germany) was added, and the tissues were finely chopped with a sharp razor blade for 40 seconds. The resulting suspension was incubated for 30–90 seconds and subsequently filtered through a 50 µm mesh filter (CellTrics, Sysmex Partec, Germany) into sample tubes. Each filtrate was then supplemented with 2000 µL of staining solution, consisting of staining buffer (CyStain UV Precise P Staining Buffer), propidium iodide, and RNAse A stock solution at a ratio of 333.333:2:1. Samples were shielded from light and incubated at room temperature (25°C) for 60 minutes. Fluorescence intensity of the isolated nuclei was measured using a flow cytometer (BD FACSCalibur, USA). The ploidy levels of both *in-vitro regenerated* and maternal *A. macrocephala* plants were determined by comparing their relative fluorescence intensities against that of the internal standard, according to the following formula:

2C DNA content of *A. macrocephala* (pg) = $\frac{Fluorescence\ intensity\ of\ test\ sample}{Fluorescence\ intensity\ of\ internal\ standard} x 16.19\ \mathrm{pg}$

Where:

- Intensity of test = Relative fluorescence peak of the sample (*A. macrocephala*)
- Intensity of standard = Fluorescence peak of the internal reference standard
- 16.19 pg = 2C DNA content of the reference standard used for comparison

Fourier Transform Near-Infrared (FT-NIR) Analysis

Leaf and root parts of *A. macrocephala* plants from both *in vitro* regenerated [IL (leaf), and IR (root)] and maternal plants [ML (leaf), and MR (root)] were collected, oven-dried at 60°C for 24 hours, and ground into a fine powder using a 250g Pulverizing Machine operating at 25,000 rpm (Model RT-N04-2V, Taiwan). The powdered samples were then analyzed using a TANGO FT-NIR spectrometer (Bruker Optics, Billerica, MA, United States). Prior to analysis, the spectrometer was calibrated using a Light Trap (Type 1002961,

ECL 00) and a Gold Standard (Type 1024957, ECL:01). For each sample, 2.0 g of powdered material were placed in a vial with a 20 mm diameter and scanned. Absorbance spectra were recorded in the wavenumber range of 12,487–3,948 cm^{-1} to identify different classes of compounds based on their functional groups as shown in (Fig. 5A). Dendrograms representing sample relationships were generated using Ward's clustering algorithm, following data preprocessing with first derivative transformation, vector normalization, and standardization of Euclidean distances within the 9,981 to 4,014 cm^{-1} range as shown in (Fig. 5B). The OPUS TANGO-R software was used to perform the Ward clustering analysis, with homogeneous groupings optimized through the minimum variance method for cluster analysis.

Figure 5

Data Analysis

The data recorded for number of shoot, shoot length, number of roots, length of roots, surface area of roots were exposed to one-way analysis of variance (ANOVA), followed by Tukey's post hoc tests using GraphPad Prism v 5.03. All means compared were considered significantly different at $p \leq 0.05$.

Results

Seed germination test

Germination of achenes began two weeks of culture, and by the end of the third week, the germination rate reached 94%, indicating the high efficiency of MS medium for seed induction in *A. macrocephala*. After three of culture, well developed zygotic plantlets were selected and used as primary explants for further experiments.

Effect of growth media on the growth of A. macrocephala.

After six weeks of culture, all tested culture media supported the regeneration and growth of *A. macrocephala* from shoot tips, as evidenced by the parameters evaluated during this period (Fig. 6). Notably, MS medium exhibited both the greatest increase in shoot length (1.47 ± 10.12%), however, the percentage increase in shoot length in MS medium did not significantly differ from the percentage increase recorded in LS (1.36 ± 13.03%) and WPM (1.35 ± 12.78%) media as indicated in (Fig. 6A). The lowest percentage increase in shoot length (0.71 ± 40.92%) occurred in CHU medium. Plants cultured in MS medium produced dark green, healthier leaves. Regarding root development, MS medium yielded the greatest root formation, with an average of (7.8 ± 1.06) roots. This was significantly different compared to other media as illustrated in (Fig. 6B). The lowest root formation was recorded in CHU medium with an average of (1 ± 0.80) root formed. However, all media tested led to an increase in both shoot length and root formation in the *A. macrocephala* plant during the experiment. MS medium was selected as the most suitable for *A. macrocephala* growth in subsequent experiments. This was because plants grown MS showed the highest overall growth index and exhibited generally healthier morphology, with broader and darker green leaves.

Figure 6

Effect of different cytokinins on shoot multiplication

To induce multiple shoots, shoot tips of *in vitro*-regenerated *A. macrocephala* were cultured on MS medium supplemented with different concentrations of cytokinins (TDZ, BAP, KIN, and 2iP), resulting in 100% shoot formation and multiplication. Among all cytokinins tested, BAP under the concentration of 1.5 mg/L was the most effective in promoting shoot initiation and multiplication from *A. macrocephala* shoot with 17.5 ± 1.21 shoots generated from 1 shoot apex as indicated in (Fig. 7A). The lowest shoot proliferation was observed at the lowest concentration of 2iP (0.1 mg/L) as indicated in (Fig. 7A), which resulted in 1.7 ± 0.8164 shoots. However, this was not significantly different from MS supplemented with 2iP 0.5, 2iP 1.0, 2iP 1.5, and KIN 0.1 mg/L. and the control MS, which produced approximately of 2.0 ± 0.89, 3.2 ± 0.75, 3.7 ± 1.75, and 3.7 ± 1.63 plants respectively. The hormone concentrations that resulted in the lowest shoot regeneration (2iP 0.1 mg/L, 2iP 0.5 mg/L, 2iP 1.0 mg/L, 2iP 1.5 mg/L, and KIN 0.1 mg/L) did not significantly increase shoot numbers compared to the control (MS medium) that produced 1.7 ± 0.82, indicating their limited effectiveness in promoting shoot proliferation in *A. macrocephala* shoot tips. In terms of shoot height increase, MS medium supplemented with BAP 1.5 mg/L exhibited the greatest increase in shoot length (320.0 ± 11.93%), making it significantly different from other MS-cytokinin combinations. The lowest increase in shoot length (177.90 ± 40.76%) occurred in the lowest concentration of 2iP (0.1 mg/L). However, the shoot length increase in the lowest concentration of 2iP did not differ significantly from 2iP (0.5 mg/L), 2iP (1.0 mg/L), 2iP (1.5 mg/L), and Control (MS), indicating their relatively limited effectiveness in inducing shoot length in *A. macrocephala* shoot tip. MS supplemented with 1.5 mg/L BAP was selected as the optimal medium–cytokinin combination because it significantly enhanced shoot initiation, multiplication, and shoot elongation in *A. macrocephala*, outperforming all other concentrations. This treatment also produced dark green plants with noticeably high growth vigor. The shoot proliferation results in this study, surpass the 14.5 and 5.61 shoots reported in previous studies (Tai et al., 2023), and (Mao et al., 2009) respectively.

Figure 7

Effect of Auxin on root proliferation

For rooting, shoots derived from proliferated shoot tip in the previous experiments were cultured on MS medium fortified with auxins (NAA, IBA, IAA, and 2-4D) in different concentrations (0.1, 0.5, 1.0, and 2.0 mg/L) to induce root multiplication and elongation. After two weeks of culture, root development began to occur in some of the auxin concentrations particularly in all IAA and IBA concentrations. After six weeks of shoot tip culture, the highest rooting mean number was observed in MS medium fortified with NAA2 with a maximum 25.8 ± 2.8 roots. This result was statistically different from all other treatments including the control (MS without auxins). The rooting mean number in NAA increased with increasing concentration. The second highest mean number of roots was observed with NAA 1.5 mg/L 22.4 ± 1.43, followed by MS medium fortified with IAA 1.5 mg/L with 21.2 ± 1.32, which was followed by NAA 1.0 mg/L 25 ± 1.58 (Fig. 8A). Overall, all concentrations of NAA produced higher root mean number

compared to other auxin concentrations except IAA 1.5 mg/L that was significantly higher than NAA 16.8 mg/L and NAA 14 mg/L. Regarding root elongation, the highest root length recorded after six weeks of shoots culture was observed in MS medium fortified with IAA 1.5 mg/L measuring 8.76 cm ± 0.85. This root length was significantly different from all other medium-auxin combinations (Fig. 8B). Regarding root surface area, the highest increase was observed in MS medium fortified with IAA 1.5 mg/L, showing a surface area increase of 252.2 cm^2 ± 4.919 after six weeks of shoot tip culture as indicated in (Fig. 8C). This was significantly different from all other treatments including the control (MS without auxins). The second highest surface area increase was observed in IAA 2.0 mg/L, which had a surface area of 226.0 cm^2. Excepts for all concentrations of 2-4D, that did not induce any root formation, there was an increase in surface area across all other MS medium and hormone combination.

Figure 8

Acclimatization of A. macrocephala

After six weeks of acclimatization, the *in vitro* regenerated *A. macrocephala* plantlets exhibited a high survival rate of 93.33%. The acclimatized plants grew well and phenotypically similar to maternal plant stock.

Measurement of Chlorophyll Content

In the first week, the chlorophyll content of *in vitro*-regenerated plants (41.27 ± 1.116) was significantly lower than that of the maternal plants (60.3 ± 1.713). However, by the fifth week, the SPAD chlorophyll content in the *in vitro*-regenerated plants increased at a slightly faster rate, reaching a level nearly equal to that of the maternal plants. From the sixth to the eighth week, both plant groups showed only a modest increase in chlorophyll content, with *in vitro*-regenerated plants rising from (67.20 ± 0.767) and maternal plants from (67.40 ± 0.760). During this period, chlorophyll levels in both groups remained comparable, indicating similar photosynthetic development in *in vitro* regenerated and maternal plants as observed in (Fig. 2C).

