Research Advances in In Vitro Culture Techniques and Applications of the Medicinal Plant Andrographis paniculata (Postprint)

Authors: Chen Dongliang, Zhong Chu, Jian Shaofen

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Abstract

Andrographis paniculata is one of the important southern Chinese medicinal herbs, used for heat-clearing and detoxifying, blood-cooling and swelling-reducing effects. Its principal active constituent, andrographolide, exhibits anticancer, anti-HIV, anti-inflammatory, and hepatoprotective properties. Chemical synthesis of andrographolide is difficult, and it is mainly extracted from cultivated plant materials. However, the quality of cultivated medicinal herbs is uneven due to various factors including soil, climate, and water-fertilizer management, while A. paniculata has a long growth cycle and occupies land resources. In vitro culture technology offers significant advantages in rapid seedling propagation and active constituent accumulation, representing an important approach for rapid and efficient production of A. paniculata active constituents. The in vitro tissue regeneration technology system for A. paniculata is increasingly perfected, with in vitro tissue regeneration from explants to intact plants becoming mature, and has found applications in seedling propagation and ploidy breeding. Meanwhile, through optimization of culture conditions and application of appropriate elicitors, the accumulation of andrographolide and other active constituents in cultures can be substantially increased during callus culture, cell suspension culture, adventitious root culture, and hairy root culture of A. paniculata. This paper comprehensively and systematically reviews recent domestic and international research progress on A. paniculata in vitro culture technology and andrographolide production from perspectives of tissue, cell, adventitious root, and hairy root culture, aiming to promote the development and application of A. paniculata in vitro culture technology and provide references for research on in vitro andrographolide production. This paper also proposes three key aspects that require focused attention in future research on A. paniculata in vitro culture technology and andrographolide production via this technology: (1) Maturation and perfection

of the A. paniculata tissue in vitro regeneration technology system and establishment of a comprehensive and systematic evaluation system. (2) Combined optimization of culture conditions and highly efficient elicitors to further increase yields of andrographolide and other important active constituents. (3) Development of bioreactor cultivation studies for andrographolide production via cell suspension culture technology.

Full Text

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Research Advances in In Vitro Culture Technology and Its Application in Medicinal Plant *Andrographis paniculata*

Dongliang Chen[1]*, Chu Zhong[2], Shaofen Jian[2]**

[1]Institute of Cash Crops, Guangxi Academy of Agricultural Sciences, Nanning 530007, China
[2]National Center for TCM Inheritance and Innovation, Guangxi Botanical Garden of Medicinal Plants, Nanning 530023, China

Abstract

Andrographis paniculata is one of the most important "Southern Medicines" in China, traditionally used for clearing heat, detoxifying, cooling blood, and reducing swelling. Its main active ingredient, andrographolide, exhibits significant anti-cancer, anti-HIV, anti-inflammatory, and hepatoprotective effects. Due to the difficulty of artificial synthesis, andrographolide is primarily obtained through extraction from cultivated plant materials. However, the quality of cultivated medicinal materials is affected by various factors such as soil, climate, and water-fertilizer management, while *A. paniculata* also has a long growth cycle and occupies substantial land resources. Plant in vitro culture technology offers significant advantages for rapid seedling propagation and active ingredient accumulation, representing an important pathway for rapid and efficient production of *A. paniculata* active ingredients. The in vitro regeneration technology system for *A. paniculata* has become increasingly refined, with tissue-to-plant regeneration techniques maturing and finding applications in seedling propagation and ploidy breeding. Meanwhile, during callus culture, cell suspension culture, adventitious root culture, and hairy root culture of *A. paniculata*, the accumulation of andrographolide and other active ingredients can be substantially increased through optimized culture conditions and appropriate elicitors. This paper comprehensively and systematically reviews recent research advances in *A. paniculata* in vitro culture technology and andrographolide production from tissue, cell, adventitious root, and hairy root cultures, aiming to promote the development and application of these technologies and provide references for in vitro andrographolide production. The paper also proposes three key areas for future research: (1) maturing and improving the tissue in vitro regeneration tech-

nology system for *A. paniculata* while establishing a comprehensive evaluation system; (2) further increasing andrographolide yield through optimized culture conditions combined with efficient elicitors; and (3) developing bioreactor-based cell suspension culture systems for andrographolide production.

