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Review

# A Review of Plants' Secondary Metabolites: Extraction Techniques and Production in In Vitro Culture

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## Abstract

The production and isolation of secondary metabolites through in vitro plant culture techniques represent a sustainable and versatile alternative to traditional cultivation. This review provides an integrative overview of the principles, methods, and advances in in vitro systems—such as callus and suspension cell cultures, hairy root cultures, micropropagation, somatic embryogenesis, and protoplast culture—highlighting their ability to exploit plant cell totipotency for the controlled synthesis of bioactive compounds with pharmaceutical, nutraceutical, and cosmetic value. These systems enable scalable, reproducible, and environmentally responsible production, supported by innovations in elicitation, metabolic engineering, and omics technologies that enhance yield and stability. Despite challenges such as high establishment costs and species-specific requirements, in vitro approaches ensure continuous metabolite production independent of climatic or ecological factors. Moreover, these biotechnological strategies open new opportunities to explore and preserve global plant biodiversity—particularly in regions rich in endemic flora, such as Ecuador—while promoting equitable access to scientific and industrial applications. Ultimately, in vitro plant culture serves as both a platform for secondary metabolite discovery and a pathway toward sustainable, inclusive, and socially impactful biotechnological development.

**Keywords:** secondary metabolite; extraction techniques; in vitro culture; plant biotechnology

## 1. Introduction

The extraction and isolation of secondary metabolites constitute fundamental steps in optimizing their application at an industrial scale, as these processes directly influence the yield, purity, and bioactivity of the resulting compounds. Traditional extraction approaches, including maceration, steam distillation, heat reflux, agitation, soaking, and liquid–solid extraction [1,2], have long been employed. Nevertheless, these methods are often associated with significant drawbacks, such as intensive solvent consumption, lengthy processing times, and the risk of thermal degradation of heat-sensitive molecules [3]. Consequently, innovative and environmentally sustainable

alternatives—such as ultrasound-assisted extraction, microwave-assisted extraction, and supercritical fluid extraction—have progressively gained prominence [4,5].

Globally, extensive research has demonstrated the therapeutic potential of plant-derived metabolites, with several findings paving the way for the development of plant-based pharmaceuticals. The global market for medicinal plant products is currently estimated to exceed USD 100 billion annually [6]. Within this context, Ecuador—one of the most biodiverse countries worldwide—has witnessed a growing body of research on the exploitation and application of secondary metabolites [7–10], underscoring the importance of expanding knowledge regarding their diverse functional and industrial uses.

A comprehensive understanding of secondary metabolite extraction from plants is essential for the development of sustainable, efficient, and globally standardized methodologies that support both research and industrial applications. Therefore, the objective of this review is to provide a concise yet integrative introduction to the phytochemical field, emphasizing the principles, methods, in vitro culture techniques applied to the production of secondary metabolites and significance of plant secondary metabolites in modern biotechnological and pharmaceutical contexts.

## 2. Primary Metabolites

Primary metabolism is essential for all living organisms, as it encompasses the biochemical processes that sustain growth, development, and reproduction. In plants, primary metabolites include diverse organic compounds such as carbohydrates, lipids, proteins, nucleic acids, and vitamins, which are synthesized through central pathways like glycolysis, the Krebs cycle, and the Calvin cycle [11,12]. These molecules are indispensable as energy sources (e.g., sucrose, starch), structural components (e.g., cellulose), informational macromolecules (e.g., DNA and RNA), and functional cofactors or pigments (e.g., chlorophyll). In addition to their fundamental roles in plant physiology, primary metabolites act as precursors for the biosynthesis of secondary metabolites, thereby providing the biochemical foundation for more specialized phytochemical pathways [13].

## 3. Secondary Metabolites

Unlike primary metabolites, secondary metabolites are not directly involved in essential metabolic processes but are crucial for plant fitness, ecological adaptation, and survival under biotic and abiotic stress. They function as defensive compounds against herbivores and pathogens, act as signaling molecules in ecological interactions, such as a pollination, seed dispersal mediated by herbivores, and contribute to phenotypic traits such as pigmentation, aroma, and flavor that facilitate pollination and reproduction [2,4,14].

