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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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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, genetic, and chemical 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 cm². 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, and biochemical characteristics to maternal plants. Chemical analysis confirmed comparable chromatographic profiles between regenerated and maternal roots, with the characteristic compound Atractylenolide I detected at similar retention times. 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.

Key message

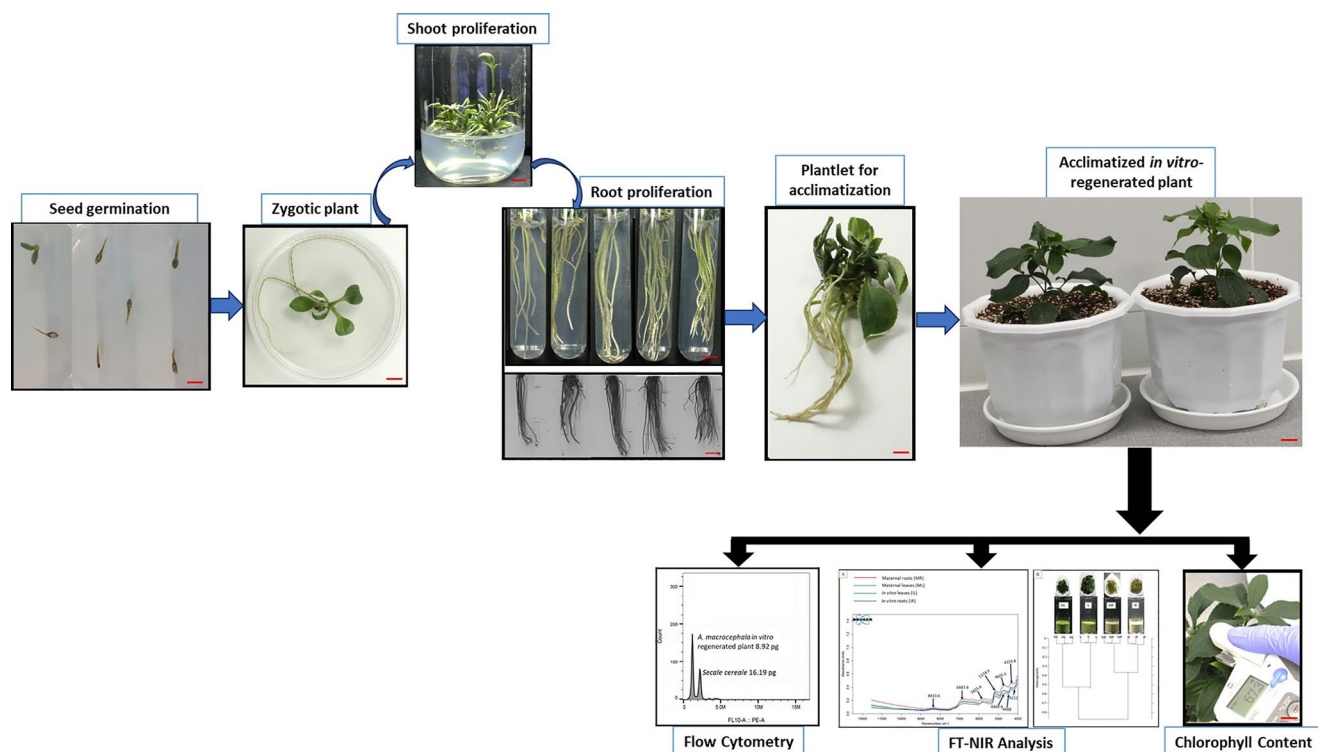
Plant growth regulators enhanced *A. macrocephala* propagation, producing 17.5 shoots and 21.2 roots per explant, with Atractylenolide I compound detected, confirming phytochemical consistency.

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Graphical abstract



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

Abbreviations

2-4D	2,4-Dichlorophenoxyacetic acid
2ip	Isopentenyl adenine
AMR	<i>Atractylodes macrocephala</i> rhizome
BAP	6-Benzylaminopurine
CHU	Chu's Nutrient Medium
CRB	Completely randomized design
DJ	De Greef and Jacobs 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

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. 2025b). *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. 2025b). 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. 2025b). 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. 2025a). 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), *Rhodiola 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 limited range of culture media and a limited evaluation of alternative plant growth regulators (PGRs). Moreover, no comprehensive evaluations have been conducted to compare in vitro-regenerated plants with their maternal counterparts in terms of genetic stability, physiological performance and biochemical characteristics. Such comparisons are important in validating the reliability of in vitro propagation, maintaining the genetic fidelity, biochemical characteristics, 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 Dută-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.

The present study introduces a more integrated approach to the micropropagation of *A. macrocephala*, by systematically optimizing multiple basal media and plant growth regulator (PGR) combinations for efficient shoot and root proliferation, with the aim of improving shoot and root number and length during in vitro propagation. The study further conducted a comprehensive comparative evaluation between in vitro-regenerated plants and their maternal counterparts. Physiological performance was assessed using Soil Plant Analysis Development (SPAD) chlorophyll

readings and FluorPen FP110 measurements to determine chlorophyll pigment content and photosynthetic efficiency, respectively. In addition, biochemical characteristics and chemical composition were examined using Fourier Transform Near-Infrared (FT-NIR) spectroscopic profiling, while high-performance liquid chromatography (HPLC) was employed to evaluate the consistency of valuable secondary metabolites between regenerated and maternal plants. To further investigate potential somaclonal variations arising from tissue culture, genomic stability of the in vitro-regenerated *A. macrocephala* plants was analyzed using flow cytometry. This multi-level assessment provides a more comprehensive validation of in vitro-derived plants than previously reported for this species and offers new insights into the physiological performance, biochemical consistency, and genetic fidelity of regenerated plants, supporting the reliability of this protocol for large-scale cultivation, conservation, and commercial production of this important medicinal species.

This research will contribute significantly to optimizing propagation techniques for *A. macrocephala*, ensuring genetic and biochemical consistency, and providing valuable insights to support the sustainable cultivation and conservation of this food-medicinal resource. 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 min 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 min followed by 1% sodium hypochlorite solution for 15 min 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 min. 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 min. Afterwards, the medium was poured into 90 × 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 (16/8 h photo period) under radiation of 35 μmol m⁻²s⁻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

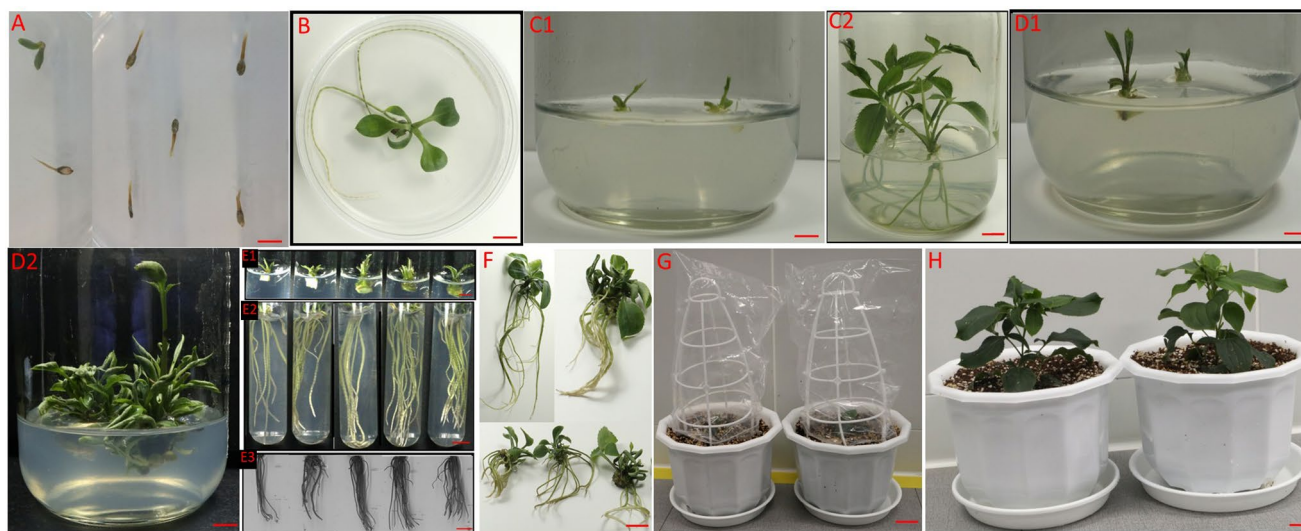


Fig. 1 A summary of in vitro propagation of *A. macrocephala* plant. (A) Seed spread on MS medium. (B) Four weeks old zygotic plantlet regenerated from seed that was later used for medium optimization test. (C1) Day one of shoot tip segment of *A. macrocephala* during optimization test experiment. (C2) 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

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 1011 C, 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 lx illumination with a 16/18 h light/dark photoperiod, using cool white fluorescent lamps providing a light intensity of 33.73 $\mu\text{mol m}^{-2} \text{s}^{-1}$ 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 min. 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 min, 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 1011 C, 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 min. After sterilization, 100 mL of the prepared medium was dispensed into polystyrene culture vessels (125 × 110 mm; Gaooze 1011 C, 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, Quebec, 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.

