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

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Abstract

Andrographis paniculata is one of the important southern traditional Chinese medicines in China, used for clearing heat and detoxifying, cooling blood and reducing swelling. Its main active component, andrographolide, possesses anticancer, anti-HIV, anti-inflammatory, hepatoprotective and other pharmacological effects. Andrographolide is difficult to synthesize artificially and mainly relies on extraction from artificially cultivated plant materials. However, the quality of cultivated medicinal materials is uneven due to various factors such as soil, climate, water and fertilizer management, and Andrographis paniculata has a long growth cycle and occupies land resources. Plant in vitro culture technology has significant advantages in rapid propagation of seedlings and accumulation of active components, representing one of the important approaches for rapid and efficient production of Andrographis paniculata active components. The tissue in vitro regeneration technology system for Andrographis paniculata is increasingly being perfected, with the technology for tissue in vitro regeneration from explants to complete plants becoming mature, and has found certain applications in seedling propagation, ploidy breeding and other aspects. Meanwhile, in the processes of Andrographis paniculata callus culture, cell suspension culture, adventitious root culture, and hairy root culture, the accumulation of andrographolide and other active components in the cultures can be significantly increased through optimizing culture conditions and using appropriate elicitors. This article comprehensively and systematically reviews the research progress on in vitro culture technology of Andrographis paniculata and its production of andrographolide in recent years both domestically and internationally, from the aspects of tissue, cell, adventitious root and hairy root culture, with the aim of promoting the development and application of in vitro culture technology for Andrographis paniculata and providing references for research on in vitro production of andrographolide. This article also proposes three aspects that

need to be focused on in future research on in vitro culture technology of Andrographis paniculata and production of andrographolide through this technology: (1) Perfect and mature the tissue in vitro regeneration technology system for Andrographis paniculata, and establish a comprehensive and systematic evaluation system; (2) Combine optimized culture conditions with efficient elicitors to further improve the yield of important active components such as andrographolide; (3) Conduct research on bioreactor cultivation for andrographolide production through cell suspension culture technology.

Full Text

Preamble

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Title: Study Advances of In Vitro Culture Technology and Its Application in Medicinal Plant *Andrographis paniculata*

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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 extracted from cultivated plant materials. However, the quality of cultivated medicinal materials varies considerably due to soil, climate, water, and fertilizer management factors, 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 components. The in vitro regeneration technology system for *A. paniculata* has become increasingly sophisticated, with tissue regeneration from explants to complete plants now well-established and applied in seedling propagation and ploidy breeding. Meanwhile, through optimization of culture conditions and application of appropriate elicitors, the accumulation of andrographolide and other active ingredients in callus cultures, cell suspension cultures, adventitious root cultures, and hairy root cultures can be substantially increased. This paper comprehensively and systematically reviews recent domestic and international 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 *A. paniculata* in vitro culture technology and provide references for in vitro andrographolide production research. The paper also proposes three key areas for future research: (1) maturing and improving the *A. paniculata* tissue regeneration system and establishing a comprehensive evaluation system; (2) further increasing the yield of andrographolide and other important active ingredients through optimized culture conditions combined with efficient elicitors; and (3) conducting bioreactor cultivation studies for andrographolide production via cell suspension culture technology.

Keywords: *Andrographis paniculata*, tissue culture, cell suspension culture, andrographolide, advances

