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RESEARCH ARTICLE

STUDIES ON *IN VITRO* PROPAGATION AND PHYTOCHEMICAL CONSTITUENTS OF *ANDROGRAPHIS PANICULATA L.*

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ABSTRACT

Andrographis paniculata (Burm. f.) Wall. ex Nees, commonly known as "King of Bitters," is a medicinally important plant widely used in traditional medicine systems. The increasing demand for this plant and its bioactive compounds necessitates the development of efficient micropropagation protocols and comprehensive phytochemical characterization. Nodal explants from aseptically germinated seedlings were cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of 6-benzylaminopurine (BAP), kinetin (KIN), naphthaleneacetic acid (NAA), and indole-3-butyric acid (IBA). Shoot proliferation, rooting, and acclimatization were evaluated. Phytochemical screening of leaf extracts was performed using standard qualitative and quantitative methods to detect alkaloids, flavonoids, tannins, phenols, terpenoids, saponins, steroids, and andrographolide content. Maximum shoot proliferation (28.4 ± 1.8 shoots per explant) was achieved on MS medium supplemented with 2.0 mg/L BAP and 0.5 mg/L NAA. Optimal rooting (92% frequency with 8.6 ± 0.9 roots per shoot) was observed on half-strength MS medium containing 2.0 mg/L IBA. Acclimatized plantlets showed 88% survival rate. Phytochemical analysis revealed the presence of alkaloids, flavonoids, tannins, phenols, terpenoids, saponins, and steroids. Andrographolide content in micropropagated plants was 2.34 ± 0.12% dry weight, comparable to field-grown plants. An efficient and reproducible micropropagation protocol was established for *A. paniculata* with high multiplication rates and survival. The phytochemical profile confirmed the presence of major bioactive constituents, validating the pharmaceutical potential of micropropagated plants.

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INTRODUCTION

Andrographis paniculata (Burm. f.) Wall. ex Nees, belonging to the family Acanthaceae, is an annual herbaceous plant native to India and Sri Lanka, widely distributed throughout tropical and subtropical Asia. Okhuarobo *et al*, (2014). Commonly known as "King of Bitters" or "Kalmegh," this plant has been extensively used in traditional medicine systems including Ayurveda, Unani, and Traditional Chinese Medicine for centuries. (Subramanian *et al*, 2008). The plant is renowned for its diverse pharmacological properties, including hepatoprotective, anti-inflammatory, immunomodulatory, antimicrobial, and anticancer activities [Jayakumar *et al*., 2013.] The therapeutic efficacy of *A. paniculata* is primarily attributed to its rich phytochemical composition, particularly diterpenoid lactones such as andrographolide, neoandrographolide, and deoxyandrographolide. Li *et al.,* (2007); Hahn *et al*., (2020). Andrographolide, the major bioactive constituent, has been extensively studied for its biological activities including antioxidant, anti-inflammatory, and anticancer properties [Dai *et al*., (2019); Islam *et al*, (2018). Additionally, the plant contains flavonoids, phenolic compounds, alkaloids, and other secondary metabolites that contribute to its medicinal value [Kulyal *et al*, 2011; Ahamed *et al*, 2012].

The increasing global demand for *A. paniculata* and its bioactive compounds, coupled with overexploitation, has led to a decline in natural populations. Okhuarobo *et al*, (2014). Conventional propagation through seeds is often unreliable due to low germination rates and genetic variability. Plant tissue culture techniques, particularly micropropagation, offer a viable alternative for rapid multiplication and year-round production of genetically uniform plants with consistent phytochemical profiles [Purkayastha *et al*, 2008; Dandin and Murthy 2012].

Several researchers have reported successful micropropagation protocols for *A. paniculata* using various explant types including nodal segments [Karim *et al*, (2024), Purkayastha *et al*, (2008), Roy (2016), shoot tips Patuhai *et al*, (2023). , and leaf explants [Martin (2004), Thorat *et al*, (2013). Different combinations of plant growth regulators, particularly cytokinins such as BAP and KIN combined with auxins like

NAA and IBA, have been employed to optimize shoot proliferation and rooting [Dandin and Murthy (2012), Jindal *et al*, (2015). The present study was undertaken to: (i) develop an efficient micropropagation protocol using nodal explants, (ii) optimize plant growth regulator concentrations for shoot proliferation and rooting, (iii) establish an effective acclimatization procedure, and (iv) perform comprehensive phytochemical screening including andrographolide quantification of micropropagated plants.