Assessment of leaf chlorophyll content

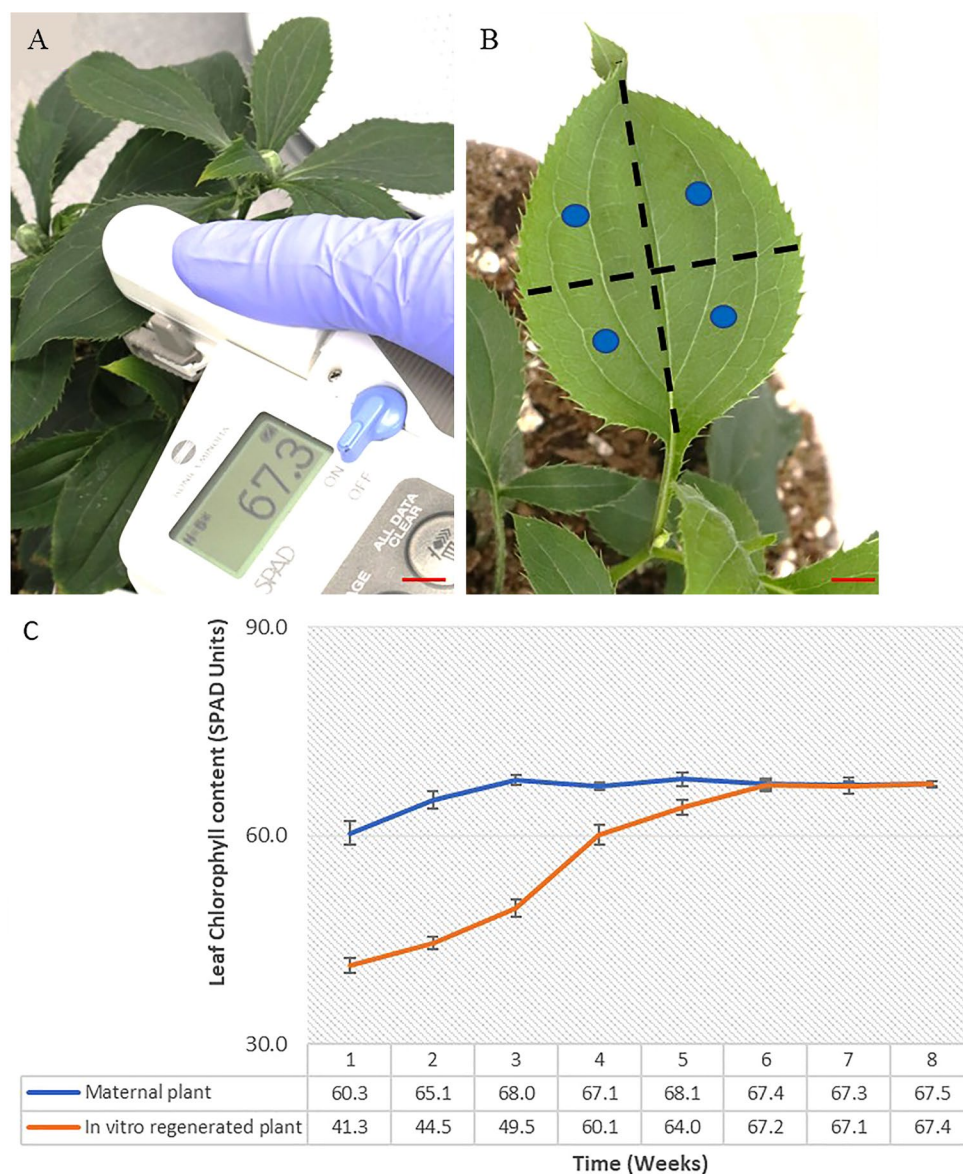
After nine weeks of acclimatization, the leaf chlorophyll content of *in vitro*-regenerated and maternal *A. macrocephala* plants was assessed using a calibrated Soil Plant Analysis Development (SPAD) chlorophyll meter (SPAD-502

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).

Chlorophyll fluorescence measurements

Chlorophyll fluorescence measurements were carried out using the FluorPen FP110 (Drásov 470, 664 24 Drásov,

Fig. 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, SPAD meter=Soil Plant Analysis Development meter



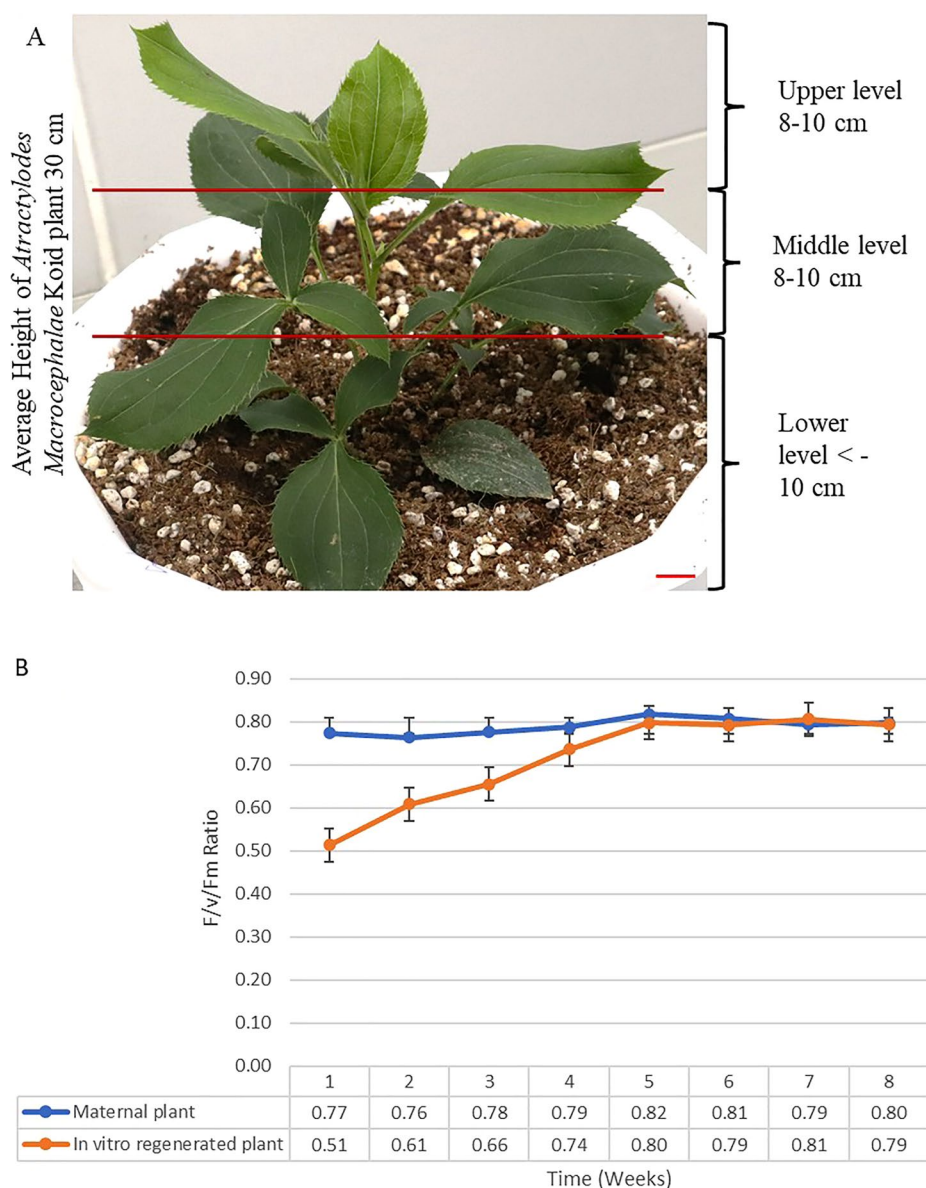
Czechia) following dark adaptation of the leaves for approximately one hour (from 9:00 to 10:00) using leaf clip gaskets. Ten fully expanded leaves were randomly selected from different sections of ten replicates of both *in vitro*-regenerated and maternal plants, and chlorophyll fluorescence was measured 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 $4,000 \mu\text{mol m}^{-2} \text{s}^{-1}$ was applied to determine the Fv/Fm values, following the FluorPen FP110 OJIP protocol. In the dark-adapted state, the minimal chlorophyll fluorescence (F_0), the maximum fluorescence (F_m), and the variable fluorescence (F_v) 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).

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 (Supplementary material 1 A). The top and bottom parts of each leaf were removed, and

Fig. 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 ratio of maternal and *in vitro*-regenerated *A. macrocephala* over a period of 8 weeks. Note. Bar=1 cm, Fv=Variable fluorescence, Fm=Maximum fluorescence



any dead or dying tissue was discarded as illustrated in (Supplementary material 1B). 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 (Supplementary material 1C). Next, 1 cm of distilled water was added to each tube as shown in (Supplementary material 1D), and the tubes were placed in a refrigerator at 4 °C in darkness for 24 h, allowing the leaves to reach full turgidity. After 24 h, the leaves were carefully removed from the tubes, blotted dried with a paper towel as illustrated in (Supplementary material 1E), and weighed (TW; turgid weight). The leaf samples were then placed in labeled envelopes and dried at 60 °C for 24 h or until they reached a constant mass as shown in (Supplementary material 1F), after which the dry weight (DW) was recorded.

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

$$FW = \text{tubeW} + FW - \text{tubeW}$$

Finally, the leaf RWC was calculated using the formula:

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

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

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 2 C 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 s. The resulting suspension was incubated for 30–90 s 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 min. 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:

$$\begin{aligned} & 2C \text{ DNA content of } A. \text{ macrocephala (pg)} \\ &= \frac{\text{Fluorescence intensity of test sample}}{\text{Fluorescence intensity of internal standard}} \times 16.19 \text{ pg} \end{aligned}$$

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=2 C DNA content of the reference standard used for comparison.