Key words: *Andrographis paniculata*, tissue culture, cell suspension culture, andrographolide, advances

1.1 Overview of Tissue Culture Technology Research in *Andrographis paniculata*

The effectiveness of plant tissue culture primarily depends on several key factors, including explant selection and sterilization, culture medium type, plant hormone ratios, and culture conditions.

1.1.1 Explants

The success of explant inoculation depends on factors such as the sampling site, timing, and sterilization method. In *A. paniculata* tissue culture, various plant parts including leaves, axillary buds, stem segments, stem segments with axillary buds, and shoot tips can serve as explants. However, most studies suggest that leaves and stem segments with axillary buds are the most effective explants for tissue culture (Purkayastha et al., 2008; Bansi & Rout, 2013; Chen, 2017; Dai et al., 2018). Ethanol, sodium hypochlorite, and mercuric chloride are commonly used for explant sterilization, though their concentration and treatment duration significantly affect explant viability. Chen (2017) and Dai (2018) found that for stem segments with axillary buds, sterilization with 75% ethanol for 15 seconds followed by 3% sodium hypochlorite for 30 minutes yielded the best results, with a survival rate of 56.33%. When using seeds as explants, treatment with 75% ethanol for 15 seconds and 3% sodium hypochlorite for 20 minutes achieved a survival rate of 78.67%.

1.1.2 Culture Medium

The basal culture medium serves as the primary nutrient source in plant tissue culture, and its composition and concentration directly affect explant growth, differentiation, and proliferation status. Different explants require different medium types. Huang et al. (2010) identified MS as the most suitable basal medium for *A. paniculata* bud proliferation and growth, achieving an 83.3% proliferation rate with robust growth. Ji et al. (2017) compared four different media—pure agar, MS, 1/2 MS, and MS + 2.0 mg $\cdot$ L^{-1} 6-BA—for seed germination, concluding that MS or 1/2 MS were optimal based on comprehensive evaluation of germination and survival rates. Yan (2016) investigated five basal media (MS, 1/2 MS, MT, H, and B5) for sterile seedling growth, determining that 1/2 MS was most suitable for *A. paniculata* seedling development. Ji (2018) compared MS, N6, and Nistch media for anther culture, finding N6 to be the most appropriate basal medium for *A. paniculata* anther culture.

1.1.3 Plant Growth Regulators

Basal culture media can only maintain minimal survival and nutritional requirements. Adding appropriate ratios of plant growth regulators to basal media can induce cell division initiation, callus growth, and root/shoot differentiation. Different types and ratios of plant growth regulators exert varying effects on explant dedifferentiation and organogenesis.