Biosynthetically, secondary metabolites are derived from or interconnected with primary metabolic pathways, often utilizing common intermediates, synthesized in specific anatomical structures, cell types, or organelles, specifically for production and storage, have relatively small molecular weights, typically less than 1500 Da. They are generally classified into three major groups based on their metabolic origin: alkaloids, terpenoids, and phenolics. These compounds exhibit remarkable structural and functional diversity, underpinning their ecological relevance and broad applicability in pharmacology, agriculture, and biotechnology [2,4,15].

It is important to emphasize that this classification is not mutually exclusive, as many plant species are capable of synthesizing multiple classes of secondary metabolites simultaneously in response to specific environmental cues, including abiotic stresses such as temperature fluctuations, UV radiation, and nutrient deficiencies, as well as biotic challenges such as pathogen infections. This metabolic plasticity highlights the presence of highly dynamic regulatory networks that facilitate efficient adaptive responses [16].

## 2.1. Terpenes

The history of terpenes extends back to ancient civilizations, where essential oils were widely employed, for example, in ceremonial practices in Egypt. Camphor was introduced by Arab around the 11th century. Systematic analyses of plant-derived oils began in 1818 with the work of J.J. Houston, and in the 19th century Jean-Baptiste Dumas proposed the term “terpene,” derived from turpentine, a resinous product of pine trees particularly rich in  $\alpha$ -pinene. Since then, terpenes have been recognized as one of the most structurally diverse and biologically relevant classes of natural products [17,18].

First, it is important to clarify a common misconception regarding the term’s terpenes and terpenoids. Terpenes, such as pinene, myrcene, limonene, terpinene, and p-cymene, are hydrocarbons composed of five-carbon isoprene units, which can be assembled in numerous structural arrangements. In contrast, terpenoids represent a modified class of terpenes that incorporate additional functional groups or structural modifications, such as oxidation or rearrangement of methyl groups. Based on the number of isoprene units in their backbone, terpenes are classified into monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, and triterpenes [19–21].

Terpenes or isoprenoids are widely distributed in animals, plants, fungi, and bacteria, and the largest class of natural products produced by terrestrial plants [19,22]. The number of isoprene units are primarily responsible for structural diversity of terpenes: hemiterpenes are formed by one isoprene unit (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), and tetraterpenes (C40) [23,24].

Structurally, terpenes may be acyclic, monocyclic, or polycyclic, and often bear functional groups such as alcohols, aldehydes, ketones, or esters, which account for their diverse polarities and biological activities [25]. For instance, limonene is a cyclic monoterpene with antioxidant and antiseptic properties, whereas  $\beta$ -caryophyllene, a sesquiterpene, binds to CB2 receptors exerting anti-inflammatory effects without psychoactivity. Their chemical structures influence volatility, bioavailability, and pharmacological potential, while the presence of multiple chiral centers complicates chemical synthesis, encouraging the use of plant cell cultures and bioreactors as alternative production systems. The hydrophobicity of terpenes further promotes their integration into biological membranes, enhancing interactions with enzymes and receptors [26–28].

Terpenes are primarily synthesized through two pathways: the mevalonate (MVA) pathway and the methylerythritol-4-phosphate (MEP) pathway. The MVA pathway originates in eukaryotes and occurs in the cytoplasm, endoplasmic reticulum, and peroxisomes, while the MEP pathway originates in prokaryotes and operates in plastids [29,30].