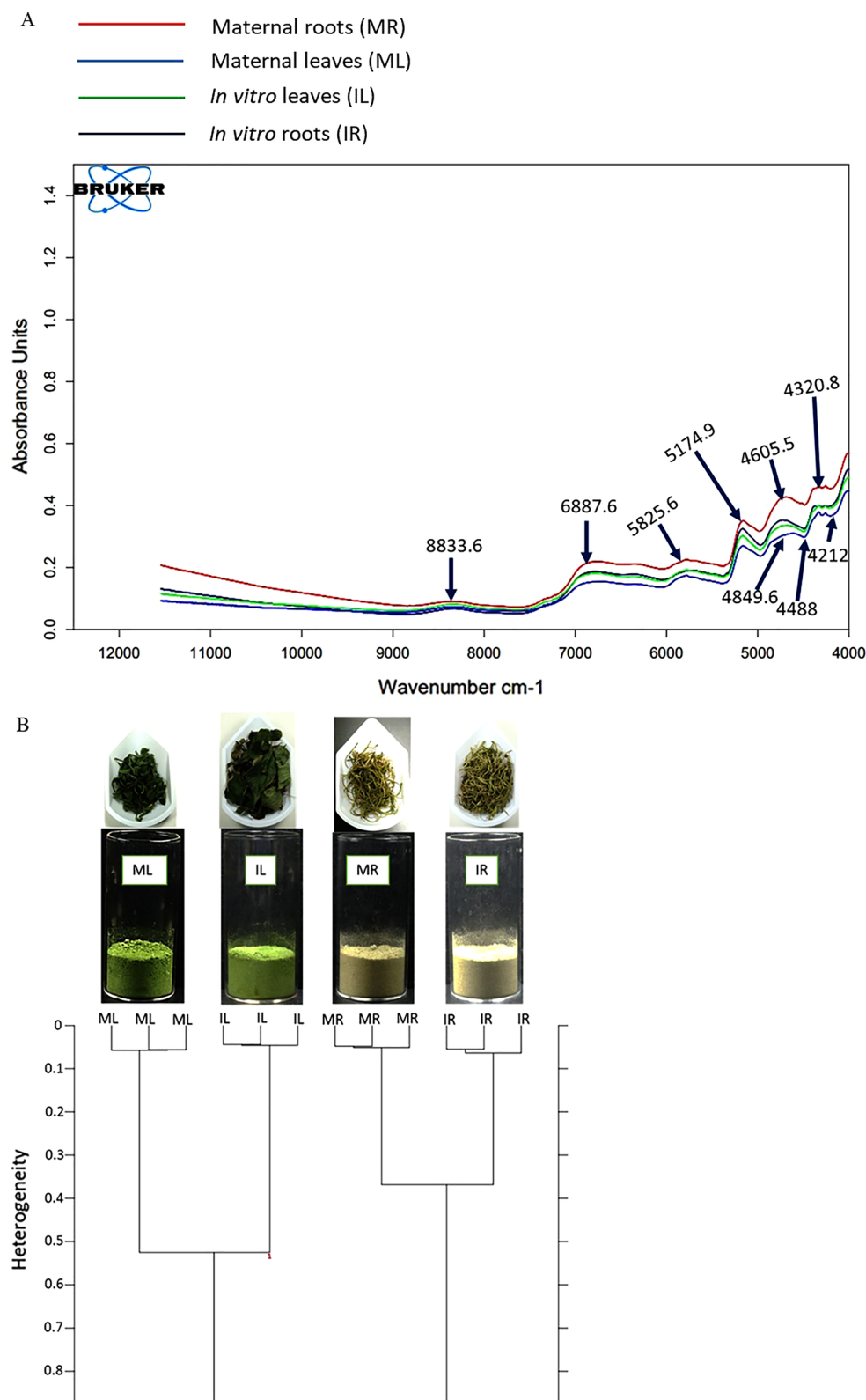
Fourier transform near-infrared (FT-NIR) analysis

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 h, and ground into a fine powder using a 250 g Pulverizing Machine operating at 25,000 rpm (Model RT-N04-2 V, 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. 4A). 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. 4B). 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.

HPLC analysis and identification of atractylenolide I in maternal and in vitro regenerated roots

HPLC analysis was performed on mother plant roots and in vitro regenerated plant roots to determine the presence

Fig. 4 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)



of Atractylenolide I, one of the major bioactive compounds of *A. macrocephala*. Extracts were prepared by dissolving the samples in 70% ethanol and subsequently analyzed using an Agilent Technologies 1260 Infinity HPLC system

(Santa Clara, CA, USA). The concentrations of the mother plant root extract and in vitro regenerated root extract were prepared at 10,000 ppm, while the commercial standard compound of Atractylenolide I was prepared at 100

ppm. HPLC-grade water containing 0.4% acetic acid was used as mobile phase A, while 100% acetonitrile served as mobile phase (B). The gradient program was as follows: 0–12 min, 98% A; 12–30 min, 98–85% A; 30–50 min, 85% A; 50–80 min, 85–5% A; 80–85 min, 5–2% A; 8–90 min, 2–90% A; and 90–110 min, 90–98% A. Chromatographic separation was performed using a C18 column (Agilent Zorbax Eclipse Plus, 250 × 4.6 mm, 5 µm) at a flow rate of 0.8 mL min⁻¹. The column temperature was maintained at 30 °C, and detection was carried out at 280 nm. Data acquisition and analysis were performed using EZChrom Elite software. Identification of Atractylenolide I bioactive compound in the sample extracts was achieved by comparing the retention time of peaks from maternal plant roots and in vitro regenerated plant roots with that of a commercial Atractylenolide I standard compound.

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.

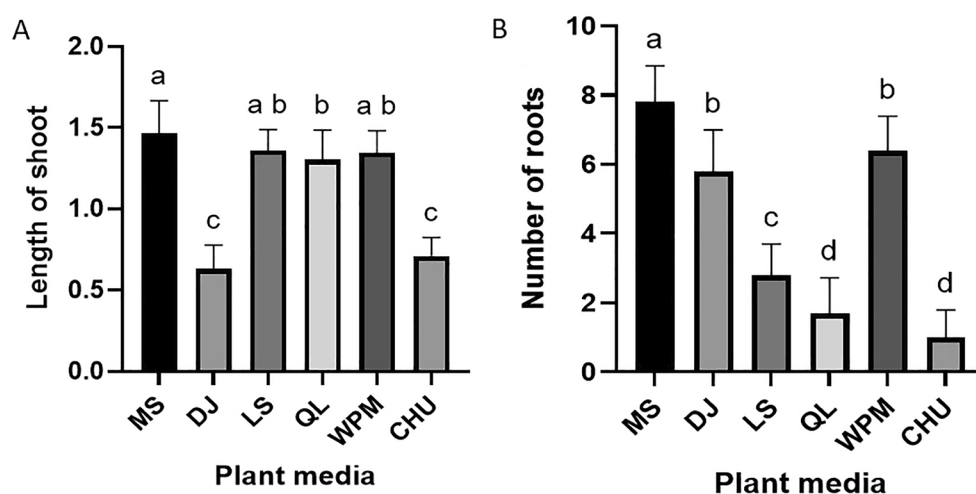
Effect of growth media on the growth of *A. macrocephala*

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. 5). Notably, MS medium exhibited both the greatest increase in shoot length ($1.47 \pm 10.12\%$), however, the percentage increase in shoot length in MS medium did not significantly differ from the percentage increase recorded in LS ($1.36 \pm 13.03\%$) and WPM ($1.35 \pm 12.78\%$) media as indicated in (Fig. 5A). The lowest percentage increase in shoot length ($0.71 \pm 40.92\%$) 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. 5B). 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.

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.*

Fig. 5 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 ($p \leq 0.05$)



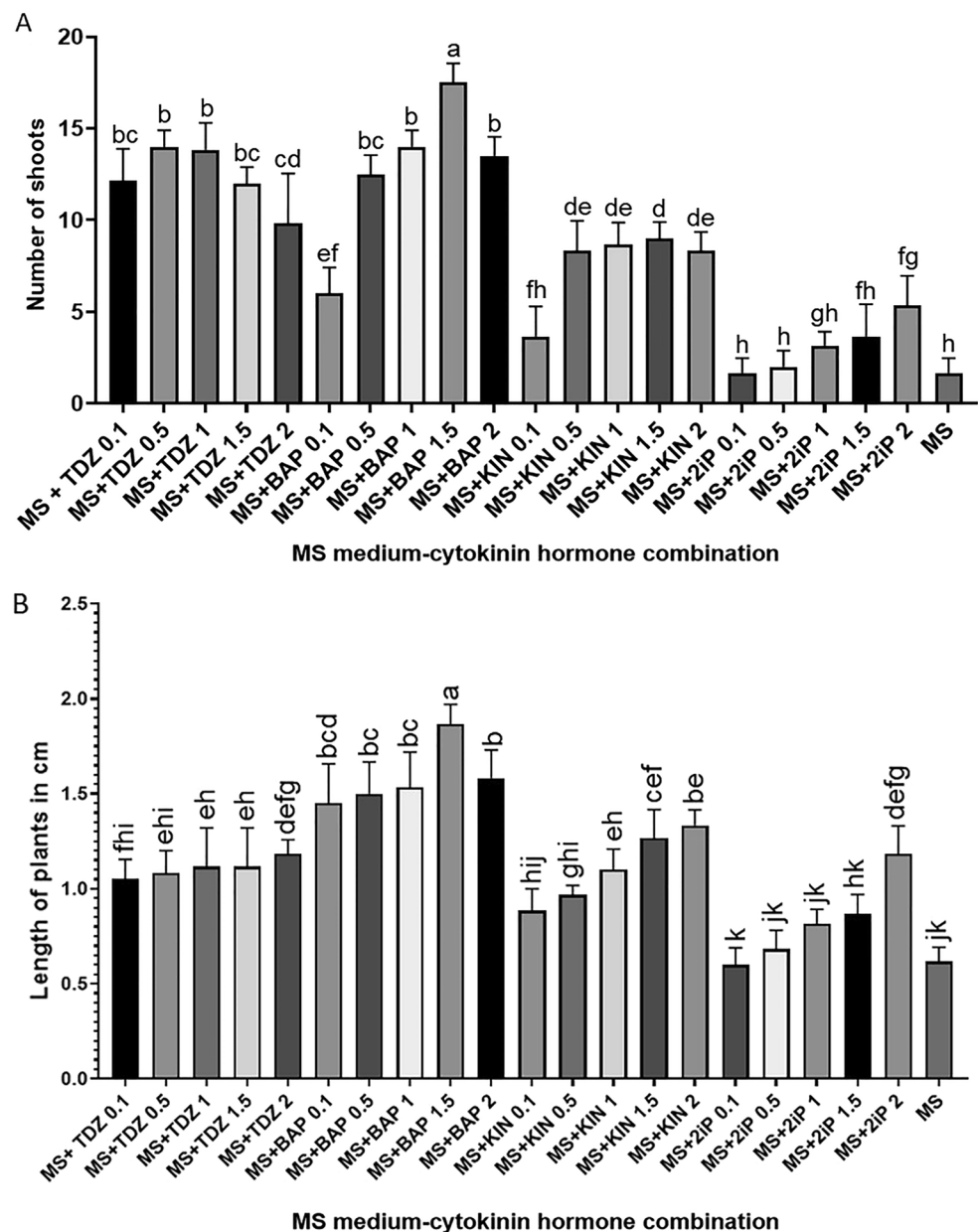
macrocephala shoot with 17.5 ± 1.21 shoots generated from 1 shoot apex as indicated in (Fig. 6A). The lowest shoot proliferation occurred with 2iP at 0.1 mg/L (1.7 ± 0.82 shoots). However, this was not significantly different from MS medium containing 2iP (0.5–1.5 mg/L), KIN (0.1 mg/L), or the control MS medium, which produced 2.0–3.7 shoots. These treatments did not significantly enhance shoot regeneration, indicating limited effectiveness in promoting shoot proliferation in *A. macrocephala* shoot tips. MS medium supplemented with BAP (1.5 mg/L) produced the greatest increase in shoot length ($320.0 \pm 11.93\%$), significantly outperforming other cytokinin treatments. The lowest shoot elongation occurred with 2iP (0.1 mg/L) at $177.90 \pm 40.76\%$. However, this was not significantly different from other 2iP