Cytokinins and auxins are commonly used plant growth regulators in tissue culture. Cytokinins promote cell division and growth, stimulate cell differentiation and bud formation, and influence callus differentiation and shoot development (Loyola-Vargas & Ochoa-Alejo, 2018). Auxins promote cell elongation and expansion and are associated with vascular bundle and root formation (Kuluev et al., 2015). For *A. paniculata* callus induction and cluster bud differentiation, combining cytokinins (6-BA, KT, etc.) with auxins (NAA, IAA, IBA, 2,4-D, etc.) as additives yields good results (Table 1). For instance, Dai et al. (2018) identified the optimal hormone combination for callus induction as MS + 1.0 $mg \cdot L^{-1}$ 6-BA + 1.5 $mg \cdot L^{-1}$ NAA. Chen (2017) found that MS + 1.0 $mg \cdot L^{-1}$ 6-BA + 0.1 $mg \cdot L^{-1}$ 2,4-D was most effective for cluster bud induction, achieving a proliferation coefficient of 18.47 (proliferation coefficient = number of new buds/original bud number). However, cluster buds can also be induced efficiently using only cytokinins without auxins. Dandin & Murthy (2012) reported that inoculating stem segments with axillary buds on MS + 0.2 $mg \cdot L^{-1}$ 6-BA produced an average of 9.25 cluster buds per explant, which increased to 39.08 buds per explant when the medium was optimized to MS + 0.2 $mg \cdot L^{-1}$ 6-BA + 1.0 $mg \cdot L^{-1}$ KT. Other studies showed that inoculating stem segments from sterile seedlings on MS + 2.0 $mg \cdot L^{-1}$ 6-BA yielded 34.1 cluster buds per explant, but these buds failed to elongate. Transferring these regenerated cluster buds to MS + 0.35 $mg \cdot L^{-1}$ GA_3 for two weeks resulted in 96% elongation with lengths up to 3.9 cm (Purkayastha et al., 2008), likely because cytokinin 6-BA promotes bud proliferation while inhibiting shoot elongation. Adenine sulfate (ADS), a cytokinin synthesis precursor, enhances cytokinin biosynthesis (Khan et al., 2014) and is widely used as a plant growth regulator to promote bud proliferation and growth (Rency et al., 2018). Bansi & Rout (2013) demonstrated that culturing *A. paniculata* stem segments and leaves on MS + 3.0 $mg \cdot L^{-1}$ 6-BA + 50 $mg \cdot L^{-1}$ ADS + 1.0 $mg \cdot L^{-1}$ NAA for six weeks facilitated callus development. After subculturing the callus on the same medium for another six weeks, the average cluster bud number per explant reached 28.6, with bud regeneration rates of 75.3% from leaf-derived callus and 63.4% from stem-derived callus. During the rooting stage, adding either 0.5 $mg \cdot L^{-1}$ IBA or 0.5 $mg \cdot L^{-1}$ NAA separately to MS or 1/2 MS media is generally considered effective for root induction in tissue-cultured plantlets (Table 1).

1.1.4 Culture Conditions

In plant tissue culture, environmental conditions such as light, humidity, and temperature in the culture room are crucial factors for inducing organogenesis

and significantly influence explant differentiation and growth. Light is a particularly important culture condition. Studies show that *A. paniculata* seeds exhibit higher germination rates under light than in darkness; seedlings germinated in darkness appear yellowish-white with thin, weak stems and pale leaves, whereas those grown under light develop green coloration with robust stems and fresh green leaves (Ji et al., 2017). Commonly used culture conditions for *A. paniculata* tissue culture include a temperature of (25 ± 2)°C, light intensity of 2,000–2,500 lx, and a photoperiod of either 12 h light/12 h dark or 16 h light/8 h dark (Huang et al., 2010; Dandin & Murthy, 2012; Bansi & Rout, 2013; Chen, 2017).

1.1.5 Acclimatization and Transplantation

Transferring test-tube seedlings grown under controlled conditions of constant temperature, high humidity, and low light to natural outdoor environments requires a gradual acclimatization process. Bansi & Rout (2013) reported that transplanting rooted *A. paniculata* plantlets into a mixture of soil, sand, and dry cow manure (1:1:1, w/v) followed by greenhouse hardening achieved a 60% survival rate, with successful growth in the field. Dandin & Murthy (2012) achieved a 95% survival rate by transplanting rooted plantlets into pots containing a 1:1 (w/v) mixture of sterile soil and vermiculite, hardening them for two weeks at 25 ± 2°C with a 16 h light/8 h dark photoperiod, 80% relative humidity, and 50 $\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light intensity, followed by two weeks of acclimatization in shade before transferring to natural outdoor conditions. Purkayastha et al. (2008) obtained 92% survival with robust plant growth by transplanting tissue-cultured seedlings into a 1:1:1 mixture of soil, vermiculite, and vermicompost. Yan et al. (2016) found that *A. paniculata* plantlets with six true leaves grew well in a 1:1 sand-to-vermiculite substrate, achieving an 86.7% survival rate.