From a pharmacological perspective, several terpenes have been employed in the treatment of infectious, inflammatory, and neoplastic diseases. Paclitaxel (taxol), a tetracyclic diterpene containing ester and oxazoline groups, is widely used as a chemotherapeutic agent due to its ability to stabilize microtubules and thereby inhibit cell division. However, its low solubility has prompted the development of semisynthetic derivatives with improved clinical efficacy [31]. Another notable example is artemisinin, a sesquiterpene lactone bearing an endoperoxide bridge responsible for its antimalarial activity; this chemical structure generates free radicals in the presence of iron, which are lethal to *Plasmodium* spp. [32]. In addition, terpenes such as linalool, geraniol, and citronellol are widely used in the cosmetic and food industries owing to their aroma, flavor, and calming properties. The increasing demand for these compounds has encouraged the use of in vitro culture techniques, genetic engineering, and microbial fermentation to enhance their sustainable production at an industrial scale [33].

In agriculture, terpenes act as natural defense compounds against insects and pathogens. Monoterpenes such as carvone and eucalyptol have demonstrated fungicidal and bactericidal activity, whereas sesquiterpenes like farnesene participate in induced defense signaling. These molecules also play a crucial role in plant–insect communication; for example, (E)- $\beta$ -ocimene is emitted by damaged plants to attract natural enemies of herbivores [34]. At the molecular level, genes involved in terpene biosynthesis, such as terpene synthases, are regulated by environmental and

hormonal factors, including jasmonic acid and ethylene. Bioprospecting strategies combined with transcriptomic and metabolomic analyses have facilitated the identification of plant species with unique terpene profiles, valuable for the development of eco-friendly agricultural products. In this context, terpenes represent a natural and sustainable alternative to synthetic pesticides, aligning with the principles of regenerative agriculture and the circular bioeconomy [35].

## 2.2. Alkaloids

Alkaloids are among the earliest identified secondary metabolites, known for their potent biological and medicinal properties. Historical evidence indicates their use in ancient civilizations across Asia, Europe, and Africa for therapeutic and toxic purposes. The scientific study of alkaloids advanced significantly in the early 19th century with the isolation of pure compounds, notably morphine by Friedrich Sertürner, marking the beginning of modern alkaloid chemistry [36]. The term “alkaloid” was introduced in 1819 by the German chemist Carl F. W. Meissner, derived from the Arabic word *al-qali*, referring to the plant source from which soda was originally obtained [37].

Alkaloids constitute a diverse group of nitrogen-containing secondary metabolites synthesized by plants, often in response to abiotic stress conditions [38,39]. Broadly, they can be divided into three main categories: (i) 1 non-heterocyclic amines, (ii) alkaloids containing nitrogen within heterocyclic rings and derived from amino acids, and (iii) steroidal alkaloids that possess nitrogen-containing heterocyclic rings but are not derived from amino acids. More specifically, alkaloids are commonly grouped into fourteen structural families, including pyrrolizidine, pyrrol and pyrrolidine, quinoline, pyridine and piperidine, tropane, aporphine, isoquinoline, indolizidine, terpenoid, norlupinane, indole, purine, steroid, and imidazole alkaloids [40,41].

Alkaloids are generally colorless compounds characterized by a distinctly bitter taste. They are predominantly found in angiosperms, particularly within plant families such as Solanaceae, Magnoliaceae, Papaveraceae, Rubiaceae, Ranunculaceae, Apocynaceae, and Fabaceae. Biologically, these compounds play essential roles in plant defense mechanisms against herbivores and pathogens, while also contributing to ecological interactions such as pollinator attraction and allelopathy [42]. Alkaloids play a crucial role in plant defense mechanisms, exhibiting potent activity against microbial pathogens and herbivorous animals. Moreover, their biosynthesis is often upregulated under abiotic stress conditions, leading to the accumulation of higher alkaloid levels and the production of diverse structural derivatives that enhance plant adaptability [38].