concentrations (0.5–1.5 mg/L) and the control MS medium, indicating limited effectiveness in promoting shoot elongation in *A. macrocephala* shoot tips. Consequently, MS with 1.5 mg/L BAP was identified as the optimal treatment. 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.

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

Fig. 6 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 ($p \leq 0.05$)



concentrations (0.1, 0.5, 1.0, and 2.0 mg/L) to induce root multiplication and elongation. 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. The rooting mean number in NAA increased with increasing concentration (Fig. 7A). 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 $8.76 \text{ cm} \pm 0.85$. This root length was significantly different from all other medium-auxin combinations (Fig. 7B). Whereas for root surface area, the highest increase was observed in MS medium fortified with IAA 1.5 mg/L, showing a surface area increase of $252.2 \text{ cm}^2 \pm 4.919$ after six weeks of shoot tip culture as indicated in (Fig. 7C). This was significantly different from all other treatments. 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.

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).

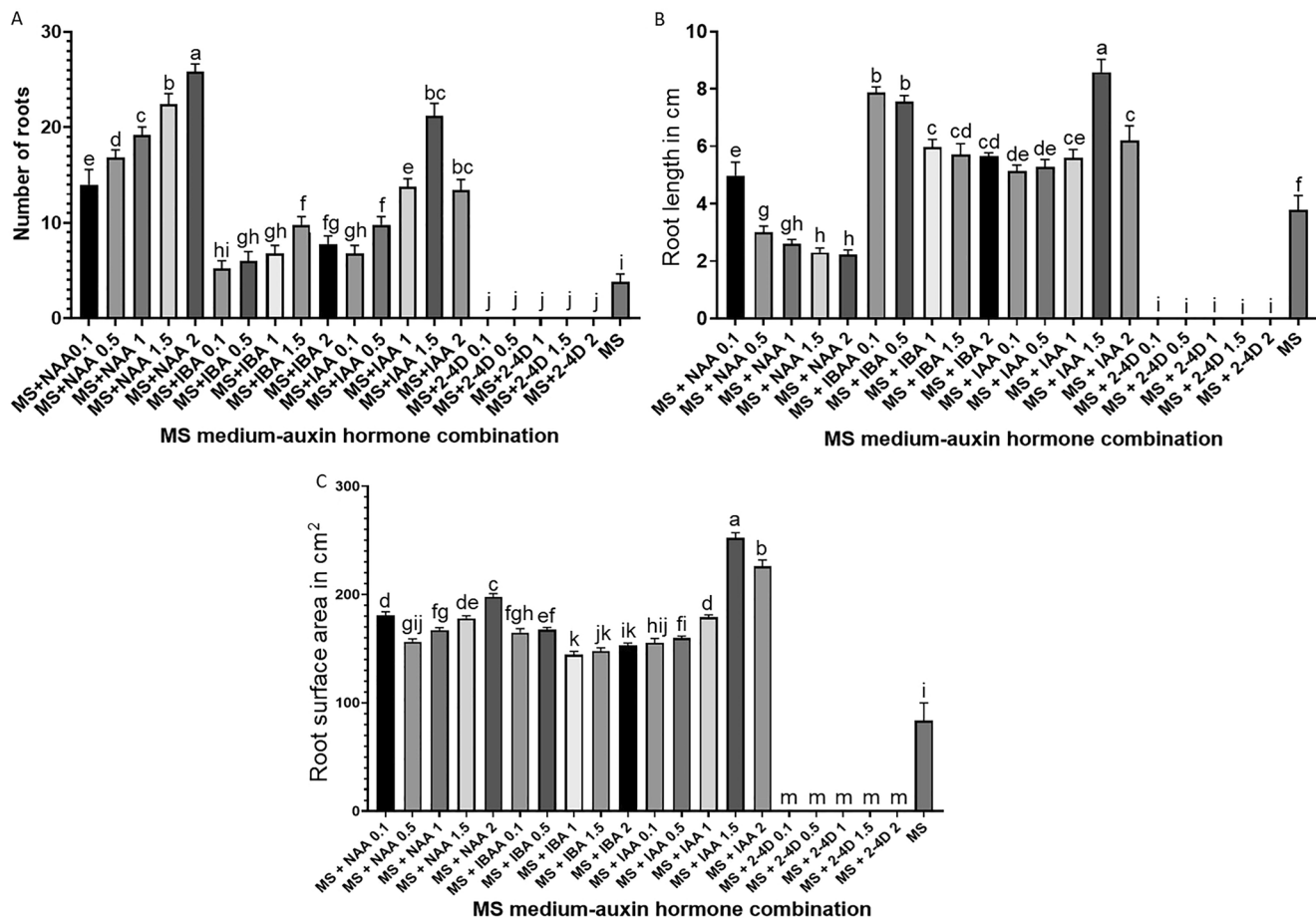


Fig. 7 Effects of auxins on in vitro rooting of regenerated *A. macrocephala* shoots; (A) Effects on percentage rooting. (B) Effects on number of roots formed per shoot. (C) Effects on root lengths. Means with the same letter do not differ significantly based on Tukey's test ($p \leq 0.05$)

Chlorophyll fluorescence 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 $80.1 \pm 82.3\%$, while those for the in vitro-regenerated plants ranged between $79.6 \pm 82.8\%$ (Table 1).

Flow cytometry analysis

The flow cytometry histogram indicated that the ploidy levels of both maternal (Fig. 8A) and in vitro regenerated (Fig. 9C) *A. macrocephala* plants were identical. The 2 C DNA content of the maternal plant was 9.0 pg (Fig. 8B), while that of the in vitro regenerated plant was 8.92 pg

(Fig. 8D). The 9.0 pg of 2 C DNA content in the maternal plant corresponded to a genome size (2 C) of 8,802.0 Mbp, whereas the 8.92 pg of 2 C DNA content in the regenerated plant equated to a genome size (2 C) 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).

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. 4). A peak at 5174.9 cm^{-1} was observed in all leaf and root samples (Fig. 4A). Additionally, both in vitro regenerated and maternal samples showed peaks at 5,826.6, 6887.6, and 8,833.6 cm^{-1} (Fig. 4A). Between 9,000 and 4,000 cm^{-1} , seven prominent peaks were detected for all samples (Fig. 4A). Clustering analysis using Ward's algorithm revealed the overall heterogeneity value for all samples being of 0.89 (Fig. 4B), which was followed by the heterogeneity values of ML and IL that was 0.53 (Fig. 4B). 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. 4B), ML and IL registered the highest dissimilarity.

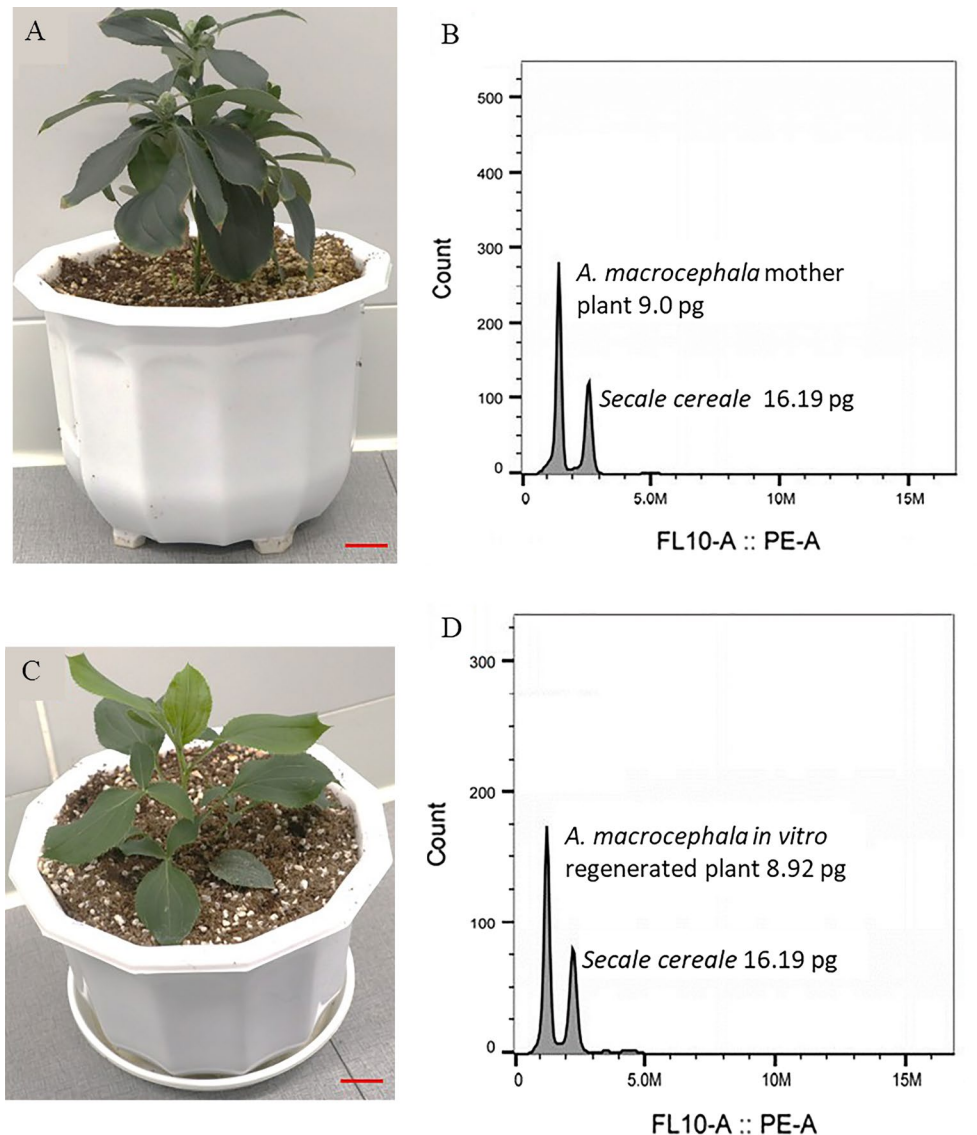
HPLC analysis and identification of atractylenolide I in maternal and in vitro regenerated roots