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Andrographis paniculata, also known as "Yijianxi," "Indian herb," or "Lanhelian," is an annual herbaceous medicinal plant belonging to the family Acanthaceae and genus *Andrographis*. Its dried aerial parts are used medicinally for clearing heat, detoxifying, cooling blood, and reducing swelling (National Pharmacopoeia Committee, 2020). *A. paniculata* serves as the primary raw material for national essential medicines such as Fuke Qianjin Pian and Xiaoyan Lidan Pian, and is a major component in various Chinese patent medicines including Andrographolide Dropping Pills, Jinji Capsules, Andrographis Tablets, Compound Andrographis Tablets, Qinghuo Zhimai Pian, and Yuye Qinghuo Capsules. Andrographolide, the main active component in the aerial parts of *A. paniculata*, has been one of the most extensively studied natural products over the past 70 years and is widely used clinically, demonstrating remarkable efficacy in anticancer, anti-HIV, anti-inflammatory, and hepatoprotective applications (Islam et al., 2018; Burgos et al., 2020; Ren et al., 2021). With continuous development of medicinal applications for *A. paniculata* and its active components, the commercial demand for andrographolide is enormous. However, due to its complex structure, artificial synthesis remains challenging, and production currently relies primarily on extraction from cultivated plant materials. Although *A. paniculata* has been widely cultivated in India, Pakistan, Sri Lanka, Thailand, Malaysia, China, and Indonesia (Kandanur et al., 2019; Chen et al., 2020), andrographolide biosynthesis and accumulation are influenced by genotype, cultivation mode, climatic factors, and geographical environment (Tajidin et al., 2019; Rafi et al., 2020; Dalawai et al., 2021; Zhong et al., 2021), while cultivation area is also constrained by increasingly limited land resources. Therefore, relying solely on agriculturally produced plant materials for andrographolide extraction has inherent limitations.

Plant tissue, organ, and cell in vitro culture has developed into one of the most attractive alternative approaches for secondary metabolite production (Murthy et al., 2014; Espinosa-Leal et al., 2018). Plant in vitro culture technology includes tissue culture, cell suspension culture, adventitious root culture, hairy root culture, and protoplast culture, offering significant advantages for maintaining medicinal plant germplasm, accumulating active ingredients, and genetic

transformation research. Research on *A. paniculata* tissue culture began in the 1970s, and the technology system has become increasingly sophisticated. In vitro regeneration from explants to complete plants is now well-established and has been applied in seedling propagation and ploidy breeding. Simultaneously, through optimization of culture conditions and application of appropriate elicitors, the accumulation of andrographolide and other active ingredients in callus, cell suspension, adventitious root, and hairy root cultures can be substantially increased.

This paper comprehensively and systematically reviews recent research advances in *A. paniculata* in vitro culture technology and production of its main active ingredients through in vitro culture, analyzes existing problems, and proposes directions for further research, aiming to promote the research, application, and further development of *A. paniculata* in vitro culture technology.

1.1 Overview of *A. paniculata* Tissue Culture Technology

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

1.1.1 Explants Successful explant inoculation depends on factors such as tissue source, collection time, 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 yield better tissue culture results (Purkayastha et al., 2008; Bansi & Rout, 2013; Chen, 2017; Dai et al., 2018). Ethanol, sodium hypochlorite, and mercuric chloride can be used for explant sterilization, but disinfectant concentration and treatment time significantly affect explant survival. Chen (2017) and Dai (2018) found that when using stem segments with axillary buds as explants, sterilization with 75% ethanol for 15 seconds followed by 3% sodium hypochlorite for 30 minutes achieved the best results, with a survival rate of 56.33%. When using seeds as explants, treatment with 75% ethanol for 15 seconds followed by 3% sodium hypochlorite for 20 minutes yielded a survival rate of 78.67%.

1.1.2 Culture Media As the primary nutrient source in plant tissue culture, the composition and concentration of basal media directly affect explant growth and differentiation status, with different explants requiring different media types. Huang et al. (2010) determined that MS medium was most suitable for *A. paniculata* bud proliferation and growth, achieving a proliferation rate of 83.3% with vigorous growth. Ji et al. (2017) compared four different media (pure agar, MS, 1/2 MS, MS + 2.0 $mg \cdot L^{-1}$ 6-BA) for seed germination and concluded that MS or 1/2 MS were suitable for *A. paniculata* seed germination based on comprehensive consideration of germination and survival rates. Yan (2016) compared five basal media (MS, 1/2 MS, MT, H, B5) for sterile seedling

growth and found 1/2 MS most suitable. Ji (2018) compared MS, N6, and Nistch media for anther culture and identified N6 as the most suitable basal medium for *A. paniculata* anther culture.