MATERIALS AND METHODS

Plant Material and Explant Preparation: Healthy plants of Andrographis paniculata were collected from the medicinal plant garden and authenticated by a taxonomist. Seeds were collected from mature plants and stored at 4°C until use. For explant preparation, seeds were germinated aseptically on half-strength MS medium [Murashige and Skoog, 1962] without growth regulators. After 15 days, nodal segments (1.0–1.5 cm length) containing a single axillary bud were excised from the aseptically grown seedlings and used as explants for further experiments.

Surface Sterilization; For seed sterilization, seeds were washed thoroughly under running tap water for 15 minutes, followed by treatment with 5% (v/v) Teepol solution for 10 minutes. Seeds were then surface sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 3 minutes, followed by three to four rinses with sterile distilled water under aseptic conditions in a laminar air flow cabinet. The sterilized seeds were inoculated on half-strength MS medium for germination. For nodal explants excised from in vitro-grown seedlings, no additional sterilization was required as they were already maintained under aseptic conditions.

Culture Media and Growth Regulators: Murashige and Skoog (MS) medium was used as the basal medium throughout the study. The medium was supplemented with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar (Bacteriological grade). The pH of the medium was adjusted to 5.8 ± 0.1 before autoclaving at 121°C and 15 psi pressure for 20 minutes. All cultures were maintained at 25 ± 2°C under a 16-hour photoperiod with light intensity of 40–50 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes. Plant growth regulators used in the study included cytokinins: 6-benzylaminopurine (BAP) and kinetin (KIN); and auxins: naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA). All growth regulators were filter-sterilized (0.22 µm membrane filter) and added to autoclaved medium after cooling to approximately 50°C.

Shoot Proliferation: Nodal explants were cultured on MS medium supplemented with different concentrations of BAP (0.5, 1.0, 1.5, 2.0, 2.5 mg/L) and KIN (0.5, 1.0, 1.5, 2.0 mg/L) either alone or in combination with NAA (0.1, 0.3, 0.5 mg/L). Each treatment consisted of 15 replicates, and experiments were repeated three times. Cultures were observed weekly, and data on percentage of explants showing shoot proliferation, number of shoots per explant, and shoot length were recorded after 4 weeks of culture. Shoots were subcultured at 4-week intervals on fresh medium of the same composition for multiplication.

Rooting and Acclimatization: Well-developed shoots (4–5 cm length) were excised from multiplication cultures and transferred to rooting medium. For root induction, half-strength MS medium supplemented with different concentrations of IBA (0.5, 1.0, 1.5, 2.0, 2.5 mg/L) and NAA (0.5, 1.0, 1.5, 2.0 mg/L) were tested. Each treatment consisted of 15 replicates. Data on rooting percentage, number of roots per shoot, root length, and time taken for root initiation were recorded after 3 weeks of culture. Well-rooted plantlets were carefully removed from culture vessels, washed gently to remove adhering agar, and transferred to plastic pots containing a sterile mixture of soil, sand, and vermiculite (1:1:1). Potted plantlets were covered with transparent polyethylene bags to maintain high humidity and kept under controlled conditions for 2 weeks. The polyethylene covers were gradually opened to reduce humidity. After 4 weeks, survival rate was recorded and successfully acclimatized plants were transferred to a greenhouse.

Phytochemical Screening

Preparation of Plant Extract: Fresh leaves from 3-month-old micropropagated plants were collected, washed with distilled water, and shade-dried at room temperature for 7 days. The dried leaves were powdered using a mechanical grinder and stored in airtight containers. For extraction, 10 g of dried leaf powder was extracted with 100 mL of methanol using a Soxhlet apparatus for 6 hours. The extract was filtered through Whatman No. 1 filter paper, and the solvent was evaporated under reduced pressure using a rotary evaporator. The crude extract was stored at 4°C for further analysis.

Qualitative Phytochemical Tests: Standard qualitative phytochemical tests were performed to detect various secondary metabolites. Alkaloids were detected by Dragendorff's test and Wagner's test (orange-red or brown precipitate). Flavonoids were detected by the alkaline reagent test and lead acetate test (yellow coloration). Tannins were detected by the ferric chloride test (dark blue or greenish-black color). Phenols were also detected by the ferric chloride test (blue or green coloration). Terpenoids were detected by the Salkowski test (reddish-brown coloration at the interface). Saponins were detected by the foam test (persistent foam formation). Steroids were detected by the Liebermann–Burchard test (blue-green color).