1.2 Evaluation of In Vitro Regeneration Systems

In vitro regeneration systems from explants to complete plants have been established for the medicinal plant *A. paniculata*. As early as 2012, Dandin & Murthy established an efficient in vitro regeneration system using stem segments with axillary buds as explants and analyzed the genetic stability of regenerated plantlets using random amplified polymorphic DNA (RAPD) markers. The results showed no genotypic variation compared to the mother plant, with andrographolide content in leaves and stems of regenerated plants exceeding that of the mother plant. Purkayastha et al. (2008), Dandin & Murthy (2012), and Bansi & Rout (2013) also established efficient rapid propagation systems using stem segments with axillary buds and leaves as explants, though they did not evaluate genetic stability or medicinal component content. Kadapatti & Murthy (2021) developed an efficient rapid propagation system for *Andrographis alata* (Vahl) Nees using stem segments with axillary buds, analyzing genetic stability with both RAPD and simple sequence repeat (SSR) markers. Their results confirmed no genotypic variation from the mother plant, while high-performance

liquid chromatography (HPLC) analysis revealed that neoandrographolide content in regenerated plants was comparable to that of the mother plant.

1.3 Breeding Applications

Andrographis paniculata exhibits limited germplasm resource diversity, with new variety breeding significantly lagging (Chen et al., 2020). Ploidy breeding based on plant tissue culture technology offers a novel approach for quality improvement and germplasm innovation in *A. paniculata*. For polyploid induction, Yan et al. (2016) used freshly germinated mature embryos as induction material with colchicine as the inducing agent, establishing preliminary methods for induction and identification of autotetraploid *A. paniculata* and obtaining four autotetraploid sterile plantlets. They determined that 0.075% colchicine treatment for 24 hours was optimal, yielding a tetraploid induction rate of 3.3%, thus laying the foundation for subsequent polyploid breeding and germplasm innovation. She et al. (2022) achieved 89% seed survival by soaking *A. paniculata* seeds in 0.05% colchicine for 48 hours, obtaining eight tetraploid plants overall. For haploid induction, Ji (2018) established an anther culture system using *A. paniculata* anthers as explants, successfully obtaining haploid embryogenic callus. This pioneering work in haploid breeding of *A. paniculata* provides a foundation for inducing homozygous diploids.

1.4 Production of Andrographolide via Callus Culture

Andrographolide and other active ingredients are present at very low concentrations in *A. paniculata* callus. Plant hormones including NAA, 2,4-D, TDZ, 6-BA, and KT, used individually or in combination, can induce andrographolide accumulation in callus cultures (Vidyalakshmi & Ananthi, 2013; Jindal et al., 2016). Jindal et al. (2016) established a callus culture system using *A. paniculata* leaves as explants, finding that leaf explants on MS + 1.0 $mg \cdot L^{-1}$ 2,4-D + 1.0 $mg \cdot L^{-1}$ NAA achieved a 92% callus induction rate with andrographolide content reaching 8.34 $mg \cdot g^{-1}$ fresh cell weight (FCW). Andrographolide is biosynthesized through the coordinated action of the cytosolic mevalonate (MAV) pathway and the plastidial deoxy-xylulose phosphate (DXP) pathway (Singh et al., 2018; Sinha et al., 2018; Das & Bandyopadhyay, 2021). Das & Bandyopadhyay (2021) discovered that treating *A. paniculata* callus with the MAV pathway inhibitor lovastatin blocked the MAV pathway, shifting andrographolide synthesis to the plastidial DXP pathway, which was upregulated, resulting in significantly increased andrographolide content and greening of the callus. Conversely, treatment with the DXP pathway inhibitor fosmidomycin blocked the DXP pathway, forcing synthesis toward the plastidial MAV pathway, which could not independently compensate for andrographolide production, leading to reduced yields. Further research revealed that silver nitrate ($AgNO_3$) could induce andrographolide production in callus cultures. Combined treatment with silver nitrate and lovastatin produced higher andrographolide yields (3.41–3.76 $mg \cdot g^{-1}$ dry cell weight, DCW) than silver nitrate with fosmidomycin, indicating