Chlorophyll florescence measurements

To assess the photosynthetic performance of maternal and *in vitro*-regenerated plants, dark-adapted F_v/F_m values, which reflect the maximum or intrinsic potential quantum efficiency of PSII, were measured. The F_v/F_m values for the maternal plants ranged from 0.76 ± 0.015 to 0.82 ± 0.0013, while those for the *in vitro*-regenerated plants ranged from 0.51 ± 0.020 to 0.81 ± 0.011 over the 8 weeks of measurement. In the first week, the photosynthetic performance of *in vitro*-regenerated plants was significantly lower than that of the maternal plants. From the third week, the photosynthetic performance of the *in vitro* regenerated plant increased faster until the sixth week where it leveled with the maternal plant. From the sixth to the eight week, the F_v/F_m values of both plant groups were within similar ranges as observed in (Fig. 3B).

Relative water content

To evaluate the relative water content (RWC) of *A. macrocephala* maternal plants and *in vitro*-regenerated plants, specimens that had been acclimatized for at least 10 weeks were utilized. Five samples were collected from both the maternal plants and the *in vitro*-regenerated plants, with each sample consisting of five fully matured leaves. The RWC of maternal plants ranged between 80.1 ± 82.3%, while those for the *in vitro*-regenerated plants ranged between 79.6 ± 82.8% (Table 1).

Table 1
Relative water content of *A. macrocephala* leaves

Samples	Tube weight (g)	Tube weight + fresh leaves weight (g)	Fresh leaves weight (g)	Turgid leaves' weight (g)	Dry leaves' weight (g)	Leaf RWC (%)
MPs 1	12.88	13.995	1.116	1.348	0.124	81.048
MPs 2	13.172	14.44	1.268	1.508	0.149	82.34
MPs 3	12.9	14.13	1.23	1.493	0.131	80.690
MPs 4	13.074	14.395	1.321	1.585	0.133	80.823
MPs 5	13.179	14.412	1.233	1.491	0.135	80.968
MPs 6	13.12	14.443	1.323	1.59	0.135	81.64
IVPs 1	13.01	14.16	1.15	1.401	0.136	80.158
IVPs 2	13.11	14.341	1.231	1.46	0.126	82.834
IVPs 3	13.011	14.616	1.605	1.841	0.544	81.804
IVPs 4	12.98	14.14	1.16	1.431	0.103	79.593
IVPs 5	13.03	14.298	1.268	1.463	0.461	80.539
IVPs 6	13.004	14.46	1.456	1.713	0.25	82.433

Table 1

Flow cytometry analysis

The flow cytometry histogram indicated that the ploidy levels of both maternal (Fig. 9A) and *in vitro* regenerated (Fig. 9C) *A. macrocephala* plants were identical. The 2C DNA content of the maternal plant was 9.0 pg (Fig. 9B), while that of the *in vitro* regenerated plant was 8.92 pg (Fig. 9D). The 9.0 pg of 2C DNA content in the maternal plant corresponded to a genome size (2C) of 8,802.0 Mbp, whereas the 8.92 pg of 2C DNA content in the regenerated plant equated to a genome size (2C) of 8723.0 Mbp. The expression in Mbp was calculated using the conversion factor 1pg DNA = 978 Mbp, as per Lepers-Andrzejewski et al. (2011).

Figure 9

Fourier Transform Near-Infrared (FT-NIR) Analysis

Overall, the FT-NIR spectra for leaf and root samples (both from *in vitro* regenerated and maternal plants), were similar with slight difference observed on maternal root spectra with other samples in the 5,000 to 4,000 cm^{-1} wavenumber range as indicated in (Fig. 5). A peak at 5174.9 cm^{-1} was observed in all leaf and root samples (Fig. 5A). Additionally, both *in vitro* regenerated and maternal samples showed peaks at 5,82.6, 6887.6, and 8,833.6 cm^{-1} (Fig. 5A). Between 9,000 and 4,000 cm^{-1}, seven prominent peaks were detected for all samples (Fig. 5A). Clustering analysis using Ward's algorithm revealed the overall heterogeneity value for all samples being of 0.89 (Fig. 5B), which was followed by the heterogeneity values of ML and IL that was 0.53 (Fig. 5B). The highest similarity was observed between maternal roots (MR) and *in vitro* regenerated roots (IR), with the heterogeneity value of 0.36. This low heterogeneity values indicates strong relationship between the MR and IR. With the heterogeneity value of 0.53 (Fig. 5B), ML and IL registered the highest dissimilarity.

Discussion

In vitro propagation, a key component of plant biotechnology, has proven to be a reliable method for the rapid mass production of high-quality, disease-free medicinal plants. It also supports the propagation of species that are challenging to reproduce through conventional methods (Gaurav et al., 2018; Anuruddi et al., 2023). Additionally, *In vitro* propagation, is known for maintaining the genetic stability of regenerated plants without exhibiting somaclonal variations while supporting the enhancement of desirable traits such as high secondary metabolites content (Bidabadi and Jain, 2020; Duta-Cornescu et al., 2023). Within the food-medicine continuum, this is particularly important, as maintaining both the medicinal efficacy and nutritional quality of species like *A. macrocephala* is essential for their dual role in healthcare and diet. Thus, *in vitro* propagation provides a sustainable approach for mass production, and conservation or medicinal plants including homologous food-medicine resources .

Effects of culture media, cytokinin, and auxins on A. macrocephala seed germination, shoot, and root growth

Following sterilization process of the seeds, they were carefully placed in MS basal medium (Murashige and Skoog, 1962) for germination. Under aseptic conditions, the seeds were evenly spread on the surface of the medium as indicated in (Fig. 1A). Germination began approximately two weeks after culture initiation, and zygotic plantlets aged 21 days were selected as primary explants for subsequent *in vitro* experiments. A similar study that used MS free from regulators for the germination of seeds is the germination of *Polygonum multiflorum* (Kang et al., 2018). To identify the optimal medium for *A. macrocephala* growth, explants from germinated seeds were transferred to and tested across different media. Plant tissue culture media serve as the primary source of mineral nutrients and water, both essential for supporting plant growth and development under *in vitro* conditions. Their composition act as key factors that influencing the success of *in vitro* propagation, as they play a vital role in directing morphogenesis by enabling plant tissues and cells to develop into whole organs or complete plants (Monfort et al., 2018; Jayusman et al., 2022). However, different culture media formulations vary in their composition, particularly in macronutrient content and total ion concentrations, which significantly

influence plant morphogenesis (Okello et al., 2021a; Gang et al., 2023). For instance, MS medium is characterized by high levels of nitrate (39.4 mM) and ammonium (20.6 mM) ions but contains a low concentration of sulfate (1.7 mM). In contrast, QL medium has a lower ammonium content (5.0 mM), while Woody Plant Medium (WPM) features both low ammonium (5.0 mM) and nitrate (9.7 mM) levels, but relatively high sulfate content (7.5 mM) (Okello et al., 2021a). Because of the significant variation in the mineral composition of the media, this study observed notable differences in individual growth parameters of *A. macrocephala* across different culture media. Nevertheless, all media contributed to the plant's growth, as each provided a distinct nutrient profile, suggesting that while nutrient availability varied, all tested media were capable of supporting plant development to varying degrees.

The present study indicates that MS exhibited the best growth parameters (height of plant and number of roots) on *A. macrocephala*. Increase in shoot length in MS medium was (1.47 ± 10.12%), however, the percentage increase in shoot length in MS medium did not significantly differ from the percentage increase recorded in LS (1.36 ± 13.03%) and WPM (1.35 ± 12.78%) media as indicated in (Fig. 6A). The smallest percentage increase in shoot length (0.71 ± 40.92%) was observed in CHU medium. Regarding root development, MS medium yielded the greatest root formation, with an average of (7.8 ± 1.06) roots. This was significantly different compared to other media as illustrated in (Fig. 6B). The CHU medium recorded the lowest root formation, with an average of (1 ± 0.80) root. The height of the plant and the number of roots are important parameters for evaluating the effectiveness of growth media in *in vitro* propagation. These indicators reflect the overall vigor and developmental success of plantlets in response to the nutrient composition of the medium. Taller plants typically signify enhanced shoot proliferation and nutrient uptake, while a greater number of roots indicate efficient root induction and adaptation to the culture environment (Hussain et al., 2022). Together, these traits provide a reliable measure of the medium’s ability to support balanced morphogenesis and healthy plantlet development. In addition to plant height and length or roots, plants cultured in MS medium produced dark green, healthier leaves compared to plants culture in other media. According to Okello et al. (2021a), leaf color serves as a visual indicator of a plants’ nutritional status and physiological health. These colors reflect availability and balance of essential nutrients within the culture medium. In this context, MS medium, known for its high concentrations of macronutrients, particularly nitrate and ammonium, often supports vigorous growth and the development of healthy, deep green foliage in many plant species (Alamgir, 2017; Isah and Umar, 2020). The richness of MS medium in essential elements contributes to enhanced chlorophyll synthesis, which is directly linked to the dark green colour of the leaves (Razavizadeh et al., 2023). Thus, the presence of vibrant green leaves in plantlets grown on MS medium can be attributed to its nutrient-rich composition, which promotes overall plant health and robust physiological performance. Successful use of MS in the propagation of in the same family “Asteracea” have been recorded in *Sausurea esthonica* (Gailīte et al., 2010), *Susurea medusa Maxim* (Liu and Saxena, 2009), *Tripleurospermum insularum* (Inceer et al., 2022), *Calendula maritima Guss* (Catalano et al., 2022), *Tripleurospermum ziganaese* (Cuce and Inceer, 2024). However, other studies have demonstrated that certain Asteracea species respond better to alternative media WPM and LS media. For instance, WPM has been effectively used in the propagation of *Rhaponticoides mykalea* (Hayta et al., 2017), and

Achyrocline satureioides (Guariniello et al., 2018), while *Stevia rebaudiana* Bertoni exhibited enhanced shoot elongation in LS medium (Miladinova-Georgieva et al., 2023). These findings align with observations in the present study, where no significant differences in shoot length were recorded for the lengths of *A. macrocephala* across MS, WPM, and LS media, suggesting that all three media are capable of supporting comparable growth performance in this species.