The initial stages of alkaloid biosynthesis are critical, as they redirect metabolic flux from primary to specialized pathways and establish the structural scaffold of the final compound. Typically, these steps involve: (i) the formation of amine and aldehyde precursors, (ii) the generation of an iminium ion, and (iii) a Mannich-like condensation that defines the core alkaloid structure. Structural diversity arises from variations in these reactions, which may occur intra- or intermolecularly, as seen in the Pictet–Spengler condensation characteristic of many benzyloquinoline alkaloids [43]. Alkaloid biosynthesis largely depends on the precursor amino acids and the specific enzymatic systems of plants, fungi, and some animals. Most alkaloids originate from amino acids such as tyrosine, tryptophan, ornithine, lysine, and histidine, each giving rise to distinct structural families—for example, isoquinoline alkaloids from tyrosine and indole alkaloids from tryptophan. In contrast, steroidal alkaloids derive from isoprenoid or sterol backbones rather than amino acids, as observed in *Aconitum* and Solanaceae species [44].

Representative examples include morphine (a pentacyclic alkaloid with two hydroxyl groups), nicotine (a pyridine–pyrrolidine alkaloid), and atropine (a tropane alkaloid with anticholinergic activity). Their stereochemical configuration and the presence of functional groups such as esters, tertiary amines, or hydroxyls determine their affinity for cellular receptors, as well as their biological activity and pharmacokinetic behavior. Alkaloids are known for their potent effects on the central nervous, cardiovascular, and gastrointestinal systems, which has led to their use as analgesics, stimulants, anesthetics, antiparasitic agents, and cardiotonics (Singh et al., 2023). Their intrinsic toxicity also makes them valuable as botanical pesticides. Due to their structural complexity, many

alkaloids cannot be efficiently synthesized in the laboratory, making plant cell and suspension cultures viable alternatives for their large-scale production.

In medicine, alkaloids have long served as an inexhaustible source of therapeutic agents. Vinblastine and vincristine, bisindole alkaloids produced by *Catharanthus roseus*, inhibit tubulin polymerization, thereby arresting mitosis and demonstrating efficacy against leukemias and lymphomas. These compounds possess multiple chiral centers and intricate three-dimensional architectures, rendering their total chemical synthesis at an industrial scale impractical [45]. Other notable examples include galantamine, extracted from *Galanthus* spp., used in the treatment of Alzheimer's disease through reversible inhibition of acetylcholinesterase [44]; and reserpine, isolated from *Rauwolfia serpentina*, employed as an antihypertensive and antipsychotic agent. In traditional and complementary medicine, alkaloids such as berberine and sanguinarine have been investigated for their antimicrobial, antitumor, and anti-inflammatory properties [44,46]. The growing prevalence of antibiotic resistance has renewed interest in these compounds as natural therapeutic alternatives, supported by modern bioprospecting platforms and *in silico* validation approaches.

Recent advances in plant biotechnology have enabled the optimization of alkaloid production through *in vitro* culture, bioreactor systems, and gene editing technologies. Factors such as precursor availability, medium pH, elicitor presence, and expression of key biosynthetic genes strongly influence their accumulation [47]. For instance, jasmonic acid treatment in *C. roseus* cultures significantly enhances the levels of indole alkaloids [48]. At the molecular level, genes such as TDC (tryptophan decarboxylase) and STR (strictosidine synthase) have been identified as key regulators in the biosynthesis of vinblastine [49]. Moreover, metabolic engineering has facilitated the heterologous production of alkaloids in systems such as yeast and *E. coli*, enabling sustainable biosynthesis and structural diversification [50]. These strategies not only improve production efficiency but also allow the generation of structural analogs with enhanced pharmacological profiles and reduced toxicity, contributing to the discovery of next-generation natural product-derived drugs.

### 2.3. Phenolic Compounds

The history of phenolic compounds dates back to the early 19th century, when chemists first isolated salicylic acid from willow bark (*Salix* spp.), marking one of the earliest discoveries of plant-derived bioactive molecules. Throughout the following decades, other phenolic substances such as tannins and flavonoids were identified, laying the foundation for modern phytochemistry. By the mid-20th century, advances in analytical chemistry allowed for the structural elucidation of diverse phenolic classes, highlighting their ubiquity in plants and their relevance to both plant physiology and human health [51–53].