that the DXP pathway plays a dominant role in andrographolide biosynthesis. Additionally, Das & Bandyopadhyay (2021) observed a positive correlation between chlorophyll content and andrographolide content during treatments with light, silver nitrate, and biosynthetic pathway inhibitors. Therefore, future research may explore targeted enhancement of chlorophyll content in tissues or organs as a strategy to increase andrographolide yields.

2 Cell Suspension Culture of *Andrographis paniculata*

Research on medicinal plant cell culture primarily focuses on reducing costs and increasing active ingredient yields through screening high-yielding tissues or cell lines, optimizing culture conditions, and selecting efficient elicitors, or by regulating secondary metabolite biosynthetic pathways to achieve the same objective.

2.1 Callus Induction

Obtaining ideal callus determines the speed and efficiency of establishing plant cell suspension culture systems. Cell suspension culture requires loose and friable callus. Since *A. paniculata* leaves accumulate more diterpenoid lactone active ingredients than other organs (Mishra et al., 2010), researchers primarily use leaves as explants to induce ideal callus for active ingredient accumulation. Gandi et al. (2012) used three-week-old sterile seedlings derived from seeds to compare callus induction from stems, leaves, and roots as explants, concluding that leaf explants on MS + 2.0 $mg \cdot L^{-1}$ 2,4-D + 0.4 $mg \cdot L^{-1}$ 6-BA produced loose, friable callus with high propagation rates, making it suitable for cell suspension culture. Sharma & Jha (2012) used young leaves from greenhouse-grown *A. paniculata* as explants, finding that MS medium supplemented with 1.0 $mg \cdot L^{-1}$ NAA and 1.0 $mg \cdot L^{-1}$ 2,4-D produced the maximum amount of milky-white, loose, friable callus suitable for cell suspension culture. Dawande & Sahay (2020) used ten-day-old sterile seedlings from germinated seeds to investigate the effects of different explants (cotyledons, primary leaves, hypocotyls, and epicotyls), media (B5 and SH), and hormone combinations on callus induction. Their results indicated that cotyledons and hypocotyls showed optimal callus induction on SH medium containing 2.0 $mg \cdot L^{-1}$ 2,4-D and 0.1 $mg \cdot L^{-1}$ 6-BA.

2.2 Andrographolide Production from Cell Culture

Andrographolide accumulation in *A. paniculata* cell suspension cultures can be substantially enhanced through optimized culture conditions and elicitor treatments. Sharma and Jha (2012) reported that cell cultures on liquid MS + 1.0 $mg \cdot L^{-1}$ NAA + 1.0 $mg \cdot L^{-1}$ 2,4-D contained up to 32.4 $mg \cdot g^{-1}$ FCW of andrographolide, representing 2.4 times the content in the original callus and 1.3 times that in leaves. Secondary metabolite accumulation in plant cell cultures can be further enhanced through appropriate elicitor-induced stress (Yue et al.,