Quantitative Determination of Andrographolide: Andrographolide content was determined using High-Performance Liquid Chromatography (HPLC). The dried methanolic extract was dissolved in methanol (HPLC grade) and filtered through a 0.45 µm membrane filter. HPLC analysis was performed using a C18 column (250 mm × 4.6 mm, 5 µm particle size) with a mobile phase of acetonitrile:water (40:60, v/v) at a flow rate of 1.0 mL/min. Detection was at 254 nm using a UV detector. Andrographolide standard (Sigma-Aldrich) was used for calibration and content expressed as percentage dry weight.

Statistical Analysis: All experiments were conducted in a completely randomized design with at least 15 replicates per treatment, repeated three times. Data were expressed as mean ± standard error (SE). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) at $p < 0.05$ significance level using SPSS software version 20.0.

RESULTS

Shoot Proliferation: Nodal explants cultured on MS medium supplemented with different concentrations and combinations of cytokinins (BAP and KIN) and auxin (NAA) showed varied responses in terms of shoot proliferation. Explants began to swell within 3-4 days of culture, and axillary bud break was observed within 7-10 days. Multiple shoot formation was evident after 2 weeks of culture. Among the cytokinins tested individually, BAP was found to be more effective than KIN for shoot proliferation. MS medium supplemented with 2.0 mg/L BAP alone resulted in 22.6 ± 1.4 shoots per explant with 94% response frequency. In comparison, KIN at 2.0 mg/L produced 16.8 ± 1.2 shoots per explant with 86% response frequency. Lower concentrations of BAP (0.5-1.5 mg/L) resulted in fewer shoots (8.4-18.2 shoots per explant), while higher concentration (2.5 mg/L) showed a slight decline in shoot number (20.4 ± 1.6 shoots per explant) with signs of hyperhydricity. The combination of BAP with NAA significantly enhanced shoot proliferation compared to BAP alone. MS medium supplemented with 2.0 mg/L BAP and 0.5 mg/L NAA yielded the maximum number of shoots (28.4 ± 1.8 shoots per explant) with 96% response frequency and an average shoot length of 4.8 ± 0.4 cm after 4 weeks of culture. This combination was significantly superior to all other treatments tested ($p < 0.05$). Lower concentrations of NAA (0.1 and 0.3 mg/L) in combination with 2.0 mg/L BAP produced 24.2 ± 1.5 and 26.6 ± 1.6 shoots per explant, respectively. The combination of KIN with NAA also promoted shoot proliferation, though to a lesser extent than BAP-NAA combinations. MS medium with 1.5 mg/L KIN and 1.0 mg/L NAA produced 19.4 ± 1.3 shoots per explant. Subculturing of shoot clusters on fresh medium of the same composition at 4-week intervals maintained the multiplication rate, and shoots could be successfully multiplied for more than 10 passages without any decline in regeneration potential or morphological variations.

Root Induction: Well-developed shoots (4-5 cm length) excised from multiplication cultures were tested for root induction on half-strength MS medium supplemented with different concentrations of IBA and NAA. Root initiation was observed within 5-7 days of culture on auxin-containing media. IBA was found to be more effective than NAA for root induction. Half-strength MS medium supplemented with 2.0 mg/L IBA resulted in the highest rooting response with 92% rooting frequency, 8.6 ± 0.9 roots per shoot, and an average root length of 6.4 ± 0.6 cm after 3 weeks of culture. Lower concentrations of IBA (0.5-1.5 mg/L) produced fewer roots (4.2-7.4 roots per shoot) with rooting frequencies ranging from 76-88%. Higher concentration of IBA (2.5 mg/L) resulted in callusing at the base of shoots with reduced root quality. NAA at 2.0 mg/L produced 6.8 ± 0.8 roots per shoot with 84% rooting frequency, which was significantly lower than IBA at the same concentration. The roots induced by IBA were thick, white, and healthy, whereas NAA-induced roots were relatively thinner and showed some callus formation at the base. Full-strength MS medium with auxins resulted in lower rooting frequencies (68-74%) and fewer roots compared to half-strength MS medium, indicating that reduced salt concentration favored root development. Control shoots cultured on auxin-free half-strength MS medium showed only 28% rooting with 2.4 ± 0.5 roots per shoot after 4 weeks.