1.1.3 Plant Growth Regulators Basal media can only maintain basic survival and minimal nutritional requirements of cultured tissues. Adding appropriate ratios of plant growth regulators to basal media can induce cell division initiation, callus growth, and root and bud differentiation. Different types and ratios of plant growth regulators have varying effects on explant dedifferentiation and organogenesis.

Cytokinins and auxins are commonly used growth regulators in plant tissue culture. Cytokinins promote cell division and growth, stimulate cell differentiation and bud formation, and influence callus differentiation and bud development (Loyola-Vargas & Ochoa-Alejo, 2018). Auxins promote cell growth and elongation and are associated with vascular tissue 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 example, 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 produced the best cluster bud induction with a proliferation coefficient of 18.47 (proliferation coefficient = number of new buds/original bud number). However, cluster buds can also be induced efficiently by adding only one or more cytokinins without auxins. Dandin & Murthy (2012) reported that inoculating stem segments with axillary buds on MS + 0.2 mg $\cdot$ L^{-1} 6-BA yielded an average of 9.25 regenerated cluster buds per explant, which further increased to 39.08 buds per explant on 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 medium with 2.0 mg $\cdot$ L^{-1} 6-BA produced an average of 34.1 cluster buds per explant, but these buds failed to elongate. Transferring the regenerated cluster buds to MS medium containing 0.35 mg $\cdot$ L^{-1} GA_3 for two weeks achieved 96% elongation with lengths up to 3.9 cm (Purkayastha et al., 2008), likely because cytokinin 6-BA promotes bud proliferation while inhibiting bud elongation. Adenine sulfate (ADS), a precursor for cytokinin synthesis, can increase cytokinin biosynthesis (Khan et al., 2014) and is widely used as a growth regulator to promote bud proliferation and growth (Rency et al., 2018). Bansi & Rout (2013) demonstrated that culturing 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 favored callus development. After subculturing the callus on the same medium for another six weeks, each explant produced an average of 28.6 cluster buds, with bud regeneration rates of 75.3% from leaf-derived callus and 63.4% from stem-derived callus. During the rooting stage, adding 0.5 mg $\cdot$ L^{-1} IBA or 0.5 mg $\cdot$ L^{-1} NAA separately to MS or 1/2 MS medium is generally considered effective for root induction in tissue-cultured seedlings (Table 1).

1.1.4 Culture Conditions In plant tissue culture, environmental conditions such as light, humidity, and temperature in the culture room are important factors for inducing organogenesis and significantly affect explant differentiation and growth. Light is a crucial culture condition. Studies show that *A. paniculata* seeds have higher germination rates under light than in darkness; seedlings germinated in darkness appear yellowish-white with thin, weak stems and yellowish leaves, while those under light appear green with robust stems and fresh green leaves (Ji et al., 2017). The commonly used culture temperature for *A. paniculata* is (25 ± 2)°C, with light intensity of 2,000–2,500 lx and photoperiod of 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 constant temperature, high humidity, and low light to natural outdoor conditions requires a gradual adaptation process. Bansi & Rout (2013) reported that transplanting rooted *A. paniculata* tissue-cultured seedlings into a mixed substrate of soil, sand, and dry cow dung (1:1:1, w/v) followed by greenhouse acclimatization achieved a survival rate of 60%, with successful field growth. Dandin & Murthy (2012) found that transplanting rooted seedlings into pots containing sterile soil and vermiculite (1:1, w/v) and acclimatizing them for two weeks at 25 ± 2°C, 16 h light/8 h dark photoperiod, 80% relative humidity, and 50 $\mathrm{mol \cdot m^{-2} \cdot s^{-1}}$ light intensity, followed by two weeks in shaded conditions before transferring to natural outdoor environments, achieved a 95% survival rate. Purkayastha et al. (2008) reported that transplanting *A. paniculata* tissue-cultured seedlings into pots with soil, vermiculite, and vermicompost (1:1:1) resulted in 92% survival after two weeks with robust plant growth. Yan et al. (2016) demonstrated that tissue-cultured seedlings with six true leaves grew well in a substrate mixture of river sand and vermiculite (1:1), achieving an 86.7% survival rate.