Phenolic compounds (PCs) represent the most widely distributed class of secondary metabolites in the plant kingdom. They are defined by the presence of at least one aromatic ring bearing one or more hydroxyl groups. PCs are generally water-soluble natural products of plant origin, with molecular weights typically ranging from 500 to 4000 Da [54,55]. The bioactivity of phenolic compounds is determined by the number and position of hydroxyl groups, the conjugation of the aromatic ring, and the presence of substituents such as sugars or methoxy groups. These chemical features confer not only antioxidant activity but also anti-inflammatory, antitumor, and cardioprotective effects. Their stability and polarity enable their incorporation into pharmaceutical, nutraceutical, and cosmetic formulations, as well as their use as natural colorants and preservatives in food products [56].

Phenolic compounds exhibit remarkable structural diversity, primarily determined by the number and arrangement of hydroxyl groups on their aromatic rings. Traditionally, they are classified according to the number of phenolic rings and the nature of the linkages between them, resulting in categories such as simple phenols, phenolic acids, flavonoids, xanthenes, stilbenes, coumarins, and lignans [57,58]. Recent comprehensive reviews have proposed updated classification frameworks. One approach distinguishes between monomeric compounds—comprising flavonoids

and non-flavonoids (e.g., stilbenes and simple phenols)—and polymeric forms, represented mainly by tannins [54]. Another system divides them into four major structural classes: phenolic acids, flavonoids, stilbenes, and lignans [55]. A third classification broadly groups them into simple phenolic compounds (simple phenols and phenolic acids) and polyphenols (flavonoids, tannins, stilbenes, and lignans) [59].

The biosynthesis of phenolic compounds in plants involves the coordinated activity of two major metabolic routes—the shikimate and aceto-malonate pathways—and one derived branch, the phenylpropanoid pathway. The shikimate pathway provides the aromatic amino acids L-phenylalanine, L-tyrosine, and L-tryptophan, which serve as primary precursors for phenolic metabolism. Through the deamination of phenylalanine by phenylalanine ammonia-lyase (PAL), the phenylpropanoid pathway emerges as an extension of the shikimate route, leading to the formation of hydroxycinnamic acids, coumarins, and other key intermediates. In parallel, the aceto-malonate pathway contributes to the biosynthesis of polyketide-derived structures by using malonyl-CoA and acetyl-CoA as building blocks. The convergence of the phenylpropanoid and aceto-malonate pathways enables the formation of complex phenolics, particularly flavonoids, in which the B-ring originates from the shikimate route and the A-ring from the aceto-malonate pathway. Together, these interconnected metabolic networks underlie the vast structural and functional diversity of plant phenolic compounds [60,61].

In addition to constitutive pathways, the biosynthesis of phenolic compounds can be rapidly induced by mechanical stress such as cutting or wounding. This response activates the phenylpropanoid metabolism through reactive oxygen species signaling, enhancing the activity of key enzymes and redirecting primary metabolites toward the synthesis of phenolic acids, flavonoids, and lignin. As a result, plants increase their antioxidant capacity and defense potential, demonstrating the close interplay between primary and secondary metabolism under stress conditions [62].

From a physiological perspective, phenolic compounds protect plants against ultraviolet radiation, pathogens, herbivores, and oxidative stress. They also participate in tissue lignification, pollinator attraction, and rhizospheric interactions [63,64]. In the pharmacological context, flavonoids such as quercetin, apigenin, and luteolin have been shown to act on multiple molecular targets involved in inflammation, apoptosis, and angiogenesis. Likewise, resveratrol, a stilbene found in grapes and peanuts, exhibits neuroprotective, anti-aging, and antidiabetic effects attributed to its ability to activate sirtuins and modulate gene expression [65]. Phenolic content varies depending on species, tissue type, developmental stage, and environmental conditions, which has encouraged the use of biotechnological strategies to regulate their production. Cell cultures, adventitious roots, and bioreactors are commonly used to induce their synthesis, as well as elicitors such as salicylic acid and UV light that activate key enzymes in the phenylpropanoid pathway.