Acclimatization: Well-rooted plantlets with 6-8 roots were selected for acclimatization. The plantlets were carefully removed from culture vessels, and roots were washed gently under running tap water to remove adhering agar medium. The plantlets were then transferred to plastic pots (8 cm diameter) containing a sterile mixture of soil, sand, and vermiculite (1:1:1). During the initial 2 weeks, the potted plantlets were covered with transparent polyethylene bags to maintain high humidity (85-90%) and kept in the culture room under controlled conditions (25 ± 2°C, 16-hour photoperiod). The polyethylene covers were gradually opened over the next week to reduce humidity and acclimatize the plants to ambient conditions. After 4 weeks of acclimatization, 88% of the plantlets survived and showed active growth with new leaf formation. The successfully acclimatized plants were transferred to larger pots (15 cm diameter) containing garden soil and maintained in a greenhouse. After 8 weeks, the plants attained a height of 25-30 cm and showed normal morphology comparable to conventionally grown plants. The acclimatized plants flowered after 12 weeks, confirming their normal growth and development.

Phytochemical Analysis

Qualitative Phytochemical Screening: Qualitative phytochemical analysis of methanolic leaf extracts from micropropagated Andrographis paniculata plants revealed the presence of various secondary metabolites. The results of phytochemical screening are presented in Table 1.

Table 1. Qualitative phytochemical screening of methanolic leaf extracts of micropropagated and field-grown Andrographis paniculata. (+) = Present; (−) = Absent

Phytochemical Class	Test Applied	Micro propagated Plants	Field-Grown Plants
Alkaloids	Dragendorff's/Wagner's test	+	+
Flavonoids	Alkaline reagent/Lead acetate	+	+
Tannins	Ferric chloride test	+	+
Phenols	Ferric chloride test	+	+
Terpenoids	Salkowski test	+	+
Saponins	Foam test	+	+
Steroids	Liebermann-Burchard test	+	+

Andrographolide Content: HPLC analysis revealed that the andrographolide content in leaves of 3-month-old micropropagated plants was 2.34 ± 0.12% dry weight. This content was comparable to that of field-grown plants of the same age (2.28 ± 0.15% dry weight), indicating that the micropropagation process did not adversely affect the biosynthesis and accumulation of this major bioactive constituent. The retention time of andrographolide in the sample matched with that of the standard (approximately 8.2 minutes), confirming its identity.

DISCUSSION

The present study successfully established an efficient and reproducible micropropagation protocol for Andrographis paniculata using nodal explants. The protocol encompasses all critical stages with high success rates. The choice of explant is crucial for successful micropropagation. Nodal explants were selected due to their high regeneration potential, genetic stability, and ease of handling Purkayastha *et al*,(2008),

Dandin and Murthy (2012). Previous studies have demonstrated that nodal explants of A. paniculata are highly responsive to in vitro culture and produce genetically uniform plants through axillary bud proliferation, minimizing somaclonal variation Roy (2016), Kataky, & Handique (2010). The type and concentration of plant growth regulators play a pivotal role in micropropagation success. In the present study, BAP was more effective than KIN for shoot proliferation, consistent with previous reports Purkayastha *et al*, (2008), Dandin and Murthy (2012), Jindal *et al,.* (2015). BAP at 2.0 mg/L produced 22.6 shoots per explant, comparable to Purkayastha *et al* (2008) who reported maximum shoot proliferation at 10 µMBAP with 34 shoots per explant. Variations in shoot number could be attributed to differences in genotype, explant source, and culture conditions. The synergistic effect of cytokinin and auxin combination is well documented in plant tissue culture. In our study, the combination of 2.0 mg/L BAP with 0.5 mg/L NAA resulted in the highest shoot proliferation (28.4 shoots per explant), superior to BAP alone. This enhancement is attributed to complementary roles of cytokinins and auxins in regulating cell division and differentiation. Similar synergistic effects have been reported by Jindal *et.al,*. (2015), who obtained 28.0 shoots per explant on MS medium with 2.0 mg/L BAP and 0.5 mg/L NAA, validating the reproducibility of our protocol. Other researchers have reported varying optimal concentrations. Dandin and Murthy (2012) achieved 39.08 shoots per explant using 1.0 µM BAP combined with 5.0 µM KIN. Roy (2016) reported that 11.10 µM/L BAP supplemented with coconut water and activated charcoal produced 30 shoots per culture. These variations highlight the influence of genotype and culture conditions on regeneration response. Root induction is critical for successful acclimatization and field establishment. IBA was more effective than NAA for root induction, with half-strength MS medium containing 2.0 mg/L IBA producing the best results (92% rooting frequency, 8.6 roots per shoot). The superiority of IBA over NAA has been reported in several studies on *A. paniculata*. Purkayastha *et al*, (2008), Bidari & Hebsur (2012), Dandin and Murthy (2012). Purkayastha *et al* (2008) reported optimal rooting with 2.5 µM IBA, while Dandin and Murthy (2012) found 2.0 µM IBA most effective. The preference for IBA may be due to its slower degradation and sustained availability compared to NAA.