1.2 Evaluation of In Vitro Regeneration Systems

Currently, an in vitro regeneration technology system from explant tissues to complete plants has been established for the medicinal plant *A. paniculata*. As early as 2012, Dandin & Murthy (2012) 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 indicated no genotypic variation compared to the mother plant, with andrographolide content in leaves and stems of regenerated plants higher than in the mother plant. Purkayastha et al. (2008), Dandin & Murthy (2012), and Bansi & Rout (2013) also established efficient in vitro 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) established an efficient in vitro rapid propagation system for *Andrographis alata* (Vahl) Nees using stem segments with axillary buds as explants, analyzed genetic stability using both RAPD

and simple sequence repeat (SSR) markers, and detected neoandrographolide content in regenerated plants using high-performance liquid chromatography (HPLC). The results showed no genotypic variation compared to the mother plant, with neoandrographolide content equivalent to that of the mother plant.

1.3 Breeding Applications

Andrographis paniculata has limited germplasm resource diversity, and new variety breeding has seriously lagged (Chen et al., 2020). Ploidy breeding based on plant tissue culture technology provides a new approach for quality improvement and germplasm innovation in *A. paniculata*. For polyploid induction, Yan et al. (2016) used freshly germinated mature embryos as induction materials and colchicine as the inducing agent to preliminarily establish induction and identification methods for autotetraploid *A. paniculata*, obtaining four autotetraploid sterile plantlets. They identified 0.075% colchicine treatment for 24 hours as optimal, achieving a tetraploid induction rate of 3.3%, which laid the foundation for subsequent polyploid breeding and germplasm innovation research. She et al. (2022) treated seeds with 0.05% colchicine for 48 hours, achieving 89% survival rate and obtaining eight tetraploid plants. For haploid induction, Ji (2018) established an anther culture system using *A. paniculata* anthers as explants, successfully obtaining haploid embryogenic callus, pioneering haploid breeding in *A. paniculata* and laying the foundation for pure diploid induction.

1.4 Andrographolide Production from Callus Culture

Andrographolide content is extremely low in *A. paniculata* callus. Plant hormones including NAA, 2,4-D, TDZ, 6-BA, and KT, used alone or in combination, can induce andrographolide accumulation in callus (Vidyalakshmi & Ananthi, 2013; Jindal et al., 2016). Jindal et al. (2016) established a callus culture system using *A. paniculata* leaves as explants and found that leaf explants on MS + 1.0 $mg \cdot L^{-1}$ 2,4-D + 1.0 $mg \cdot L^{-1}$ NAA achieved 92% callus induction rate with andrographolide content up to 8.34 $mg \cdot g^{-1}$ fresh cell weight (FCW). Andrographolide is produced through coordinated action of the cytosolic mevalonate (MAV) pathway and plastidial deoxy-xylulose phosphate (DXP) pathway (Singh et al., 2018; Sinha et al., 2018; Das & Bandyopadhyay, 2021). Das & Bandyopadhyay (2021) found that when *A. paniculata* callus was treated with the MAV pathway blocker lovastatin, the MAV pathway was blocked, shifting andrographolide synthesis to the plastidial DXP pathway, which was upregulated, significantly increasing andrographolide content and causing callus greening. Conversely, treatment with the DXP pathway blocker fosmidomycin shifted synthesis to the plastidial MAV pathway, which could not independently compensate for andrographolide production, resulting in reduced accumulation. Further research revealed that silver nitrate ($AgNO_3$) could induce andrographolide production in callus, with combined silver nitrate and lovastatin treatment yielding higher andrographolide production (3.41–3.76 $mg \cdot g^{-1}$ dry cell weight, DCW) than silver nitrate and fosmidomycin combination, indicating that the DXP

pathway plays a dominant role in andrographolide biosynthesis. Additionally, Das & Bandyopadhyay (2021) found a positive correlation between chlorophyll content and andrographolide content during light, silver nitrate, and biosynthetic pathway inhibitor treatments. Therefore, purposefully increasing chlorophyll content in tissues or organs may represent another strategy for enhancing andrographolide yield in future research.