Cytokinins, a group of essential plant hormones are classified into two main types: purine-type cytokinins and phenyl urea-type cytokinins (Grzegorczyk-Karolak and Hnatuszko-Konka, 2021). Both types play a central role in *in vitro* propagation by regulating key processes involved in plant growth and development. Their primary function is to promote cell division and shoot formation, making them essential components in most plant tissue culture protocols (Schoene and Yeager, 2005; Asghar et al., 2023). In the present study, both types were used. The purine-type cytokinins applied included BAP, TDZ, and 2iP, while TDZ, a phenyl urea-type cytokinins was also applied. Despite their structural differences, both types effectively promote shoot proliferation and multiplication in the *in vitro* propagation of medicinal plants (Kapchina-Toteva et al., 2000). Effective shoot proliferation and multiplication largely depends on culture medium enriched with cytokinins, which serve as key regulators in tissue differentiation and morphogenesis (Cavallaro et al., 2022). According to (Kumari et al., 2018), Plant growth regulators (cytokinins and auxins) affect various physiological functions in plants, particularly the initiation and multiplication of shoots and roots.

In this study, addition of various cytokinins at different concentrations in MS medium promoted shoot proliferation and multiplication. Considering the mean number of shoot per explants and the length of initiated shoot the best results were achieved in BAP under the concentration of 1.5 mg/L was the most effective in promoting shoot initiation and multiplication from *A. macrocephala* shoot with 17.5 ± 1.21 shoots generated from 1 shoot apex as indicated in (Fig. 7A). The same concentration (MS medium supplemented with BAP 1.5 mg/L) exhibited the greatest increase in shoot length (320.0 ± 11.93%), making it significantly different from other MS-cytokinin combinations. Additionally, plants in MS medium-BAP 1.5 mg/L combination developed dark-green leaves-an indicator of good physiological health, making them more suitable for further development. Su et al. (2023) indicates that leaf colour is an indicator of healthy plant as dark-green leaves exhibit higher expression of photosynthesis-related genes and greater chlorophyll content, which correlated with improved photosynthetic capacity and potential crop yield, while (Dutta Gupta et al., 2013) illustrates that dark green leaves typically reflect adequate or high levels of essential nutrients like nitrogen and magnesium, key components for chlorophyll synthesis whereas pale or yellowing leaves signal nutrient deficiencies. Overall, MS medium supplemented with 1.5 mg/L BAP was identified as the optimal medium-hormone combination for enhancing shoot proliferation, multiplication, and elongation in *A. macrocephala*. Plants grown under this treatment developed dark-green leaves-an indicator of good physiological health and consistently outperformed those in all other treatments across all evaluated parameters. A similar observation of BAP producing healthy plants with dark-green leaves were observed in the propagation of *Ficus benjamina* (Benedetto et al., 2020). Studies that indicate the superiority BAP concentrations over other cytokinins include the propagation of; *Aspilia Africana* (Okello et al., 2021a), *Hedyyotis biflora* (Linn.) Lam

(Revathi et al., 2019), *Ipomoea batatas* (L.) Lam. (Dewir et al., 2020), and *Crataeva nurvala* (Buch Ham) (Kher and Nataraj, 2020). The results of this study indicate that the mean number of shoots increased as BAP concentrations rose, reaching an optimum at 1.5 mg/L. Beyond this level, additional increases in BAP led to a reduction in shoot proliferation. This trend aligns with the findings of Wang et al. (2018) who reported that exceeding the optimum concentration can hinder shoot regeneration.

A similar optimized concentration of BAP 1.5 mg/L was reported by Khan et al. (2015) in a study on shoot regeneration in grapes (*Vitis vinifera* Vitaceae family), where the highest shoot regeneration frequency (53.33%) was achieved on MS medium supplemented with 1.5 mg/L BAP and 0.5 mg/L NAA. The regenerated shoots subsequently rooted successfully on a hormone-free half-strength MS medium. Similarly, Jafari et al. (2017) observed optimum shoot proliferation in *Passiflora caerulea* L. (Passifloraceae family), where the highest regeneration frequency (90%) and a maximum of 8.86 shoots per cotyledonary node explants were recorded on MS medium supplemented with BAP 1.5 mg/L. Although these species belong to different families other than *A. macrocepha* (Asteraceae), the consistent effectiveness of 1.5 mg/L BAP across these studies supports that this concentration could be broadly applicable for shoot induction, suggesting that optimal cytokinin levels may transcend family boundaries under certain culture conditions. However, the findings of the current study differ from several previous reports on members of the Asteraceae family, where different concentrations of BAP have been reported as the optimum for shoot proliferation. For instance Pandey et al. (2014) identified an optimal BAP concentration 1.15 mg/L for *Spilanthes calva*, (a members of Asteraceae family), producing up to 4.17 shoots per explant. Additionally, Okello et al. (2021a) found that on 1.0 mg/L resulted in the highest shoot proliferation in *Aspilia africana*, (a member of Asteraceae family), with an average of 13 shoots per nodal segment after six weeks of culture. This deviation in *A. macrocephala* suggests a species-specific response to cytokinins, even within the same family, emphasizing the need for empirical optimization rather than relying solely on trends within a taxonomic group.

Auxin hormones are essential for initiating meristematic competence in plant cells during *in vitro* propagation. They enable cells to regain the ability to divide and differentiate into root-forming cells, an important step for successful rooting (Pacurar et al., 2014; Ribeiro et al., 2022). By inducing the formation of root primordia and promoting the growth of functional roots, auxins are fundamental to the rooting process. Their careful manipulation in tissue culture media ensures efficient plant regeneration from small explants, making auxins key to the success of *in vitro* propagation and root development (Du and Scheres, 2018). These hormones promote root elongation, stimulate the formation of additional roots, and enhance the root surface area of the plant during *in vitro* propagation (Nibau et al., 2008; Komakech et al., 2020b). Several other studies have demonstrated varied rooting responses to different auxin hormones at varying concentrations in *in vitro* plant propagation, even within the same genus (Okello et al., 2021b).

In the current study, excised *A. macrocephala* shoot tips were rooted in MS medium with vitamins, supplemented with 30 g/L of sucrose and various auxin hormones at different concentrations. After six weeks of shoot tips induction, 100% rooting success was observed at all concentrations of NAA, IBA,

IAA and MS as control, while no rooting occurred at any concentration of 2,4-D. Notably, root elongation, root number and root surface area varied across the different concentrations of auxin hormones. Results in this study indicated that the highest rooting mean number was observed in MS medium fortified with NAA2 with a maximum 25.8 ± 2.8 roots. This result was statistically different from all other treatments including the control (MS without auxins). The rooting mean number in NAA increased with increasing concentration. The second highest mean number of roots was observed with NAA 1.5 mg/L 22.4 ± 1.43, followed by MS medium fortified with IAA 1.5 mg/L with 21.2 ± 1.32, which was followed by NAA 1.0 mg/L 25 ± 1.58 (Fig. 8A). However, the highest root length recorded after six weeks of shoot tip culture was observed in MS medium fortified with IAA 1.5 mg/L measuring 8.76 cm ± 0.85. This root length was significantly different from all other medium-auxin combinations (Fig. 8B). This aligns with the findings by (Komakech et al., 2020b) that registered 1.5 mg/L in IAA with the longest roots. The significance of IAA concentrations to regenerate longest roots as compared to other auxin hormones have been recorded in the study about *Polygonum multiflorum* (Kang et al., 2018). In IAA treatments, root length initially increased with rising concentrations up to the optimum point, beyond which it declined as indicated in (Fig. 8B). The trend of increasing length of roots in IAA until the optimized concentration then start to decline was registered in studies *Polygonum multiflorum* (Kang et al., 2018), and *Apios americana* Medik (Gang et al., 2024b). Regrading root surface area, the highest value was observed in shoots treated with IAA 1.5 mg/L showing a surface area increase of 252.2 cm^2 ± 4.92 after six weeks of culture. This was significantly different from all other hormone concentrations. Similar findings identifying IAA as the optimal auxin for enhancing root surface area have also been reported in studies on *Bellis perennis L.* (Karakas and Turker, 2013), *Stevia rebaudiana* Bertoni (Ahmed et al., 2007).