2016), with elicitors including biological, abiotic, and signaling molecules. Gandi et al. (2012) first reported andrographolide induction methods in *A. paniculata* cell suspension culture, finding that biological elicitors (yeast, *Escherichia coli*, *Bacillus subtilis*, *Agrobacterium rhizogenes* 532, and *Agrobacterium* C58) were more effective than abiotic elicitors ($CdCl_2$, $AgNO_3$, $CuCl_2$, and $HgCl_2$) for inducing andrographolide accumulation, with yeast elicitation being optimal at 13.5 $mg \cdot g^{-1}$ DCW—an 8-fold increase over controls. Additional studies showed that treatment with 1.5 mL of *Aspergillus niger* for 10 days maximized andrographolide accumulation at 13.2 $mg \cdot g^{-1}$ DCW in cell suspension cultures derived from leaf callus, representing a 6.94-fold increase over controls. Treatment with 0.6% *Penicillium expansum* for 8 days yielded 8.1 $mg \cdot g^{-1}$ DCW, a 6.23-fold increase (Vakil & Mendhulkar, 2013a). Salicylic acid at 7.0 $mg \cdot L^{-1}$ for 24 hours resulted in 3.7 $mg \cdot g^{-1}$ DCW, an 18.5-fold increase, while 20 mg chitosan treatment for 48 hours produced 11.9 $mg \cdot g^{-1}$ DCW, a 59.5-fold increase over controls (Vakil & Mendhulkar, 2013b). Sharma et al. (2014) demonstrated that 1.0 $mg \cdot L^{-1}$ methyl jasmonate (MJA) treatment for 24 hours during cell suspension culture increased andrographolide content by 5.25-fold. Dawande & Sahay (2020) found that culturing on 1/2 MS liquid medium with 20 $g \cdot L^{-1}$ sucrose under 20 $h \cdot d^{-1}$ illumination for three weeks yielded callus with andrographolide content up to 4.60 $mg \cdot g^{-1}$ DCW. Under these conditions, elicitation with copper sulfate, methyl jasmonate, chitin, and fungal mycelium all significantly enhanced andrographolide production, with 80 $mg \cdot L^{-1}$ copper sulfate achieving the highest yield at 29.42 $mg \cdot g^{-1}$ DCW. These results demonstrate that combining optimized culture conditions with elicitor treatments can substantially improve overall andrographolide yields. While most studies suggest antagonistic effects between salicylic acid (SA) and jasmonic acid (JA) in plant adaptive regulation (Brooks et al., 2005; Zheng et al., 2012), some indicate synergistic interactions (Mur et al., 2006). Both SA and JA can individually induce andrographolide accumulation in callus (Zaheer & Giri, 2015). Ahmed & Praveen (2023) found that SA and JA effects on andrographolide content in cell cultures increased with concentration. Specifically, 13.8 $mg \cdot L^{-1}$ SA induced 0.083 $mg \cdot g^{-1}$ DCW, an 0.18-fold increase over controls, while 21.0 $mg \cdot L^{-1}$ JA yielded 0.211 $mg \cdot g^{-1}$ DCW, a 3-fold increase. Combined treatment with 10.35 $mg \cdot L^{-1}$ SA and 15.75 $mg \cdot L^{-1}$ JA produced 0.28 $mg \cdot g^{-1}$ DCW, a 3.8-fold increase. These findings demonstrate that combined use of multiple positive elicitors is more effective than single elicitor treatments in *A. paniculata* cell suspension culture.

3.1 Accumulation of Andrographolide in Adventitious Root Culture

Adventitious roots are induced by wounding or external stimuli such as hormones and pathogenic microorganisms, developing abnormally in terms of timing and location, typically emerging from stems, leaves, and hypocotyls. Adventitious root culture can accumulate substantial secondary metabolites, providing a novel pathway for obtaining active ingredients from medicinal plants

(Paek et al., 2005). Currently, most medicinal plants have successfully induced adventitious roots for shake-flask or bioreactor culture (Miao, 2022).