2. *Andrographis paniculata* Cell Suspension Culture

The main focus of medicinal plant cell culture research is to reduce costs and increase active ingredient yield through screening high-yield tissue or cell lines, optimizing culture conditions, and selecting efficient elicitors, or to achieve the same goal by regulating secondary metabolite biosynthetic pathways.

2.1 Callus Induction Obtaining ideal callus determines the rapidity and efficiency of establishing plant cell suspension culture systems. Loose and friable callus should be selected for cell suspension culture. Since *A. paniculata* leaves accumulate more diterpene lactone active ingredients than other organs (Mishra et al., 2010), researchers typically select leaves as explants for inducing ideal callus for active ingredient accumulation. Gandi et al. (2012) used three-week-old sterile seedlings from seeds as materials and compared stem, leaf, and root explants for callus induction, 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, suitable for cell suspension culture. Sharma & Jha (2012) used young leaves from greenhouse-grown *A. paniculata* as explants and found 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 sterile seedlings germinated from seeds for 10 days to study the effects of different explants (cotyledons, primary leaves, hypocotyls, and epicotyls), media (B5, SH), and hormone combinations on callus induction, finding 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 increased through optimized culture conditions and elicitor induction. 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 andrographolide, 2.4 times higher than in parent callus and 1.3 times higher than in leaves. Secondary metabolite accumulation in plant cell cultures can be further enhanced after induction by appropriate elicitors (Yue et al., 2016), including biotic elicitors, abiotic elicitors, and signaling molecules. Gandi et al. (2012) first reported andrographolide induction methods in *A. paniculata* cell suspension culture, finding that biotic 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 the control. Other 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 from leaf-derived callus, a 6.94-fold increase, while 0.6% *Penicillium expansum* treatment for 8 days yielded 8.1 $mg \cdot g^{-1}$ DCW, a 6.23-fold increase (Vakil & Mendhulkar, 2013a). Treatment with 7.0 $mg \cdot L^{-1}$ salicylic acid for 24 hours resulted in 3.7 $mg \cdot g^{-1}$ DCW andrographolide (18.5-fold increase), and 20 mg chitosan treatment for 48 hours yielded 11.9 $mg \cdot g^{-1}$ DCW (59.5-fold increase) (Vakil & Mendhulkar, 2013b). Sharma et al. (2014) reported that 24-hour induction with 1.0 $mg \cdot L^{-1}$ methyl jasmonate (MJA) in cell suspension culture increased andrographolide content 5.25-fold compared to the control. Dawande & Sahay (2020) found that culturing callus on 1/2 MS liquid medium with 20 $g \cdot L^{-1}$ sucrose under 20 $h \cdot d^{-1}$ light for three weeks yielded 4.60 $mg \cdot g^{-1}$ DCW andrographolide. Under these conditions, copper sulfate, methyl jasmonate, chitin, and fungal mycelium elicitation all significantly increased andrographolide yield, with 80 $mg \cdot L^{-1}$ copper sulfate producing the highest yield at 29.42 $mg \cdot g^{-1}$ DCW. This demonstrates that combining optimized culture conditions with elicitor induction can significantly improve overall andrographolide yield. While most studies suggest that salicylic acid (SA) and jasmonic acid (JA) act antagonistically in plant adaptive regulation (Brooks et al., 2005; Zheng et al., 2012), some indicate synergistic effects (Mur et al., 2006). Both SA and JA can individually induce andrographolide accumulation in callus (Zaheer & Giri, 2015). Ahmed & Praveen (2023) found that the effects of SA and JA on andrographolide content in cell cultures increased with concentration, with 13.8 $mg \cdot L^{-1}$ SA inducing 0.083 $mg \cdot g^{-1}$ DCW (0.18-fold increase) and 21.0 $mg \cdot L^{-1}$ JA inducing 0.211 $mg \cdot g^{-1}$ DCW (3-fold increase). Combined treatment with 10.35 $mg \cdot L^{-1}$ SA and 15.75 $mg \cdot L^{-1}$ JA yielded 0.28 $mg \cdot g^{-1}$ DCW (3.8-fold increase), demonstrating that combined use of multiple positive elicitors is more effective than single elicitor treatment in *A. paniculata* cell suspension culture.