According to Palta and Watt (2009), a robust and extensive root system is essential for supporting resilient, healthy, and vigorous plant growth. Based on this principle, IAA 1.5 mg/L was selected as the most effective hormone for the subsequent experiments in *A. macrocephala*, as it produced the healthiest roots, with long roots and roots with high surface area. In contrast, MS medium supplemented with 2 mg/L NAA induced a higher number of roots, but these were shorter. As illustrated by (Loveys et al., 2003), numerous but short roots are less effective for plant acclimatization because they provide limited anchorage, reduced nutrient absorption capacity, and poorer water uptake during the transition to ex vitro conditions. For this reason, plants with longer, well-developed roots were preferred over those with many short roots.

Acclimatization of rooted *in vitro*-regenerated plants is an important step to ensure their successful transition from the *in vitro* environment to field conditions, requiring a period of adaptation (Sharma et al., 2023). The process helps the plants adjust to fluctuating external factors such as temperature, humidity, and light intensity, which they may not have encountered in the sterile culture environment. This adaptation phase is essential for reducing transplant shock and enhancing the plant's ability to thrive and grow in the field, ensuring high survival rates and improved growth post-transplant (Shiwani et al., 2022; Sharma et al., 2023). In the current study, thirty well-rooted and healthy *A. macrocephala* plantlets, derived from the optimized conditions of the Auxin optimization experiments, were carefully removed from the culture medium. The roots were thoroughly washed to eliminate residual agar as illustrated in

(Fig. 1F), and the plantlets were then transferred to sterile horticulture soil mixed with perlite at a ratio of 2:1, placed in plastic pots (22 cm diameter) for the acclimatization process as illustrated in (Fig. 1G). After two months, twenty eight out of thirty plants (93.33%) successfully survived as observed by plant representations in (Fig. 1H). The success of the acclimatization demonstrated the effectiveness of the acclimatization protocol. Furthermore, the plants remained healthy and robust after eight months of continuous growth, indicating their successful transition to *ex vitro* conditions. High survival rates have also been reported for other plant species within the same family, such as 100% survival for *E. scaber* (Abraham and Thomas, 2016), 95.7 for *Aspilia Africana* (Okello et al., 2021a), and 90–100% for *E. alba* (Singh et al., 2012).

Assessment of chlorophyll content, chlorophyll fluorescence and relative water content

Chlorophyll is a key photosynthetic pigment in green plants that plays an essential role in determining a plant's photosynthetic capacity, growth, and overall health. It is located in specialized cellular organelles known as chloroplasts, which are found exclusively in plant cells. Chlorophyll absorbs light energy across different wavelengths to excite electrons, thereby driving the production of chemical energy and nicotinamide adenine dinucleotide phosphate (NADPH). Its content is commonly used as a key indicator of leaf photosynthetic capacity, overall plant health, and growth status (Mandal and Dutta, 2020; Sun et al., 2024). The SPAD-502 plus meter, a portable, non-destructive tool, is commonly employed to measure chlorophyll content due to its rapid and accurate readings (Nurbaiti et al., 2025),. Previous studies have shown that SPAD values correlate well with destructive chlorophyll measurements in several plant species, including *Aspilia Africana* (Okello et al., 2021a), *Prunus Africana (*Komakech et al., 2020b), and Apios Americana Medik (Gang et al., 2024b).

In this study, chlorophyll content in *A. macrocephala* plants was measured using the SPAD-502 Plus meter during the acclimatization period in the greenhouse. In the first week of measurement, the chlorophyll content of *in vitro* regenerated *A. macrocephala* plants was significantly lower than that of the maternal plants. This reduced chlorophyll content was likely due to the decreased photochemical activity in the *in vitro* leaves during the initial stages of acclimatization, similar to observations in *Apios americana* Medik (Gang et al., 2024a). Additionally, the *in vitro*-regenerated plants had younger leaves and were still adapting to the in vivo environment. Mature leaves typically have more stable and higher chlorophyll content than younger leaves, as shown in studies on *A. africana* (Okello et al., 2021a)d *Africana* (Komakech et al., 2020a). As the *in vitro* regenerated *A. macrocephala* plants absorbed minerals from the soil, they adapted to their new environment, and their leaves matured, and their chlorophyll content increased rapidly. Over the 8-week experiment, the *in vitro*-regenerated *A. macrocephala* plants showed a substantial increase in chlorophyll content, which eventually stabilized in later stages, meanwhile, the maternal plant showed a slight increase in chlorophyll content. Initially, maternal plants had higher chlorophyll levels compared to *in vitro*-regenerated plants. However, by the sixth week, the *in vitro*-regenerated plants showed similar level of chlorophyll content with the maternal plant. From weeks

six to eight, chlorophyll content remained relatively stable for both groups. These observations suggest that, like the maternal plants, the *in vitro* regenerated *A. macrocephala* plants exhibited similar rates of photosynthesis and nitrogen content, vital for optimal plant growth and development. A similar trend in chlorophyll content was observed in *A. africana*, where both maternal and *in vitro*-regenerated plants exhibited similar levels of chlorophyll over time (Okello et al., 2021a). The similarity of chlorophyll content between maternal plants and *in vitro*-regenerated plants can be attributed to both plant types exhibiting similar photosynthetic rates and nitrogen assimilation abilities, which directly influence chlorophyll production (Ahmad et al., 2025), (Happy et al., 2025). This indicates that the *in vitro* plants, through the acclimatization process and optimal culture conditions, are able to replicate the metabolic processes of the maternal plants, resulting in similar chlorophyll content and photosynthetic performance.

Variable fluorescence/Maximum fluorescence (F_v/F_m) is a sensitive indicator of photosynthetic activity, representing the maximum efficiency of Photosystem II (PSII) under optimal conditions. It reflects the plant's capacity to capture light energy and convert it into chemical energy during photosynthesis (Lootens et al., 2004). A higher F_v/F_m ratio signifies efficient energy conversion and overall plant health, while a lower ratio suggests potential stress or damage to the photosynthetic apparatus (Song et al., 2022). Chlorophyll fluorescence, which is directly associated with the activity of chlorophyll in the reaction centers of photosystems, is commonly used to assess the rate of photosynthesis. Any disruption or structural changes in the photosystem II pigments result in reduced quantum performance under dark conditions. In this study, the photochemical efficiency of PSII (F_v/F_m) for dark adapted leaves were determined by calculating the measured parameters, following the guidelines by Maxwell and Johnson (2000). The observed F_v/F_m values of 0.51 ± 0.020 to 0.81 ± 0.011 for *in vitro* regenerated and 0.76 ± 0.015 to 0.82 ± 0.0013 for *A. macrocephala* maternal plants, at 8 weeks, indicated efficient photosynthetic performance and were consistent with F_v/F_m values reported for other plant species. The minimal variation in F_v/F_m values between the two plant types suggests that their photosynthetic rates are closely aligned. According to Roux et al. (2017), plants that are not experiencing stress in their development, have F_v/F_m values normally ranging between 0.75 and 0.85. This indicates that both the *in vitro* regenerated and *A. macrocephala* maternal plants grew under favorable tress free conditions. The lower F_v/F_m values observed in the first two weeks for *in vitro* regenerated *A. macrocephala* plants can be attributed to poorly differentiated photosynthetic tissues in the early stages of culture. However, as the plants were exposed to the external environment and leaf mesophyll differentiated, photosynthetic tissues became more developed, allowing the plants to fully adapt and perform photosynthesis. Previous studies have utilized chlorophyll fluorescence in PSII to evaluate photosynthetic performance in other plants like *Lissoclinum patella* (Schreiber et al., 1997), and *Aspilia africana* (Okello et al., 2021a). The dark-adapted F_v/F_m values, therefore, serve as a reliable measure of photosynthetic potential.

The relative water content (RWC) of a leaf, also known as relative turgidity, is an indicator of the leaf's hydration level, representing the actual water content in relation to its maximum water-holding capacity when fully turgid (Yin and Dong, 2024). According to Ehrler and Nakayama (1984), high RWC (ranging

between 80% to nearly 100%) indicates that the plant is well-hydrated, with its cells filled with water, and is able to maintain turgor pressure, which is typical of healthy, well-watered plants. While low RWC (ranging from 60% below) suggest that the plant is experiencing water loss or dehydration, meaning that it has less water content that it could potentially hold. This often happens due to water stress or drought, damage of cell structures and reduced turgor pressure (Farooq et al., 2009; Trueba et al., 2019). Additionally, according to Polivanova and Bedarev (2022), performing an RWC test helps quantify the degree of hyperhydricity and assess plant water balance in tissue culture conditions. In this experiment, the RWC of *A. macrocephala* maternal plants ranged between 80.1–82.3%, while those for the *in vitro*-regenerated plants ranged between 79.6–82.8%. Given that both sets of plants show high RWC values, the experiment suggests that both the maternal plants and *in vitro*-regenerated plants are maintaining adequate hydration and are not experiencing significant water stress or dehydration. Importantly, the results also suggest that the *in vitro* regeneration process did not induce hyperhydricity in the regenerated plants, as their water-holding capacity remains comparable to that of the maternal plants. This is important for their growth and development. Maintaining an appropriate RWC is essential for normal plant growth and development (Nam et al., 2020), therefore, the absence of hyperhydricity in this study, indicates that the *in vitro*-regenerated plants are physiologically healthy, capable of normal growth and development, demonstrating the reliability of this *in vitro*-propagation protocol of *A. macrocephala*.