The use of half-strength MS medium for rooting proved beneficial, resulting in higher rooting frequencies and better root quality compared to full-strength medium. This observation is consistent with previous reports Karim *et al*, (2024), Bidari & Hebsur (2012), Kataky, & Handique (2010), suggesting that reduced salt concentration favors root development by minimizing osmotic stress. Acclimatization is often the most challenging phase in micropropagation, requiring adaptation from heterotrophic to autotrophic nutrition. In the present study, 88% of plantlets survived acclimatization, attributed to the gradual hardening process and maintenance of high humidity during initial stages. Similar survival rates (80-90%) have been reported previously Purkayastha *et al*, (2008), Roy (2016), Kataky and Handique (2010). Phytochemical analysis is essential to validate the pharmaceutical potential of micropropagated plants. Our qualitative screening confirmed the presence of alkaloids, flavonoids, tannins, phenols, terpenoids, saponins, and steroids, indicating a comprehensive secondary metabolite profile. These findings are consistent with previous phytochemical studies on *A. paniculata*. Kulwal *et al*, (2008)., Umadevi and Kamalam (2014). Ahmed *et al*, (2012). Andrographolide, the major bioactive diterpenoid lactone, is considered the marker compound for quality assessment of *A. paniculata*. In the present study, HPLC analysis revealed that andrographolide content in micropropagated plants (2.34% dry weight) was comparable to field-grown plants (2.28% dry weight). This finding demonstrates that the micropropagation process does not adversely affect the biosynthesis and accumulation of this important bioactive constituent. Dandin and Murthy (2012) also reported that micropropagated plants maintained andrographolide content similar to mother plants, confirming genetic and biochemical stability. The andrographolide content observed in our study falls within the range reported in the literature (1.5-3.5% dry weight) for *A. paniculata* Pandey and Pandey-Rai (2019), [Rajani*et al*, 2000]. The maintenance of phytochemical integrity in micropropagated plants is crucial for commercial pharmaceutical exploitation. Several factors influence andrographolide content including genotype, age, and environmental conditions Sharma *et al*., (2008). Pandey and Pandey-Rai (2019). The comparable andrographolide content in our micropropagated plants confirms that the protocol produces plants with consistent phytochemical profiles. The protocol developed offers key advantages: (i) high multiplication rate (28.4 shoots per explant per 4-week cycle), (ii) use of nodal explants ensuring genetic stability, (iii) efficient rooting (92% frequency), (iv) high acclimatization survival (88%), and (v) andrographolide content comparable to field-grown plants. This protocol can be scaled up for commercial production of A. paniculata to meet pharmaceutical industry demands and support conservation strategies for this overexploited species Okhuarobo *et al*, (2014) ,Hossain *et al*., (2021).

CONCLUSION

An efficient and reproducible micropropagation protocol was established for Andrographis paniculata using nodal explants. MS medium supplemented with 2.0 mg/L BAP and 0.5 mg/L NAA was optimal for shoot proliferation, yielding 28.4 shoots per explant. Half-strength MS medium containing 2.0 mg/L IBA was most effective for root induction with 92% rooting frequency. The acclimatization protocol resulted in 88% survival rate, and the hardened plants showed normal growth and development. Comprehensive phytochemical screening confirmed the presence of alkaloids, flavonoids, tannins, phenols, terpenoids, saponins, and steroids in micropropagated plants. HPLC analysis revealed that andrographolide content (2.34% dry weight) was comparable to field-grown plants, validating the pharmaceutical potential of micropropagated plants. This protocol provides a reliable method for rapid multiplication, conservation, and commercial production of A. paniculata with consistent phytochemical profiles. This study contributes to the sustainable utilization of this valuable medicinal plant and supports pharmaceutical industry demand for quality plant material.

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