3.1 Andrographolide Accumulation in Adventitious Root Culture

Adventitious roots are induced by wounding or external factors such as hormones and pathogenic microorganisms, developing abnormally in terms of timing and location, typically on stems, leaves, and hypocotyls. Plant adventitious root culture can accumulate large amounts of secondary metabolites, providing a new approach for obtaining medicinal plant active ingredients (Paek et al., 2005). Most medicinal plants have been successfully induced to form adventitious roots for shake flask or bioreactor culture (Miao, 2022). For *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, achieving 7-fold higher biomass and 3.5-fold higher andrographolide content after four weeks in liquid MS medium with 0.5 $mg \cdot L^{-1}$ NAA and 0.3% sucrose. Sharma et al. (2013) induced an average of 26.7 adventitious roots per explant with 83%

induction rate from leaf explants on modified MS medium containing 1.0 mg · L^{-1} NAA, reaching 133.3 mg · g^{-1} DCW andrographolide after five weeks in liquid medium of the same composition (3.5–5.5 times higher than the control). Das & Bandyopadhyay (2015) directly induced adventitious roots from leaves and roots on MS medium with 2.0 mg · L^{-1} NAA, achieving maximum andrographolide content of 1.06 mg · L^{-1} DCW after four weeks in the same liquid medium.

3.2 Elicitor-Induced Andrographolide Accumulation

Elicitor induction not only increases andrographolide accumulation in cell cultures but also induces substantial accumulation 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, finding that various JA concentrations could induce andrographolide accumulation, with 3.45 mg · L^{-1} JA being optimal and increasing andrographolide content 10.8-fold after one week. Treatment with different SA concentrations and derivatives for one week showed that only 15.2 mg · L^{-1} methyl salicylic acid (MSA) increased andrographolide content 2.6-fold compared to the control, indicating that JA is significantly more effective than SA for andrographolide accumulation in adventitious root culture. Additionally, Srinath et al. (2022) found that ethylene (ETH) elicitation significantly increased adventitious root culture biomass 4-fold and andrographolide content 5-fold, while light increased andrographolide content 4.29-fold. Although plant growth regulators as elicitors can induce andrographolide accumulation in adventitious roots, currently known chemical elicitors for this purpose are limited and require further screening and exploration.

4. *Andrographis paniculata* Hairy Root Culture

Hairy roots are a pathological state produced after *Agrobacterium rhizogenes* infection, characterized by rapid growth, ease of large-scale culture, hormone autonomy, high secondary metabolite yield, and stable physiological, biochemical, and genetic properties, offering great industrial potential. In recent years, hairy root culture technology has gained attention as a new approach for secondary metabolite development in medicinal plants, becoming another cultivation system following tissue and cell culture. Marwani et al. (2015) showed that using cotyledons as explants and strain ATCC 15834 for 2-day infection achieved optimal hairy root induction, with maximum andrographolide content of 5.4 mg · g^{-1} DCW during the second week of culture in 1/2 MS liquid medium with 1.0 mg · L^{-1} IBA. Mahobia & Jha (2015) used MTCC 532 as the *Agrobacterium* strain to study the effects of different explants (leaves and root tip meristems), infection methods (immersion and injection), and media on hairy root induction, concluding that the highest induction rate of 58.76% was achieved using the immersion method for 3-day co-culture on 1/2 MS medium with 80.0 mg · L^{-1} acetosyringone and 0.3% sucrose.