Genetic stability (Flow cytometry) analysis of A. macrocephala

Flow cytometry (FCM) has been a valuable tool for estimating plant DNA content since the 1980s, particularly in plant breeding, including polyploid and hybrid breeding (Sliwinska, 2018; Jedrzejczyk and Rewers, 2020). It has been widely applied to analyze various medicinal plant species in tissue culture that include edible and medicinal *Malva* species (Jedrzejczyk and Rewers, 2020), *Apios americana* (Gang et al., 2024b), and medicinal species in *Kadsura* (Xu et al., 2021). According to Rohela et al. (2022), *In vitro* propagation, a common tissue culture technique for producing uniform plantlets (clones), requires careful attention to genetic stability to ensure the production of genetically consistent and true plantlets. This is because, some studies have indicated that in tissue culture, plant material may exhibit instability in DNA content due to somaclonal variation, which includes mutations, chromosome rearrangements, and polyploidization (Bairu et al., 2011; Ghosh et al., 2021). Therefore, FCM analysis is essential to confirm genetic consistency between *in vitro*-regenerated plants and their maternal counterparts. FCM can accurately assess the genetic stability ploidy level or genome size (Loureiro et al., 2023).

In this study, flocytometry (FCM) analysis revealed that the genome size/ploidy levels of maternal plant (9.0 pg/8,802 Mbp) and *in vitro*-regenerated *A. macrocephala* plant (8.92 pg/ 8,723 Mbp) were identical, as indicated in (Fig. 9A and 9B). These results confirm that the regenerated plants maintained the same ploidy and genome size as the maternal plants. The expression in Mbp was calculated using the conversion factor 1pg DNA = 978 Mbp, as per Lepers-Andrzejewski et al. (2011). These results support the reliability of the current study for producing genetically stable *A. macrocephala* plants.

FT-NIR spectra of A. macrocephala tissues

FT-NIR spectroscopy is a non-destructive analytical technique that measures the absorption of near-infrared light by chemical bonds in a sample. It is widely used to assess major chemical composition of plants by identifying key compounds (Manley, 2014). Several studies have used FT-NIR spectroscopy comparing samples from different plant parts (leaves, roots, stems) of *in vitro*-regenerated plants with their maternal counterparts *Aspilia africana* (Okello et al., 2021a), *Prunus africana* (Komakech et al., 2020b), and *Codonopsis pilosula* (Gang et al., 2023). By analyzing the chemical profiles of these samples, researchers can evaluate the biochemical similarities and differences between *in vitro*-regenerated plants and maternal plants. This comparison is important in assessing metabolic consistency, and the potential for producing high-quality, standardized plant material. Such comparative analysis helps refine *in vitro* propagation protocols, ensuring that regenerated plants exhibit similar biochemical characteristics to the maternal plants. This ensures consistency in secondary metabolite production, which is essential for the medicinal value of the plant, and helps optimize culture conditions, media compositions, and other variables for successful and reproducible plant regeneration (Li et al., 2022). Chemical bonds such as C–H, O–H, and N–H exhibit molecular vibrations that produce detectable overtones and combination bands within the NIR region, specifically between 10,000 and 4,000 cm-1. As a result, the unique patterns observed in the NIR region provide insights into the chemical and physical properties of the tested material (Czarnecki et al., 2021; Fiore and Pellerito, 2021).

In this study, the broad band observed at the wavenumber of 8833.6 cm–1 was attributed to the second overtone of C-H stretching vibrations, which are associated with CH_2 and CH_3 groups (Weyer and Lo, 2002). Absorption peak between 6800 and 7,000 cm-1 was attributed to the overtones of O-H stretching, originating from phenolic groups, carboxyl O-H groups, carbohydrates, and water. The absorption at 5,825.6 cm-1 was linked to C–H stretching modes of aliphatic chains and aromatic rings (Weyer and Lo, 2002). A sharp peak at 5,174 cm-1 corresponded to O-H stretching, primarily from water (Jiang et al., 2024). Peaks in the range of 5,000–4,500 cm-1 were associated with a combination of N-H, C-H stretching, and O-H stretching modes linked to proteins (Amanah et al., 2022). Absorption peaks between 4000 and 4545 cm-1 are attributed to combination of band of C-H stretching and bending in ring deformation. The two strongest bands near 4334 and 4260 cm-1 i.e. (4320 and 4212 cm–1) respectively, are assigned to the asymmetric and symmetric modes of the combination of CH stretch and CH_2 bending motions (Weyer and Lo, 2002), while the peak at 4488 cm-1 could be due to the presence of hydroxyl groups containing O-H functional groups (Siddique et al., 2020). The similarity in the spectra of both *in vitro* regenerated and *A. macrocephala* maternal plant samples suggests chemical homogeneity between the two. Additionally, Ward's algorithm was employed to cluster leaf and root samples from both *in vitro* regenerated and maternal *A. macrocephala* plants. This clustering method is widely used to characterize various plant samples. It was found that the roots (MR and IR) *A. macrocephala* exhibited higher homogeneity with each other than with the leaves (ML and IL), forming the first cluster at 0.36%. The *in vitro* regenerated and *A. macrocephala* maternal plant parts showed a high degree of similarity, with roots displaying the least heterogeneity at 0.052, while the leaves had 0.059. The high homogeneity observed in the samples can be attributed to the similarity in chemical composition, as reflected in the FT-NIR spectra, which resulted in smaller separation distances in the

dendrogram. Different plant parts, however, may contain varying concentrations of major compounds, contributing to the observed heterogeneity.

In a similar study by (Okello et al., 2021a), FT-NIR analysis of *A. africana* revealed comparable patterns, with roots of both *in vitro*-regenerated and maternal plants showing the highest homogeneity (0.14), while leaves exhibited slightly greater variation (0.17). The minor heterogeneity observed between similar plant parts may be attributed to age differences, as plant chemical composition is known to vary with developmental stage. Consistently, earlier studies employing Ward's clustering algorithm have also reported heterogeneity when comparing samples from plants of different ages, including findings by (Komakech et al., 2020a).

Conclusion

The present study describes a standard protocol for the mass propagation of a valuable medicinal plant *A. macrocephala* from shoot tips as explants. MS containing vitamins supplemented with 30 g L^{-1} sucrose and 1.5 mg/L BAP regenerated the overall best shoot proliferation results with 17.5 ± 1.21 dark green, healthy shoots after six weeks of shoot tip induction, exceeding the 14.5 and 5.61 shoots reported in previous studies. While MS containing vitamins supplemented with 30 g L^{-1} sucrose and IAA 1.5 mg/L provided the optimum rate 21.2 ± 1.32 of *in vitro* root proliferation, facilitating root elongation of 8.76 cm ± 0.85, and root surface area of 252.2 ± 4.92 after 6 weeks explant induction. The observed similarity in the physiological and chemical composition between the *in vitro* regenerated and *A. macrocephala* maternal plants further confirms that this protocol produces genetically and physiologically stable plants. Importantly, this *in vitro* propagation method enables the mass production of high quality plantlets, offering an effective solution associated with seed-based propagation such as low seed germination rates, slow multiplication cycles, limited seed set even under manual pollination, sensitivity to environmental stress during germination, and the short viability period of *A. macrocephala*. By producing large numbers of uniform plants through *in vitro* propagation, this protocol reduces dependance on natural habitat as a seed source, thereby supporting both conservation efforts and large-scale commercial cultivation. Overall, the study presents a rapid, efficient, and replicable method for direct shoot and root multiplication from shoot tip explants. Its simplicity, high multiplication rate, and ability to produce uniform, disease-free plants make it suitable for commercial-scale propagation, germplasm preservation, and domestication of *A. macrocephala*, meeting the increasing demand in the herbal industry. Additionally, this protocol strengthens the food-medicine continuum, where *A. macrocephala* serves as both a therapeutic and dietary resource. This reliable *in vitro* propagation thus secures a consistent supply of high-quality raw material for herbal medicines and functional food products, meeting growing demand while safeguarding biodiversity and sustainability.

Abbreviations

2-4D: 2,4-Dichlorophenoxyacetic acid, 2ip: Isopentenyl adenine, AMR: *Atractylodes macrocephala* rhizome, BAP: 6-Benzylaminopurine, CHU: Chu's Nutrient Medium, CRB: Completely randomized design,

DJ: Driver and Kuniyuki Walnut Medium, DW: Dry weight, FCM: Flow cytometry, Fm: Maximum fluorescence, FT-NIR: Fourier Transform Near-Infrared Spectroscopy, Fv: Variable fluorescence, FW: Fresh weight, IBA: Indole-3-butyric acid, IAA: Indole-3-acetic acid, KIN: Kinetin, LS: Linsmaier and Skoog Medium, MS: Murashige and Skoog Medium, NAA: Naphthaleneacetic acid, NADPH: Nicotinamide adenine dinucleotide phosphate, PGRs: Plant growth regulators, QL: Quoirin and Lepoivre Medium, RWC: Relative water content, SPAD: Soil Plant Analysis Development, TDZ: Thidiazuron, Tube W: Tube weight, TW: Turgid weight, WPM: Woody Plant Medium.

Declarations

Funding statement This work was supported by Development of Innovative Technologies for the Future Value of Herbal Medicine Resources (KSN2511030), Korea Institute of Oriental Medicine through the Ministry of Science and ICT, Republic of Korea.

Author contribution KH planned, designed and drafted the original research, and formalized the analysis. JM conducted the experimentation of SPAD and chlorophyll content, reviewed and edited the manuscript. RG; supported in research designing, reviewed and edited the manuscript. AH conducted FTNIR analysis, reviewed and edited the manuscript, SY conducted Flowcytometry analysis, YK Supervised research process, supported in project administration, reviewed and edited the manuscript, and supported in funding acquisition. All authors read and approved the final manuscript.