The HPLC chromatograms of maternal and in vitro regenerated roots showed similar peak patterns and retention times. A major peak at 78.1 min, corresponding to Atractylenolide I, matched the retention time of the commercial standard

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

Fig. 8 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, 2 C-DNA=Diploid DNA content



compound detected at 280 nm (Fig. 9). These results indicate that both samples contain the same key compound and exhibit consistent phytochemical profiles.

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

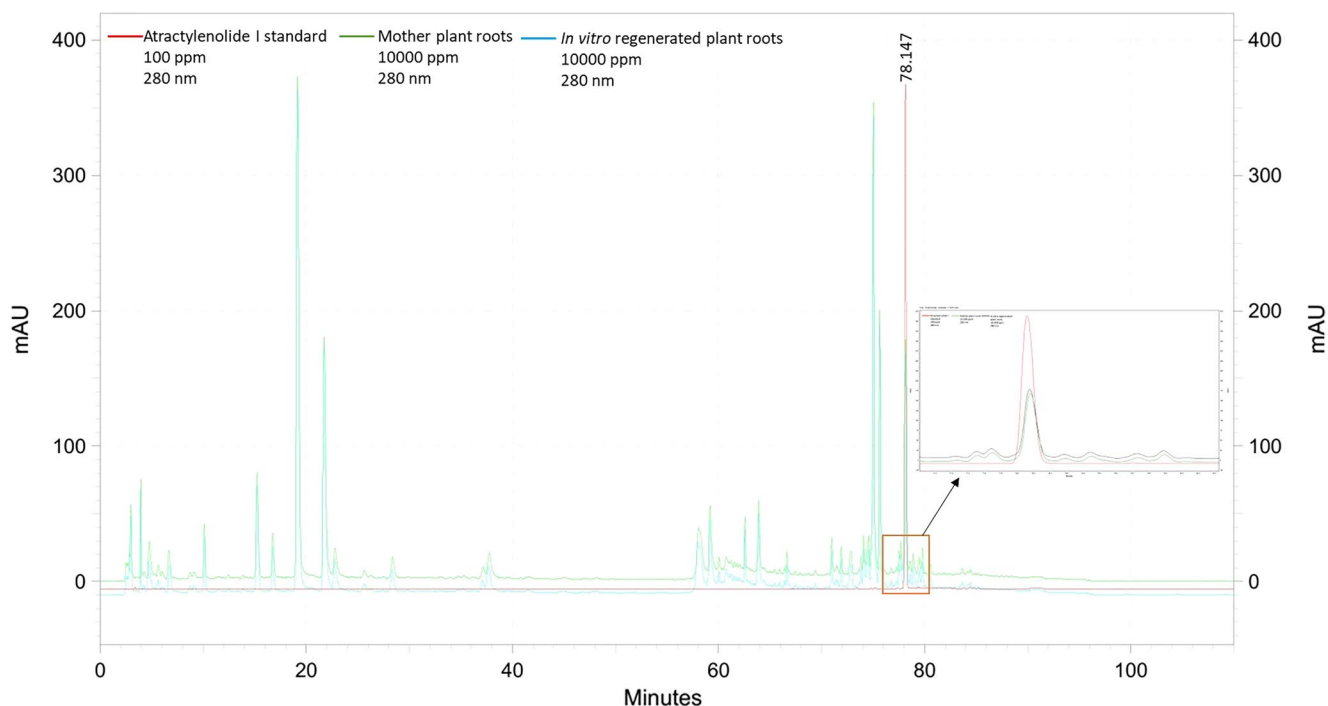


Fig. 9 High-Performance Liquid Chromatography chromatograms of 70% ethanol extracts from roots of in vitro regenerated and maternal *Atractylodes macrocephala*, indicating the presence of the characteristic compound Atractylenolide I detected at 280 nm

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. 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 provide essential mineral nutrients and water required for plant growth and development under in vitro conditions. The composition of the medium is a critical factor influencing the success of in vitro propagation, as it plays a significant role in regulating morphogenesis and enabling plant tissues or cells to develop into 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 example, MS medium contains high concentrations of nitrate and ammonium ions but relatively low sulfate levels. In contrast, QL medium has lower ammonium content, while WPM contains lower nitrate and ammonium but higher sulfate concentrations (Okello et al. 2021a). These compositional differences explain the variations observed in the growth parameters of *A. macrocephala* cultured on different media. Nonetheless, all media supported plant growth to varying extents.

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 ($1.47 \pm 10.12\%$), however, the percentage increase in shoot length in MS medium did not significantly differ from the percentage increase recorded in LS ($1.36 \pm 13.03\%$) and WPM ($1.35 \pm 12.78\%$) media as indicated in (Fig. 5A). 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). Plant height and root numbers 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 “Asteraceae” have been recorded in *Sausurea esthonica* (Gailite et al. 2010), *Susurea medusa Maxim* (Liu and Saxena 2009), *Tripleurospermum insularum* (Inceer et al. 2022), among other plants. However, other studies have demonstrated that certain Asteraceae 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 (Mila-dinova-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 (Grzegorzczak-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).

In this study, addition of various cytokinins at different concentrations in MS medium promoted shoot proliferation and multiplication. Based on shoot number and elongation, MS medium supplemented with BAP (1.5 mg/L) produced the best results for *A. macrocephala*. This treatment generated 17.5 ± 1.21 shoots per shoot apex and showed the greatest shoot length increase ($320.0 \pm 11.93\%$). Plantlets under this condition also developed dark-green leaves, indicating good physiological health and making them suitable for further growth and 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*. 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. Although the species belong to different family other than *A. macrocephala* (Asteraceae), the consistent effectiveness of 1.5 mg/L BAP beyond the current study supports that this concentration could be broadly applicable for shoot induction, suggesting that optimal cytokinin levels may transcend family boundaries under certain culture conditions.

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 precise manipulation in tissue culture media is essential for efficient plant regeneration from small explants, making auxins key to the success of in vitro propagation. Auxin promote root elongation, stimulate the formation of additional roots, and enhance the root surface area of the plant during in vitro propagation (Du and Scheres 2018). Several 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 NAA 2 mg/L with a maximum 25.8 ± 2.8 roots. This result was statistically different from all other treatments. The rooting mean number in NAA increased with increasing concentration (Fig. 7A). 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 $8.76 \text{ cm} \pm 0.85$. This root length was significantly different from all other medium-auxin combinations (Fig. 7B). In this study, under IAA treatments, root length initially increased with rising concentrations up to the optimum point, beyond which it declined as indicated in (Fig. 7B). 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. 2024a). Regrading root surface area, the highest value was observed in shoots treated with IAA 1.5 mg/L showing a surface area increase of $252.2 \text{ cm}^2 \pm 4.92$ 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 these observations, IAA at 1.5 mg/L was selected as the most effective treatment for subsequent experiments in *A. macrocephala*, as it produced the healthiest roots characterized by greater length and a larger 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).

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. 2024b). 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) and *P. 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 stress 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 *A. 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 and 82.3%, while those for the *in vitro*-regenerated plants ranged between 79.6 and 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. 2024a), 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, floctometry (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. 8A and B). 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 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).

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 CH₂ and CH₃ 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 CH₂ 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.

HPLC analysis and identification of atractylenolide I in maternal and in vitro regenerated plant roots

High-performance liquid chromatography (HPLC) is widely used to evaluate the chemical consistency of plants across generations by comparing their metabolite profiles. This technique separates and detects compounds based on their retention times and absorbance, allowing researchers to determine whether regenerated plants maintain the same chemical composition as their maternal source (Allwood and Goodacre 2010).

In the current study, the HPLC chromatograms revealed a strong similarity between the chemical profiles of maternal and in vitro regenerated plant roots of *A. macrocephala*. Both samples exhibited peaks at nearly identical retention times, particularly around 78.1 min, corresponding to Atractylenolide I detected at 280 nm (Fig. 9). The similarity in peak position and pattern suggests that the in vitro regenerated roots retained a secondary metabolite composition comparable to that of the maternal plant. This consistency indicates that the micropropagation protocol did not significantly alter the biosynthesis of key bioactive compounds. Previous studies have reported that Atractylenolide I as one of the major pharmacologically active constituents of *A. macrocephala*, contributing to its anti-inflammatory, gastroprotective, and immunomodulatory properties (Jeong et al. 2019). Therefore, maintaining this compound in regenerated plants is essential for preserving the medicinal value of the species. The comparable chromatographic profiles also suggest that the in vitro propagation system maintained metabolic stability in regenerated plants. Vanisree et al. (2004) reported on several plant species propagated through tissue culture, showing that they consistently produced secondary metabolites comparable to those found in their parent plants. The study highlighted that tissue culture propagation can maintain similar phytochemical profiles across regenerated plants. These results suggest that tissue culture techniques not only preserve metabolite production but may also enhance it under certain conditions. Overall, the HPLC results in the present study demonstrate that the in vitro regenerated plants of *A. macrocephala* possess phytochemical characteristics consistent with the maternal plants. This supports the reliability of this micropropagation protocol for producing uniform plant material suitable for large-scale cultivation and pharmaceutical utilization.