In *A. paniculata* adventitious root induction, Praveen et al. (2009) induced adventitious roots from leaf explants on MS medium containing 1.0 $mg \cdot L^{-1}$ NAA and 0.3% sucrose. Culturing in liquid MS with 0.5 $mg \cdot L^{-1}$ NAA and 0.3% sucrose for four weeks yielded 7 times higher biomass and 3.5 times higher andrographolide content than controls. Sharma et al. (2013) used leaf explants on modified MS with 1.0 $mg \cdot L^{-1}$ NAA, achieving an average of 26.7 adventitious roots per explant with an 83% induction rate. After five weeks in liquid culture of the same composition, andrographolide content reached 133.3 $mg \cdot g^{-1}$ DCW, 3.5–5.5 times higher than controls. Das & Bandyopadhyay (2015) directly induced adventitious roots from leaf and root explants on MS with 2.0 $mg \cdot L^{-1}$ NAA, achieving maximum andrographolide content of 1.06 $mg \cdot L^{-1}$ DCW in adventitious roots after four weeks in liquid culture.

3.2 Elicitor-Induced Accumulation of Andrographolide

Elicitor treatment can enhance andrographolide accumulation not only in cell cultures but also substantially in adventitious root cultures. Zaheer & Giri (2017) first reported the effects of chemical elicitors salicylic acid (SA) and jasmonic acid (JA) on andrographolide accumulation in *A. paniculata* adventitious root culture. They found that various JA concentrations could induce andrographolide accumulation, with 3.45 $mg \cdot L^{-1}$ JA being optimal, achieving 10.8 times the control content after one week. In contrast, treatment with different SA concentrations and derivatives for one week showed that only 15.2 $mg \cdot L^{-1}$ methyl salicylic acid (MSA) increased andrographolide content by 2.6 times over controls, indicating that JA is significantly more effective than SA for inducing andrographolide accumulation in adventitious root culture.

Furthermore, Srinath et al. (2022) found that ethylene (ETH) elicitation significantly increased adventitious root culture biomass by 4-fold and andrographolide content by 5-fold, while light exposure increased andrographolide content by 4.29-fold. These results demonstrate that plant growth regulators as elicitors can induce andrographolide accumulation in *A. paniculata* adventitious roots. However, the currently known chemical elicitors capable of inducing andrographolide accumulation in adventitious roots are limited, necessitating further screening and identification.

4 Hairy Root Culture of *Andrographis paniculata*

Hairy roots are a pathological state induced by *Agrobacterium rhizogenes* infection, characterized by rapid growth, ease of large-scale culture, hormone autonomy, high secondary metabolite yields, and stable physiological, biochemical, and genetic properties, offering significant industrial potential. In recent years, hairy root culture technology has gained attention as a novel approach for secondary metabolite development in medicinal plants, becoming an important

culture system alongside tissue and cell culture systems. Marwani et al. (2015) demonstrated that using cotyledons as explants and strain ATCC 15834 for two-day infection produced optimal hairy root induction, with maximum andrographolide content of 5.4 $mg \cdot g^{-1}$ DCW achieved during the second week of culture in 1/2 MS liquid medium with 1.0 $mg \cdot L^{-1}$ IBA. Mahobia & Jha (2015) used MTCC 532 as the *Agrobacterium* strain to investigate the effects of different explants (leaves and root tip meristems), infection methods (immersion and droplet inoculation), and media on hairy root induction. They found that using the immersion method for three-day co-culture on 1/2 MS medium with 80.0 $mg \cdot L^{-1}$ acetosyringone and 0.3% sucrose yielded the highest hairy root induction rate of 58.76%.

5.1 Existing Problems

Although numerous reports have documented *A. paniculata* tissue culture regeneration of complete plants (Purkayastha et al., 2008; Dandin & Murthy, 2012; Bansi & Rout, 2013), the in vitro regeneration efficiency requires further improvement and hormone ratios need optimization for large-scale production applications. For medicinal plants, the genetic stability of regenerated plants and their active ingredient content represent crucial evaluation indicators for in vitro regeneration systems and are key prerequisites for large-scale commercial application (Miao et al., 2017). However, few studies have incorporated genetic stability and active ingredient content as evaluation criteria for regeneration systems. This review also reveals that while optimizing culture conditions and using elicitors can significantly enhance andrographolide content in callus, cell, adventitious root, and hairy root cultures, and that combining multiple elicitors with optimized culture conditions can further improve yields, such studies remain extremely rare.