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

Ethics declaration

Ethics declaration not applicable

Competing interests The authors declare that they have no known competing financial interests or personal relationships that could appear to influence the work reported in this paper.

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Figures

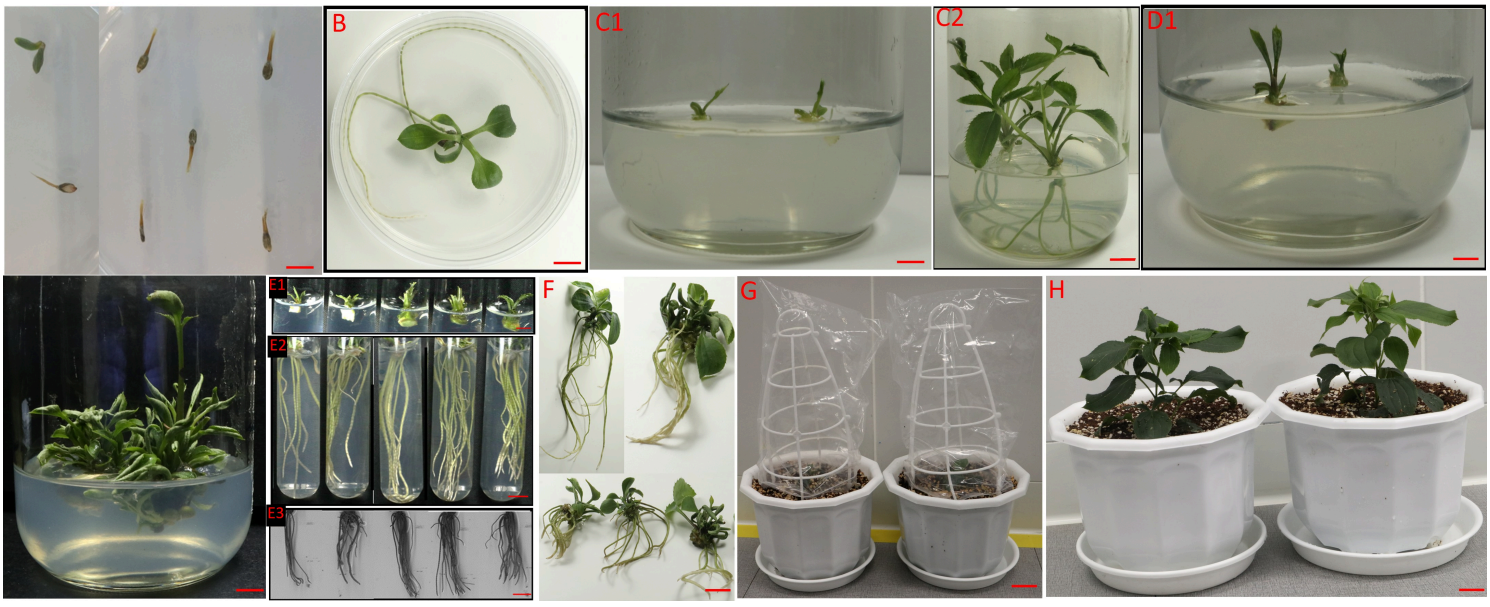


Figure 1

A summary of *in vitro* propagation of *A. macrocephala* plant. (A) Seed spread on MS medium. (B) Four weeks old zygotic plantlet regenerated from seed that was later used for medium optimization test. (C1) Day one of shoot tip segment of *A. macrocephala* during optimization test experiment. (C2) Week six of *A. macrocephala* in optimization test. (D1) Day one of shoot tip segment of *A. macrocephala* during shoot proliferation test. (D2) Week six of *A. macrocephala* in shoot proliferation test. (E1) Day one of shoot tip segment of *A. macrocephala* during root proliferation test. (E2) Week six of *A. macrocephala* in root proliferation test. (E3) Surface area test for week six of *A. macrocephala* during root proliferation test. (F) Plantlets removed from medium and root washed. (G) Regenerated *A. macrocephala* plantlets transferred to soil and covered with polythene bags to maintain moisture (H) Acclimatized potted plants. Note. Bar=1 mm in A. Bar=1 cm in B, C1 and C2, D1 and D2, E1, E2 and E3, F, G, and H.

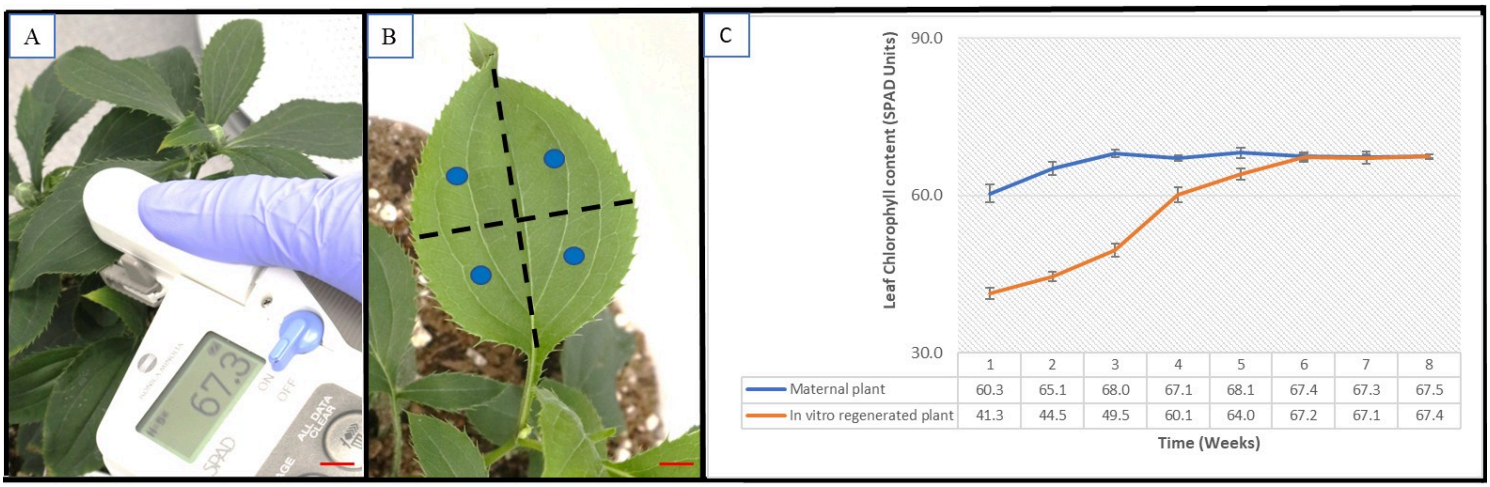


Figure 2

Chlorophyll content measurements of *A. macrocephala in vitro* regenerated and maternal plant leaves; (A) Chlorophyll content of leaf being measured with SPAD meter. (B) Various points on a leaf considered for the average chlorophyll content of each leaf. (C) Chlorophyll contents of *A. macrocephala*maternal plant in comparison to that of the *in vitro* regenerated across 8 weeks. Note. Bar=1 cm.

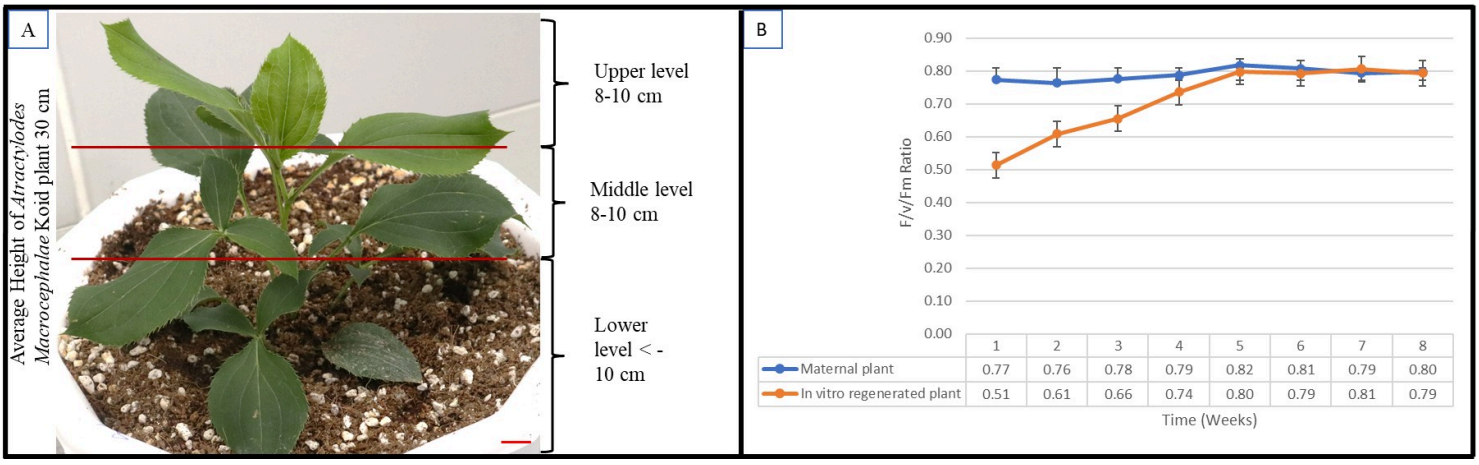


Figure 3

Chlorophyll fluorescence measurements of *A. macrocephala in vitro* regenerated and maternal plant leaves; (A) Sections of the plant where leaves were sourced for measurement of chlorophyll content, (B), Comparison of mean Fv/Fm ration of maternal and *in vitro*-regenerated *A. macrocephala* over a period of 8 weeks. Note. Bar=1 cm.