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 needs further improvement and hormone ratios require optimization for large-scale production applications. For medicinal plants, the genetic stability of regenerated plants and their active ingredient content are important evaluation indicators for in vitro regeneration systems and key prerequisites for large-scale production applications (Miao et al., 2017). However, few studies have evaluated genetic stability and active ingredient content as assessment criteria for regeneration systems. This review also reveals that while optimizing culture conditions and using elicitors can significantly increase andrographolide content in callus, cell, adventitious root, and hairy root cultures, and that combining multiple elicitors with optimized culture conditions can further improve yield, such studies are extremely rare.

5.2 Future Perspectives

Although previous research has achieved certain technical breakthroughs in *A. paniculata* in vitro culture, with increasingly mature tissue regeneration systems and improving callus, cell suspension, adventitious root, and hairy root culture systems, and although active ingredient accumulation (such as andrographolide) has been substantially increased through optimized culture conditions and elicitor induction, these achievements remain at the basic research stage with a considerable gap from large-scale production application (Murthy & Dalawai, 2021). Compared with major Chinese medicinal materials, basic research on *A. paniculata* in vitro culture technology and active ingredient production lacks systematicity, and key technologies for producing important secondary metabolites based on plant organ, tissue, and cell culture require further development. Research approaches for other major medicinal plants regarding in vitro culture and important active ingredient production, especially those already scaled up for industrial application, offer valuable references for *A. paniculata* research. The authors propose that future research on *A. paniculata* in vitro culture technology and active ingredient production should focus on three key aspects:

5.2.1 Maturing and Improving the *A. paniculata* Tissue Regeneration System and Establishing a Comprehensive Evaluation System The *A. paniculata* tissue regeneration system should be matured and improved by optimizing plant growth regulator ratios, combinations, and culture conditions to enhance tissue culture efficiency. Studies on other medicinal plants have shown that using molecular markers such as ISSR, EST-SSR, RAPD, and AFLP, or combinations of two or more markers, is reliable and feasible for evaluating genetic stability of regenerated plants (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 studies on genetic stability and tracking of main active ingredients in regenerated plants to establish a comprehensive and systematic evaluation system.

5.2.2 Combining Optimized Culture Conditions with Efficient Elicitors to Further Increase Andrographolide and Other Important Active Ingredient Yields Elicitor induction has enhanced secondary metabolite accumulation in many medicinal plants including *Panax ginseng* (Huang et al., 2013), *Cistanche deserticola* (Cheng et al., 2005), *Hypericum perforatum* (Conceição 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), but targeted regulation of andrographolide content based on its biosynthetic pathway remains understudied. Therefore, future research on in vitro andrographolide production should focus on purposefully combining optimized culture conditions with efficient elicitors based on the biosynthetic pathway to further increase andrographolide and other important active ingredient content, providing reliable and efficient technical support for large-scale production applications.

5.2.3 Conducting Bioreactor Cultivation Studies for Andrographolide Production via Cell Suspension Culture Technology Bioreactor cultivation offers large production scale, year-round production, independence from seasonal and regional constraints, high automation, and low production costs, showing broad prospects for large-scale production of medicinal plant secondary metabolites. Bioreactor cultivation 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 roots (Kochan et al., 2018), *Panax quinquefolius* hairy roots (Kochan et al., 2017), and *Angelica acutiloba* adventitious roots (Hwang et al., 2022), with important active ingredients such as ginsenosides already produced at scale via bioreactors (Thanh et al., 2014; Luthra et al., 2021). In contrast, research on *A. paniculata* in this area seriously lags behind. However, cell suspension culture for andrographolide production is relatively well-studied and technologically mature. Therefore, future bioreactor cultivation research for andrographolide production via *A. paniculata* cell suspension culture holds promising prospects.

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