5.2 Future Prospects

Although previous research has achieved technical breakthroughs in *A. paniculata* in vitro culture, with maturing tissue regeneration systems and increasingly refined callus, cell suspension, adventitious root, and hairy root culture technologies, and although active ingredient accumulation (e.g., andrographolide) has been substantially improved through optimized culture conditions and elicitor treatments, these advances remain at the basic research stage with significant gaps to large-scale production application (Murthy & Dalawai, 2021). Compared with major Chinese medicinal materials, basic research on *A. paniculata* in vitro culture and active ingredient production lacks systematic approaches, and key technologies for producing important secondary metabolites through organ, tissue, and cell culture require further development. Research approaches from other major medicinal plants, particularly those with established large-scale production technologies, offer valuable insights for *A. paniculata* studies. The authors propose that future research on *A. paniculata* in vitro culture and active ingredient production should focus on three key areas:

5.2.1 Maturing and Improving the Tissue In Vitro Regeneration System for *A. paniculata* While Establishing a Comprehensive Evaluation System

The tissue in vitro regeneration system for *A. paniculata* should be matured and improved through optimizing plant growth regulator ratios, combinations, and culture conditions to enhance tissue culture efficiency. Studies on other medicinal plants have demonstrated that evaluating genetic stability of regenerated plants using molecular markers such as ISSR, EST-SSR, RAPD, and AFLP, either individually or in combination, is reliable and feasible (Mamdouh et al., 2021; Sharma et al., 2022; Babanina et al., 2023). Therefore, future research should integrate multiple molecular marker detection methods while improving the efficiency of *A. paniculata* tissue regeneration systems, strengthening tracking studies on genetic stability and active ingredient content to establish a comprehensive and systematic evaluation framework.

5.2.2 Combining Optimized Culture Conditions with Efficient Elicitors to Further Increase Andrographolide and Other Active Ingredient Yields

Elicitor-induced enhancement of secondary metabolite accumulation has been successfully demonstrated in numerous medicinal plants including *Panax ginseng* (Huang et al., 2013), *Cistanche deserticola* (Cheng et al., 2005), *Hypericum perforatum* (Conceiçao et al., 2006), *Silybum marianum* (Sánchez-Sampedro et al., 2005), and *Calendula officinalis* (Wiktorowska et al., 2010). The andrographolide biosynthetic pathway has been gradually elucidated (Das & Bandyopadhyay, 2021; Zhong et al., 2021), yet targeted regulation of andrographolide content based on this pathway remains underexplored. Therefore, future research on *A. paniculata* in vitro culture for andrographolide production should focus on purposefully combining optimized culture conditions with efficient elicitors based on the biosynthetic pathway to further enhance andrographolide and other active ingredient content, providing reliable and efficient technical support for large-scale production applications.

5.2.3 Developing Bioreactor Culture Systems for Andrographolide Production via Cell Suspension Culture

Bioreactor culture offers large-scale production, year-round operation, independence from seasonal and regional constraints, high automation, and low production costs, making it highly promising for large-scale production of medicinal plant secondary metabolites. Bioreactor systems for active ingredient production have been established for *Panax ginseng* cell suspension cultures (Thanh et al., 2014), adventitious roots (Song et al., 2017), and hairy root cultures (Kochan et al., 2018), as well as for *Panax quinquefolius* hairy root culture (Kochan et al., 2017) and *Angelica acutiloba* adventitious root culture (Hwang et al., 2022). Ginsenosides and other important active ingredients have achieved large-scale production through bioreactors (Thanh et al., 2014; Luthra et al., 2021). In

contrast, research in this area for *A. paniculata* lags significantly behind. Currently, cell suspension culture for andrographolide production in *A. paniculata* is relatively well-studied and technologically mature. Therefore, future research on bioreactor-based cell suspension culture for andrographolide production in *A. paniculata* holds considerable promise.

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