Figure 4

Measuring leaf relative water content (RWC) of *A. macrocephala*: (A) leaves of *A. macrocephala* with petiole; (B) leaves with top and bottom cut; (C) Weighing leaf and tube; (D) tubes containing leaf sample filled with 1cm of distilled water; (E) carefully blot dry turgid leaf samples picked from the refrigerator; (F) dried leaf samples. Note. Bar=1 cm.

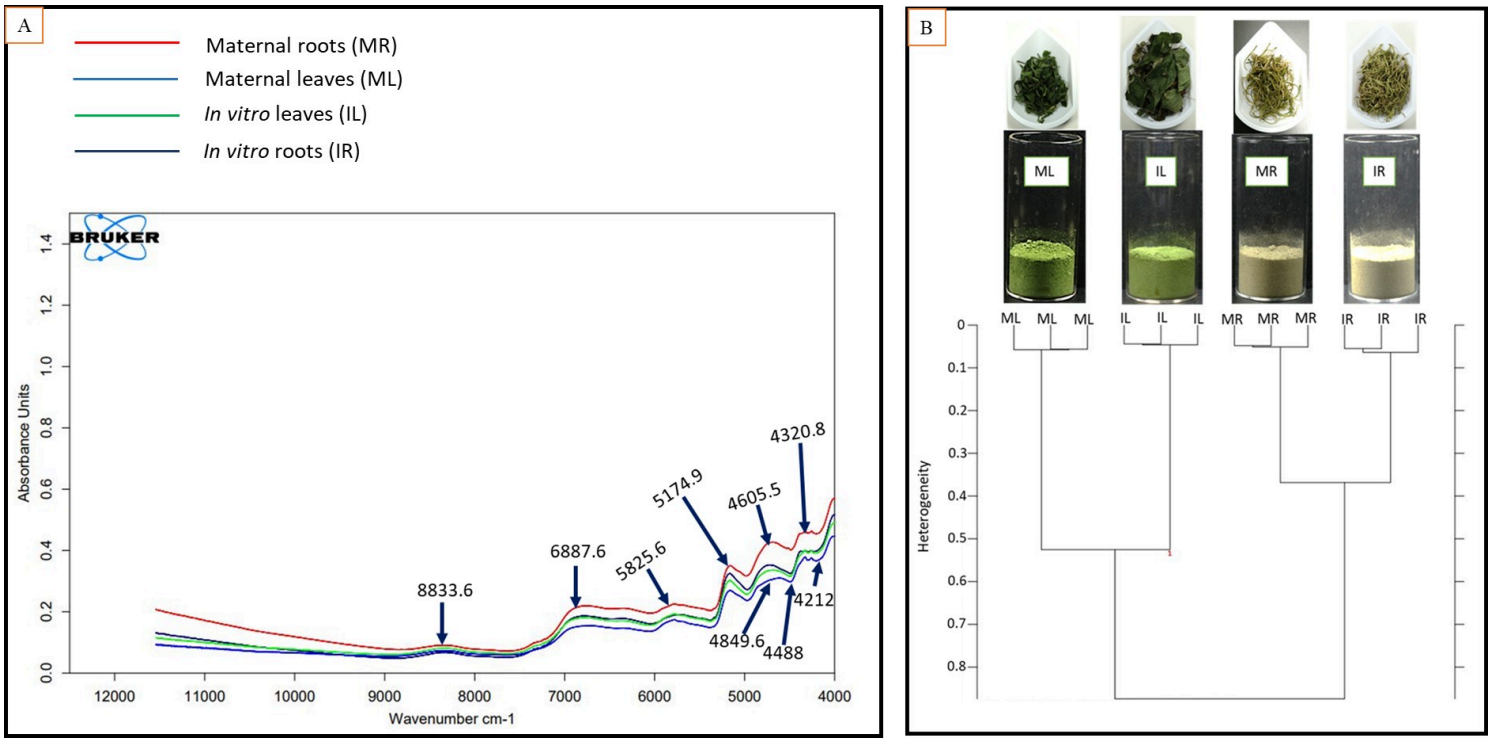


Figure 5

Fourier Transform Near-Infrared (FT-NIR) Analysis; (A) Comparison of FT-NIR spectral profiles of samples from different tissues of *in vitro* regenerated and *A. macrocephala* maternal plants. (B) Clustering Dendrogram of the FT-NIR analysis for different samples. Maternal plant samples: ML (leaf) and MR (root). *In vitro*-regenerated plant samples: IL (leaf) and IR (root).

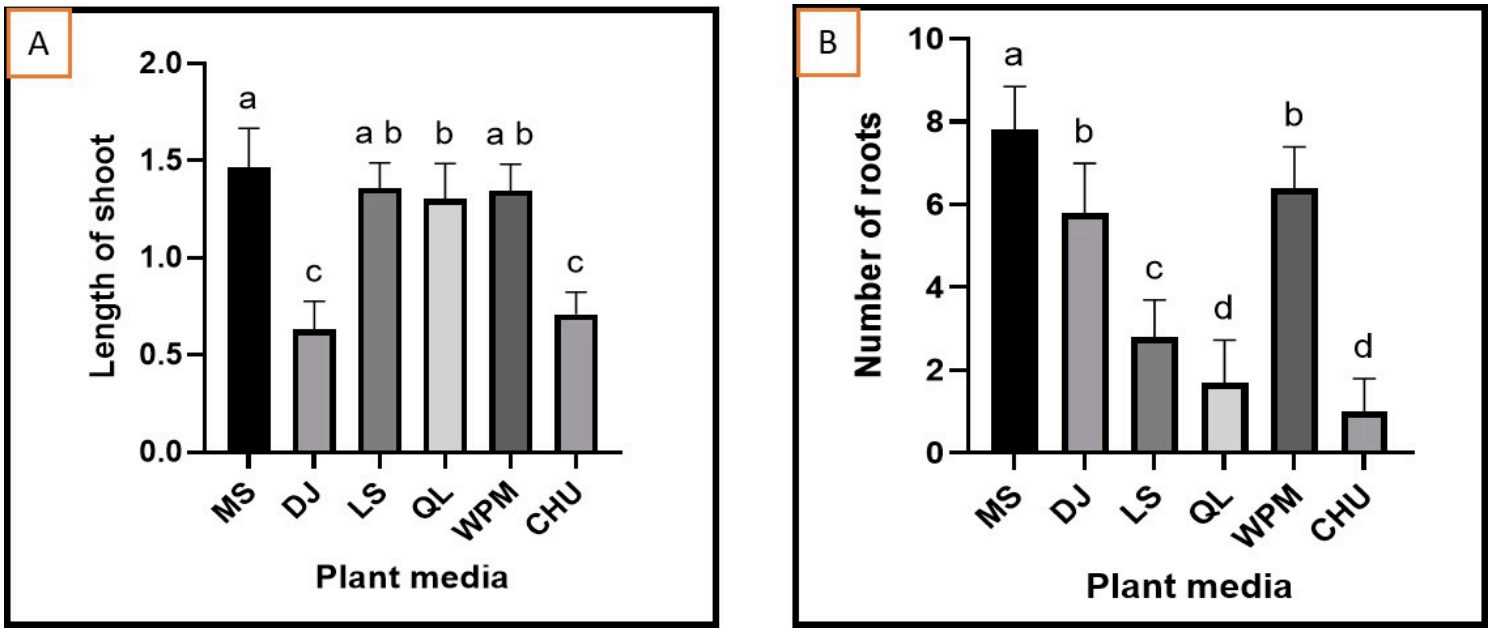


Figure 6

Plant tissue culture media effects on shoot growth of *A. macrocephala* plants from shoot tip explants. (A) Effect on shoot lengths. (B) Effect on root number. Means with the same letter do not differ significantly based on Tukey's test ($p \leq 0.05$).

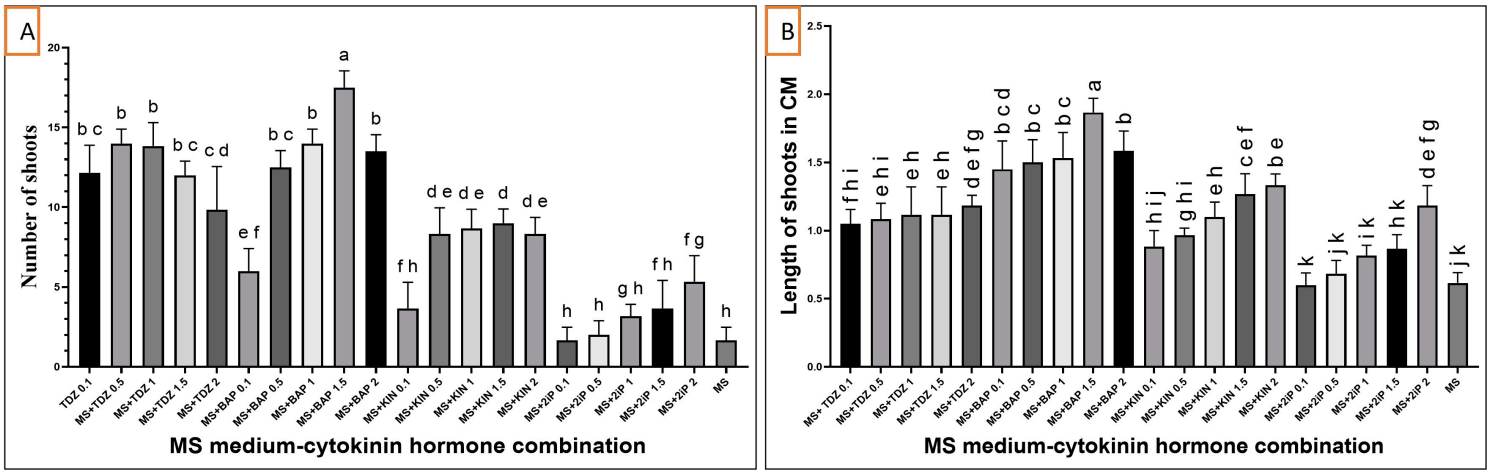


Figure 7

Effects of cytokinin on shoot proliferation of *A. macrocephala* plants from shoot tip explants; (A) Effect on number of shoots; (B) Effect on shoot length. Means with the same letter do not differ significantly based on Tukey's test ($p \leq 0.05$).