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 dependence 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.

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

Declarations

Ethical approval 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.

References

- Abdolinejad R, Shekafandeh A, Jowkar A, Gharaghani A, Alemzadeh A (2020) Indirect regeneration of *Ficus carica* by the TCL technique and genetic fidelity evaluation of the regenerated plants using flow cytometry and ISSR. *Plant Cell Tissue Organ Cult (PCTOC)* 143:131–144. <https://doi.org/10.1007/s11240-020-01903-5>
- Abraham J, Thomas TD (2016) Recent Advances in Asteraceae Tissue Culture. In: Anis M, Ahmad N (eds) *Plant Tissue Culture: Propagation, Conservation and Crop Improvement*. Springer Sing., Singapore, pp 161–195
- Ahmad Z, Babich O, Sukhikh S, Yadav V, Ramakrishnan M, Firdaus F, Shahzad A (2025) Unlocking the biotechnological potential of *Decalepis arayalpathra*: exploring synthetic seed production, metabolic profiling, genetic stability, and the impact of photosynthetic photon flux density on acclimatization. *BMC Plant Biol* 25:189. <https://doi.org/10.1186/s12870-025-06174-w>
- Ahmed M, Salahin M, Karim R, Razvy M, Hannan M, Sultana R, Hosain M, Islam R (2007) An efficient method for in vitro clonal propagation of a newly introduced sweetener plant (*Stevia rebaudiana* Bertoni.) in Bangladesh. *American-Eurasian J Sci Res* 2:121–125
- Alamgir ANM (2017) Cultivation of Herbal Drugs, Biotechnology, and In Vitro Production of Secondary Metabolites, High-Value Medicinal Plants, Herbal Wealth, and Herbal Trade. *Therapeutic Use of Medicinal Plants and Their Extracts: Volume 1: Pharmacognosy*, A.N.M. Alamgir, ed. Springer Int. Publ., Cham, pp 379–452
- Allwood JW, Goodacre R (2010) An introduction to liquid chromatography–mass spectrometry instrumentation applied in plant metabolomic analyses. *Phytochem Anal* 21:33–47. <https://doi.org/10.1002/pca.1187>
- Amanah HZ, Tunny SS, Masithoh RE, Choung M-G, Kim K-H, Kim MS, Baek I, Lee W-H, Cho B-K (2022) Nondestructive prediction of isoflavones and oligosaccharides in intact soybean seed Using Fourier Transform Near-Infrared (FT-NIR) and Fourier Transform Infrared (FT-IR) Spectroscopic techniques. *Foods*
- Anuruddi HIGK, Nakandalage N, Fonseka DLCK (2023) New Insights for the Production of Medicinal Plant Materials: Ex Vitro and in Vitro Propagation. In: Singh RS (ed) *Biosynthesis of Bioactive Compounds in Medicinal and Aromatic Plants: Manipulation by Conventional and Biotechnological Approaches*, N. Kumar and. Springer Nature Switz., Cham, pp 181–212
- Asghar S, Ghorri N, Hyat F, Li Y, Chen C (2023) Use of auxin and cytokinin for somatic embryogenesis in plant: a story from competence towards completion. *Plant Growth Regul* 99:413–428. <https://doi.org/10.1007/s10725-022-00923-9>
- Bahmanbiglo FA, Eshghi S (2021) The effect of hydrogen sulfide on growth, yield and biochemical responses of strawberry (*Fragaria* × *ananassa* cv. Paros) leaves under alkalinity stress. *Sci Hort* 282:110013. <https://doi.org/10.1016/j.scienta.2021.110013>
- Bairu MW, Aremu AO, Van Staden J (2011) Somaclonal variation in plants: causes and detection methods. *Plant Growth Regul* 63:147–173. <https://doi.org/10.1007/s10725-010-9554-x>
- Bidabadi SS, Jain SM (2020) Cellular, molecular, and physiological aspects of in vitro plant regeneration. *Plants*
- Bopana N, Saxena S (2008) In vitro propagation of a high value medicinal plant: *Asparagus racemosus* Willd. *Vitro Cell Develop Biol - Plant* 44:525–532. <https://doi.org/10.1007/s11627-008-9137-y>
- Chen C, Zheng L, Ma Q, Zhou W-B, Lu Y, Zhao Y-P, Fu C-X (2019) Impacts of domestication on population genetics of a traditional Chinese medicinal herb, *Atractylodes macrocephala* (Asteraceae). *J Syst Evol* 57:222–233. <https://doi.org/10.1111/jse.12446>
- Du Y, Scheres B (2018) Lateral root formation and the multiple roles of auxin. *J Exp Bot* 69:155–167. <https://doi.org/10.1093/jxb/erx223>
- Duta-Cornescu G, Constantin N, Pojoga D-M, Nicuta D, Simon-Grita A (2023) Somaclonal variation—advantage or disadvantage in micropropagation of the medicinal plants. *Int J Mol Sci*
- Dutta Gupta S, Ibaraki Y, Pattanayak AK (2013) Development of a digital image analysis method for real-time estimation of chlorophyll content in micropropagated potato plants. *Plant Biotechnol Rep* 7:91–97. <https://doi.org/10.1007/s11816-012-0240-5>
- Ehrler WL, Nakayama FS (1984) Water stress status in guayule as measured by relative leaf water content. *Crop Sci* 24. <https://doi.org/10.2135/cropsci1984.0011183X002400010014x>
- Farooq M, Wahid A, Kobayashi N, Fujita D, Basra SMA (2009) In: *Sustainable Agriculture E*, Lichtfouse M, Navarrete P, Debaeke S, Véronique, Alberola C (eds) *Plant Drought Stress: Effects, Mechanisms and Management*. Springer Netherlands, Dordrecht, pp 153–188
- Gailite A, Kļaviņa D, Ievinsh G (2010) In vitro propagation of an endangered plant *Saussurea esthonica*. *Environ Exp Bot* 8:43–48
- Gang R, Komakech R, Chung Y, Okello D, Kim WJ, Moon BC, Yim N-H, Kang Y (2023) In vitro propagation of *Codonopsis pilosula* (Franch.) Nannf. using apical shoot segments and phytochemical assessments of the maternal and regenerated plants. *BMC Plant Biol* 23:33. <https://doi.org/10.1186/s12870-022-03950-w>
- Gang R, Rahmat E, Yang S, Okello D, Ban Y, Chung Y, Lee J, Kang Y (2024a) In vitro multiplication and phytochemical evaluation of *Apios americana* Medik for enhanced production of the staple food and tissues with versatile bioactivities. *Sci Hort* 331:113130. <https://doi.org/10.1016/j.scienta.2024.113130>
- Gang R, Rahmat E, Yang S, Okello D, Ban Y, Chung Y, Lee J, Kang Y (2024b) In vitro multiplication and phytochemical evaluation of *Apios americana* Medik for enhanced production of the staple food and tissues with versatile bioactivities. *Sci Hort* 331. <https://doi.org/10.1016/j.scienta.2024.113130>
- Gaurav N, Juyal P, Tyagi M, Chauhan N, Kumar A (2018) A review on in vitro propagation of medicinal plants. *J pharmacognosy phytochemistry* 7:2228–2231
- Ghosh A, Igamberdiev AU, Debnath SC (2021) Tissue culture-induced DNA methylation in crop plants: a review. *Mol Biol Rep* 48:823–841. <https://doi.org/10.1007/s11033-020-06062-6>
- Grzegorzczak-Karolak I, Hnatuszko-Konka K, Krzeminska, Olszewska M, Owczarek MA (2021) A. cytokinin-based tissue cultures for stable medicinal plant production: regeneration and phytochemical profiling of *salvia bulleyana* shoots. *biomolecules* 11:1513 (s Note: MDPI stays neutral with regard to jurisdictional claims in published ...)
- Guariniello J, Iannicelli J, Peralta PA, Escandón AS (2018) In vivo and in vitro propagation of macela: a medicinal-aromatic native plant with ornamental potential. *Ornam Hort* 24:361–370. <https://doi.org/10.14295/oh.v24i4.1238>