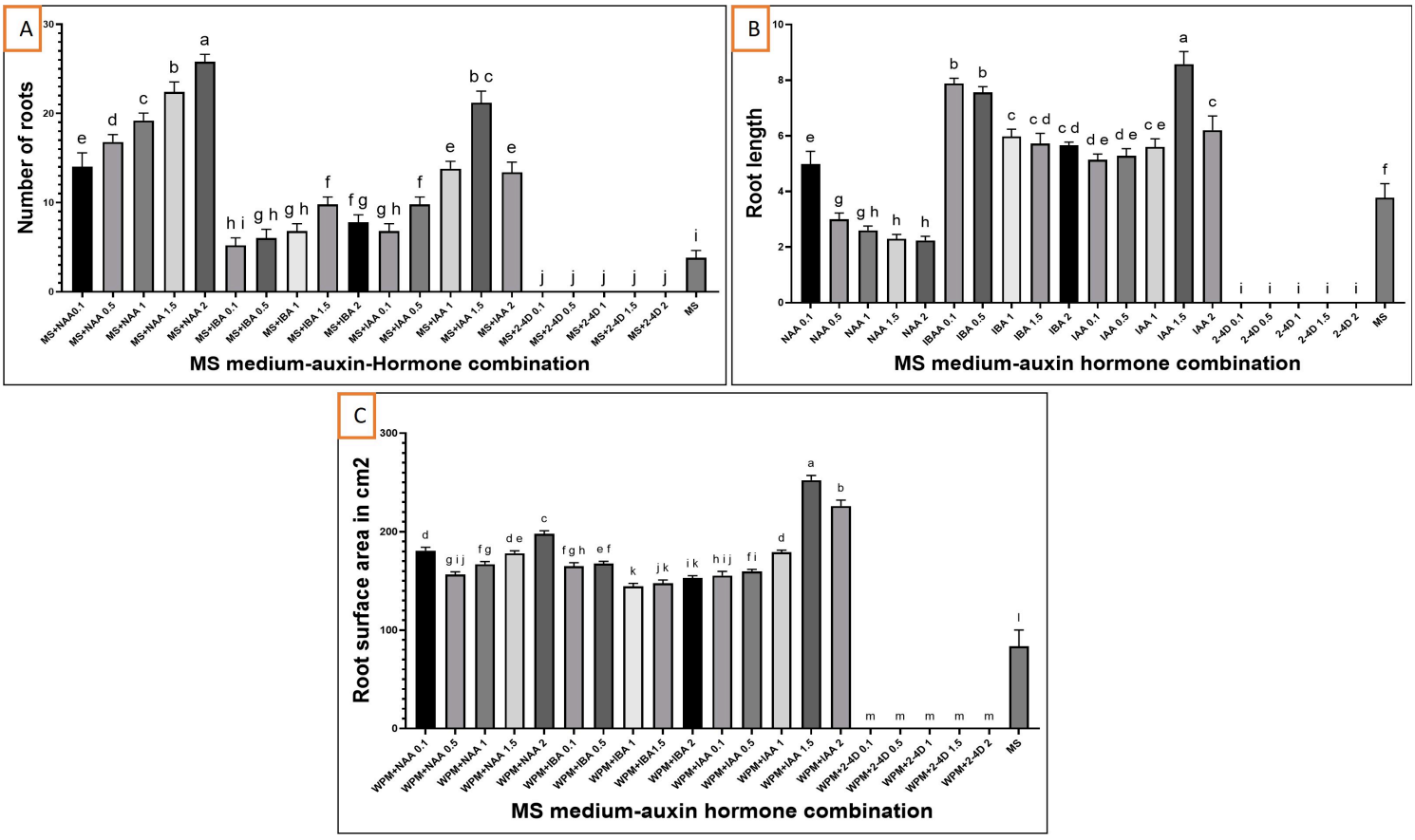


Figure 8

Effects of auxins on *in vitro* rooting of regenerated *A. macrocephala* shoots; (A) Effects on percentage rooting. (B) Effects on number of roots formed per shoot. (C) Effects on root lengths. Means with the same letter do not differ significantly based on Tukey's test ($p \leq 0.05$).

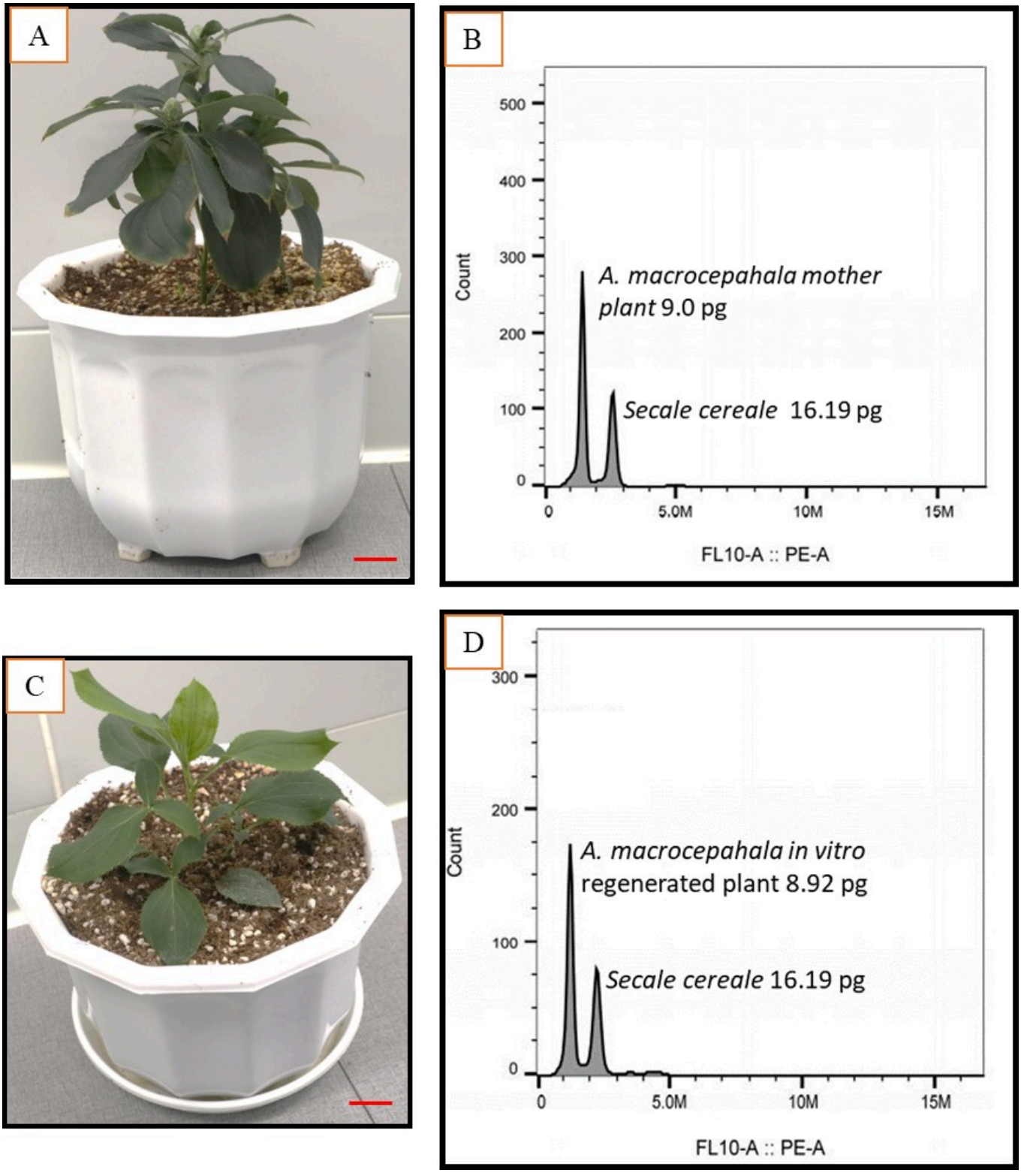


Figure 9

Flow cytometry histogram of fluorescent nuclei isolated from *A. macrocephala*: (A) maternal plant; (B) 2 C-DNA content from maternal plant. (C) *In vitro* regenerated plant. (D) 2 C-DNA content from *in vitro*

regenerated *A. macrocephala* plant. Note. Bar=1 cm.

Supplementary Files

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- GraphicalAbstract.jpg