- Happy K, Ban Y, Mudondo J, Haniffadli A, Gang R, Choi K-OK, Rahmat E, Okello D, Komakech R, Kang Y (2025) SMART-HERB-ALOMICS: An innovative multi-omics approach to studying medicinal plants grown in controlled systems such as phytotrons. *Phytomedicine* 148:157303. <https://doi.org/10.1016/j.phymed.2025.157303>
- Hayta S, Bayraktar M, Baykan erel S, Gurel A (2017) Direct plant regeneration from different explants through micropropagation and determination of secondary metabolites in the critically endangered endemic *Rhaponticoides mykalea*. *Plant Biosystems - Int J Dealing all Aspects Plant Biology* 151:20–28. <https://doi.org/10.1080/11263504.2015.1057267>
- Hazarika BN (2006) Morpho-physiological disorders in in vitro culture of plants. *Sci Hort* 108:105–120. <https://doi.org/10.1016/j.scienta.2006.01.038>
- Hewlett JD, Kramer PJ (1963) The measurement of water deficits in broadleaf plants. *Protoplasma* 57:381–391. <https://doi.org/10.1007/BF01252067>
- Hussain MJ, Abbas Y, Nazli N, Fatima S, Drouet S, Hano C, Abbasi BH (2022) Root cultures, a boon for the production of valuable compounds: a comparative review. *Plants*
- Inceer H, Cuce M, Imamoglu KV, Ergin T, Ucler AO (2022) In vitro propagation and cytogenetic stability of *Tripleurospermum insularum* (Asteraceae) – a critically endangered insular endemic species from Turkey. *Plant Biosystems - Int J Dealing all Aspects Plant Biology* 156:1213–1221. <https://doi.org/10.1080/11263504.2022.2029969>
- Isah T, Umar S (2020) Influencing in vitro clonal propagation of *Chenemorpha fragrans* (moon) Alston by culture media strength, plant growth regulators, carbon source and photo periodic incubation. *J Forestry Res* 31:27–43. <https://doi.org/10.1007/s11676-018-0794-3>
- Jain P, Pandey R, Shukla SS (2015) Natural Sources of Anti-inflammation. In: Jain P, Pandey R, Shukla SS (eds) *Inflammation: Natural Resources and Its Applications*. Springer India, New Delhi, pp 25–133
- Jayusman, Hakim L, Dalimunthe A (2022) Season, basal media and plant growth regulators effect in wood plant in vitro propagation: a comprehensive review. *IOP Conference Series: Earth and Environmental Science* 1115, 012051 <https://doi.org/10.1088/1755-1315/1115/1/012051>
- Jedrzezyk I, Rewers M (2020) Identification and genetic diversity analysis of edible and medicinal malva species using flow cytometry and ISSR molecular markers. *Agronomy*
- Jeong D, Dong G-z, Lee HJ, Ryu J-H (2019) Anti-Inflammatory Compounds from *Atractylodes macrocephala*. *Molecules* 1859
- Jiang H, Zhang C, Yuan W, Zhou Y, Jiang X, Zhou H (2024) The utility of Fourier transform near-infrared spectroscopy to identify geographical origins of Chinese pears. *J Food Meas Charact* 18:2674–2684. <https://doi.org/10.1007/s11694-023-02346-0>
- Kang Y, Lee K, Choi J, Komakech R, Min J, Ju S, Kim SW, Youn C, Kim YG, Moon BC (2018) Maximizing seedling and root tuber production in *Polygonum multiflorum* for use in ethnomedicine. *South Afr J Bot* 119:119–131. <https://doi.org/10.1016/j.sajb.2018.08.016>
- Kapchina-Toteva V, van Telgen H-J, Yakimova E (2000) Role of Phenylurea Cytokinin CPPU in Apical Dominance Release in In Vitro Cultured *Rosa hybrida* L. *J Plant Growth Regul* 19:232–237. <https://doi.org/10.1007/s003440000013>
- Karakas FP, Turker AU (2013) An efficient in vitro regeneration system for *Bellis perennis* L. and comparison of phenolic contents of field-grown and in vitro-grown leaves by LC-MS/MS. *Ind Crops Prod* 48:162–170. <https://doi.org/10.1016/j.indcrop.2013.04.008>
- Khan N, Ahmed M, Hafiz I, Abbasi N, Ejaz S, Anjum M (2015) Optimizing the concentrations of plant growth regulators for in vitro shoot cultures, callus induction and shoot regeneration from calluses of grapes. *Oeno One* 49:37–45. <https://doi.org/10.20870/oeno-one.2015.49.1.95>
- Komakech R, Kim Y-G, Kim WJ, Omujal F, Yang S, Moon BC, Okello D, Rahmat E, Kyeyune GN, Matsabisa MG (2020a) A micropropagation protocol for the endangered medicinal tree *Prunus africana* (Hook f.) Kalkman: genetic fidelity and physiological parameter assessment. *Front Plant Sci* 11. <https://doi.org/10.3389/fpls.2020.548003>
- Komakech R, Kim Y-g, KIM WJ, Omujal F, Yang S, Moon BC, Okello D, Rahmat E, Kyeyune N, Matsabisa G, M.G., and, Kang Y (2020b) A micropropagation protocol for the endangered medicinal tree *Prunus africana* (Hook f.) Kalkman: genetic fidelity and physiological parameter assessment. *Front Plant Sci* 11–2020. <https://doi.org/10.3389/fpls.2020.548003>
- Lee DH, Son Y, Jang JH, Jeong DH, Kim H-J, Kim JA (2025) Environmental effects on *Atractylodes macrocephala* rhizome growth and compounds. *Agriculture*
- Lepers-Andrzejewski S, Siljak-Yakovlev S, Brown SC, Wong M, Dron M (2011) Diversity and dynamics of plant genome size: An example of polysomaty from a cytogenetic study of Tahitian vanilla (*Vanilla x tahitensis*, Orchidaceae). *Am J Bot* 98:986–997. <https://doi.org/10.3732/ajb.1000415>
- Li L, Zhao Y, Li Z, Wang Y (2022) A strategy of fast evaluation for the raw material of Tiepi Fengdou using FT-NIR and ATR-FTIR spectroscopy coupled with chemometrics tools. *Vib Spectrosc* 123:103429. <https://doi.org/10.1016/j.vibspec.2022.103429>
- Liu C-Z, Saxena PK (2009) *Saussurea medusa* Cell Suspension Cultures for Flavonoid Production. In: Jain SM, Saxena PK (eds) *Protocols for In Vitro Cultures and Secondary Metabolite Analysis of Aromatic and Medicinal Plants*. Humana, Totowa, NJ, pp 53–59
- Liu C, Wang S, Xiang Z, Xu T, He M, Xue Q, Song H, Gao P, Cong Z (2022) The chemistry and efficacy benefits of polysaccharides from *Atractylodes macrocephala* Koidz. *Front Pharmacol* 13–2022. <https://doi.org/10.3389/fphar.2022.952061>
- Lootens P, Van Waes J, Carlier L (2004) Effect of a Short Photoinhibition Stress on Photosynthesis, Chlorophyll a Fluorescence, and Pigment Contents of Different Maize Cultivars. Can a Rapid and Objective Stress Indicator be Found? *Photosynthetica* 42:187–192. <https://doi.org/10.1023/B:PHOT.0000040589.09614.a0>
- Loureiro J, Čertner M, Lučanová M, Sliwinska E, Kolář F, Doležel J, Garcia S, Castro S, Galbraith DW (2023) The Use of Flow Cytometry for Estimating Genome Sizes and DNA Ploidy Levels in Plants. In: Heitkam T, Garcia S (eds) *Plant Cytogenetics and Cytogenomics: Methods and Protocols*. Springer US, New York, NY, pp 25–64
- Loveys BR, Atkinson LJ, Sherlock DJ, Roberts RL, Fitter AH, Atkin OK (2003) Thermal acclimation of leaf and root respiration: an investigation comparing inherently fast- and slow-growing plant species. *Glob Change Biol* 9:895–910. <https://doi.org/10.1046/j.1365-2486.2003.00611.x>
- Lu X, Fei L, Li Y, Du J, Ma W, Huang H, Wang J (2023) Effect of different plant growth regulators on callus and adventitious shoots induction, polysaccharides accumulation and antioxidant activity of *Rhodiola dumulosa*. *Chin Herb Med* 15:271–277. <https://doi.org/10.1016/j.chmed.2022.07.005>
- Luo W, Zhang K, Wang Y, Ye M, Zhang Y, Xu W, Chen L, Li H (2025a) The Rhizome of *Atractylodes macrocephala* Koidz.: A Comprehensive Review on the Traditional Uses. *Phytochemistry Pharmacol Chem Biodivers* 22:e202401879. <https://doi.org/10.1002/cbdv.202401879>
- Luo W, Zhang K, Wang Y, Ye M, Zhang Y, Xu W, Chen L, Li H (2025b) The Rhizome of *Atractylodes macrocephala* Koidz.: A Comprehensive Review on the Traditional Uses, Phytochemistry and Pharmacology. *Chem Biodivers* 22:e202401879. <https://doi.org/10.1002/cbdv.202401879>

- Mandal R, Dutta G (2020) From photosynthesis to biosensing: Chlorophyll proves to be a versatile molecule. *Sens Int* 1:100058. <https://doi.org/10.1016/j.sintl.2020.100058>
- Manley M (2014) Near-infrared spectroscopy and hyperspectral imaging: non-destructive analysis of biological materials. *Chem Soc Rev* 43:8200–8214. <https://doi.org/10.1039/C4CS00062E>
- Mao B, He B, Chen Z, Wang B, Pan H, Li D (2009) Effects of plant growth regulators on the rapid proliferation of shoots and root induction in the Chinese traditional medicinal plant *Atractylodes macrocephala*. *Front Biology China* 4:217–221. <https://doi.org/10.1007/s11515-009-0006-9>
- Maxwell K, Johnson GN (2000) Chlorophyll fluorescence—a practical guide. *J Exp Bot* 51:659–668. <https://doi.org/10.1093/jexbot/51.345.659>
- Miladinova-Georgieva K, Geneva M, Stancheva I, Petrova M, Sichanova M, Kirova E (2023) Effects of different elicitors on micropropagation, biomass and secondary metabolite production of *Stevia rebaudiana* Bertoni—a review. *Plants*
- Monfort LEF, Bertolucci SKV, Lima AF, de Carvalho AA, Mohammed A, Blank AF, Pinto JEBP (2018) Effects of plant growth regulators, different culture media and strength MS on production of volatile fraction composition in shoot cultures of *Ocimum basilicum*. *Ind Crops Prod* 116:231–239. <https://doi.org/10.1016/j.indcrop.2018.02.075>
- Murashige T, Skoog F (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiol Plant* 15:473–497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
- Nam S, Kang S, Kim J (2020) Maintaining a constant soil moisture level can enhance the growth and phenolic content of sweet basil better than fluctuating irrigation. *Agric Water Manage* 238:106203. <https://doi.org/10.1016/j.agwat.2020.106203>
- Okello D, Yang S, Komakech R, Rahmat E, Chung Y, Gang R, Kim Y-G, Omuja F, Kang Y (2021a) An in vitro propagation of *Aspilia africana* (Pers.) C. D. Adams, and evaluation of its anatomy and physiology of acclimatized plants. *Front Plant Sci* 12–2021. <https://doi.org/10.3389/fpls.2021.704896>
- Okello D, Yang S, Komakech R, Chung Y, Rahmat E, Gang R, Omuja F, Lamwaka AV, Kang Y (2021b) Indirect in vitro regeneration of the medicinal plant, *Aspilia africana*, and histological assessment at different developmental stages. *Front Plant Sci* 12:797721. <https://doi.org/10.3389/fpls.2021.797721>
- Pacurar DI, Perrone I, Bellini C (2014) Auxin is a central player in the hormone cross-talks that control adventitious rooting. *Physiol Plant* 151:83–96. <https://doi.org/10.1111/ppl.12171>
- Palta J, Watt M (2009) Vigorous crop root systems: form and function for improving the capture of water and nutrients. *Applied crop physiology: boundaries between genetic improvement and agronomy*. Academic, San Diego, pp 309–325
- Pence VC (2011) Evaluating costs for the in vitro propagation and preservation of endangered plants. *Vitro Cell Dev Biology - Plant* 47:176–187. <https://doi.org/10.1007/s11627-010-9323-6>
- Polivanova OB, Bedarev VA (2022) Hyperhydricity in plant tissue culture. *Plants*
- Razavizadeh R, Adabavazeh F, Mosayebi Z (2023) Titanium dioxide nanoparticles improve element uptake, antioxidant properties, and essential oil productivity of *Melissa officinalis* L. seedlings under in vitro drought stress. *Environ Sci Pollut Res* 30:98020–98033. <https://doi.org/10.1007/s11356-023-29384-x>
- Ribeiro YRdS, Aragão VPM, Sousa KRd, Macedo AF, Floh EIS, Silveira V, Santa-Catarina C (2022) Involvement of differentially accumulated proteins and endogenous auxin in adventitious root formation in micropropagated shoot cuttings of *Cedrela fissilis* Vellozo (Meliaceae). *Plant Cell, Tissue and Organ Culture (PCTOC)*. 148:119–135. <https://doi.org/10.1007/s11240-021-02171-7>
- Rohela GK, Jogam P, Saini P, Sandhya D, Peddaboina V, Shekhawat MS (2022) Assessing the Genetic Stability of In Vitro Raised Plants. In: Gupta S, Chaturvedi P (eds) *Commercial Scale Tissue Culture for Horticulture and Plantation Crops*. Springer Nature Sing.), Singapore, pp 245–276
- Roux D, Alnaser O, Garayev E, Baghdikian B, Elias R, Chiffolleau P, Ollivier E, Laurent S, El Maataoui M, Sallanon H (2017) Eco-physiological and phytochemical characterization of wild populations of *Inula montana* L. (Asteraceae) in Southeastern France. *Flora* 236–237:67–75. <https://doi.org/10.1016/j.flora.2017.09.012>
- Schoene G, Yeager T (2005) Micropropagation of sweet viburnum (*Viburnum odoratissimum*). *Plant Cell Tissue Organ Cult* 83:271–277. <https://doi.org/10.1007/s11240-005-7015-4>
- Schreiber U, Gademann R, Ralph PJ, Larkum AWD (1997) Assessment of Photosynthetic Performance of Prochloron in *Lissoclinum patella* in hospite by Chlorophyll Fluorescence Measurements. *Plant Cell Physiol* 38:945–951. <https://doi.org/10.1093/oxfordjournals.pcp.a029256>
- Sharma N, Kumar N, James J, Kalia S, Joshi S (2023) Strategies for successful acclimatization and hardening of in vitro regenerated plants: Challenges and innovations in micropropagation techniques. *Plant Science Today*. <https://doi.org/10.14719/pst.2376>. (Early Access).
- Shiwani K, Sharma D, Kumar A (2022) Improvement of Plant Survival and Expediting Acclimatization Process. In: Gupta S, Chaturvedi P (eds) *Commercial Scale Tissue Culture for Horticulture and Plantation Crops*. Springer Nature Sing.), Singapore, pp 277–291
- Siddique HS, Nadeem F, Inam S, Kazerooni EA (2020) Recent production methodologies and advanced spectroscopic characterization of biodiesel: A review. *Gas* 400:500
- Singh SK, Rai MK, Sahoo L (2012) An improved and efficient micropropagation of *Eclipta alba* through transverse thin cell layer culture and assessment of clonal fidelity using RAPD analysis. *Ind Crops Prod* 37:328–333. <https://doi.org/10.1016/j.indcrop.2011.12.005>
- Singh K, Singh G, Bhushan B, Kumar S, Dhurandhar Y, Dixit P (2024) A comprehensive pharmacological review of *Atractylodes Macrocephala*: Traditional uses, phytochemistry, pharmacokinetics, and therapeutic potential. *Pharmacol Res - Mod Chin Med* 10:100394. <https://doi.org/10.1016/j.prmcm.2024.100394>
- Sliwinska E (2018) Flow cytometry—a modern method for exploring genome size and nuclear DNA synthesis in horticultural and medicinal plant species. *Folia Horticulturae* 30:103
- Song X, Mo F, Yan M, Zhang X, Zhang B, Huang X, Huang D, Pan Y, Verma KK, Li Y-R (2022) Effect of smut infection on the photosynthetic physiological characteristics and related defense enzymes of sugarcane. *Life*
- Su X, Yue X, Kong M, Xie Z, Yan J, Ma W, Wang Y, Zhao J, Zhang X, Liu M (2023) Leaf Color Classification and Expression Analysis of Photosynthesis-Related Genes in Inbred Lines of Chinese Cabbage Displaying Minor Variations in Dark-Green Leaves. *Plants (Basel)*. <https://doi.org/10.3390/plants12112124>
- Sun D, Wu S, Li X, Ge B, Zhou C, Yan X, Ruan R, Cheng P (2024) The structure, functions and potential medicinal effects of chlorophylls derived from microalgae. *Marine Drugs*
- Tai NT, Quyen PT, Nhi PT, Thang PQ, Hung LT (2023) Effects of plant growth regulators and basal media on *Atractylodes macrocephala* Koidz. 's shoot multiplication. *Tạp Chí Khoa học Trường Đại học Quốc tế Hồng Bàng*, pp 95–100
- Trueba S, Pan R, Scoffoni C, John GP, Davis SD, Sack L (2019) Thresholds for leaf damage due to dehydration: declines of hydraulic function, stomatal conductance and cellular integrity precede those for photochemistry. *New Phytol* 223:134–149. <https://doi.org/10.1111/nph.15779>

- Vanisree M, Lee C-Y, Lo S-F, Nalawade SM, Lin CY, Tsay H-S (2004) Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures. *Bot Bull Acad Sin* 45:1–22
- Wang L, Du Y, Rahman MM, Tang B, Fan L-J, Kilaru A (2018) Establishment of an efficient in vitro propagation system for *Iris sanguinea*. *Sci Rep* 8:17100. <https://doi.org/10.1038/s41598-018-35281-y>
- Weyer L, Lo S (2002) Spectra-structure correlations in the near-infrared. *Handb Vib Spectrosc* 3:1817–1837
- Wu Y-X, Lu W-W, Geng Y-C, Yu C-H, Sun H-J, Kim Y-J, Zhang G, Kim T (2020) Antioxidant, Antimicrobial and Anti-Inflammatory Activities of Essential Oil Derived from the Wild Rhizome of *Atractylodes macrocephala*. *Chem Biodivers* 17:e2000268. <http://doi.org/10.1002/cbdv.202000268>
- Xu J, Wei X-P, Liu J-S, Qi Y-D, Zhang B-G, Liu H-T, Xiao P-G (2021) Genome sizes of four important medicinal species in Kadsura by flow cytometry. *Chin Herb Med* 13:416–420. <https://doi.org/10.1016/j.chmed.2021.05.002>
- Yan P, Li Y (2023) Treatment of bone hyperplasia from the perspective of spleen and kidney with *Atractylodes macrocephala* as the main method. *Front Med Sci Res* 5
- Yang R, Fan H, He B, Ruan Q, Wei B, Han B, Hao X, Mao I, Kai G (2023) Current progress of *Atractylodes macrocephala* Koidz.: A review of its biogeography, PAO-ZHI processing, biological activities, biosynthesis pathways, and technology applications. *Med Plant Biology* 2. <https://doi.org/10.48130/MPB-2023-0005>
- Yang P, Qin L-L, Yu M, Zou Z-M (2025) Rhizome of *Atractylodes macrocephala* alleviates spleen-deficiency constipation in rats by modulating gut microbiota and bile acid metabolism. *J Ethnopharmacol* 348:119884. <https://doi.org/10.1016/j.jep.2025.119884>
- Ye H, Li C, Ye W, Zeng F, Liu F, Liu Y, Wang F, Ye Y, Fu L, Li J (2022) Medicinal Angiosperms of Compositae. In: Ye H, Li C, Ye W, Zeng F (eds) *Common Chinese Materia Medica: Volume 7*. Springer Nature Singapore, Singapore, pp 201–286
- Yin S, Dong L (2024) Plant Tattoo Sensor Array for Leaf Relative Water Content, Surface Temperature, and Bioelectric Potential Monitoring. *Adv Mater Technol* 9:2302073. <https://doi.org/10.1002/admt.202302073>
- Zhu B, Zhang Q-L, Hua J-W, Cheng W-L, Qin L-P (2018) The traditional uses, phytochemistry, and pharmacology of *Atractylodes macrocephala* Koidz.: A review. *J Ethnopharmacol* 226:143–167. <https://doi.org/10.1016/j.jep.2018.08.023>
- Zwyrtková J, Němečková A, Čížková J, Holušová K, Kapustová V, Svačina R, Kopecký D, Till BJ, Doležel J, Hříbová E (2020) Comparative analyses of DNA repeats and identification of a novel Fesreba centromeric element in fescues and ryegrasses. *BMC Plant Biol* 20:280. <https://doi.org/10.1186/s12870-020-02495-0>

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