Advances in transcriptomics and metabolomics have deepened the understanding of phenolic biosynthesis regulation. In this pathway, key enzymes such as phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and chalcone synthase (CHS) are regulated by transcription factors including MYB, bHLH, and WRKY, whose expression can be induced by hormonal or environmental signals. This regulatory network allows the modulation of specific compound production in *in vitro* cultures, optimizing yield and phytochemical profiles. In agricultural applications, phenolic compounds act as natural fungicides and plant growth regulators, whereas in the food industry they are used as preservatives due to their antioxidant and antimicrobial properties. In cosmetics, extracts rich in flavonoids and tannins are incorporated for their astringent, anti-inflammatory, and anti-aging effects [54,66]. Their safety profile, efficacy, and plant-derived origin position them as natural alternatives to synthetic ingredients, fostering the development of clean, sustainable, and value-added products.

### 3. Extraction Techniques of Secondary Metabolites

The history of extraction is marked by the development of more sophisticated techniques to overcome the limitations of early methods, driven by the need for more comprehensive and accurate metabolite profiling. Key developments include the use of cold solvents (like methanol), the creation of solvent systems to isolate different polarity metabolites, and the introduction of advanced extraction techniques for increased efficiency and reduced solvent use [67,68].

The selection of an appropriate extraction method is critical, as it depends on the intended application of the extract and the physicochemical properties of the target compounds. Extraction enables the isolation of secondary metabolites from complex plant matrices, and its efficiency is influenced by several factors, including solvent polarity, extraction technique, temperature, and time. Conventional methods are categorized in three methods (1) immersion methods that include: maceration, digestion, infusion, and decoction, (2) reflux method that includes: percolation, (3) distillation methods that include: hydro distillation, hydro-steam distillation, steam distillation. In contrast, modern or non-conventional techniques—such as Soxhlet extraction, accelerated solvent extraction (ASE), microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE), enzyme-assisted extraction (EAE), and pressurized hot water extraction (PHWE)—offer higher efficiency, reduced solvent consumption, and greater environmental sustainability [2,4,6,69].

*Maceration*: is one of the simplest and most widely applied extraction techniques, particularly suitable for thermolabile plant materials. In this method, coarsely powdered plant material—such as leaves, stem bark, or roots—is soaked in a suitable solvent (menstruum) at room temperature for at least three days, with intermittent agitation to ensure complete extraction. The process is typically conducted in a closed container to minimize solvent loss through evaporation. After extraction, the liquid phase (micelle) is separated from the solid residue (marc) by filtration or decantation, and the extract is often concentrated under reduced pressure or by gentle evaporation. The choice of solvent is crucial, as it determines the range and yield of phytochemicals recovered from the plant matrix, enabling the efficient extraction of thermolabile and polar compounds [4,70–72].

*Digestion*: is an extraction technique similar to maceration but performed under mild heating to enhance solvent efficiency and metabolite recovery. In this method, the powdered plant material is immersed in a suitable solvent and maintained at a controlled temperature—typically between 35 °C and 50 °C—using a water bath or oven. Gentle heating reduces solvent viscosity, facilitating the dissolution and diffusion of secondary metabolites without compromising thermolabile phytochemicals. The extraction process generally lasts from 30 minutes to 24 hours, with intermittent agitation to ensure uniform contact between solvent and plant material. This technique is particularly effective for plant matrices with low solubility or dense tissues such as barks and roots, allowing improved extraction yields under moderate thermal conditions [4,70–72].

*Infusion*: is a simple extraction method in which plant material is immersed in boiling water and left to stand briefly before filtration. It is mainly used for easily soluble constituents such as caffeine, phenols, and flavonoids, commonly found in herbal teas and medicinal preparations [4,71,72].

*Decoction*: involves boiling powdered plant material in water for a short period, typically between 15 and 60 minutes, depending on the toughness of the plant tissue. This method is suitable for extracting water-soluble and heat-stable compounds such as phenols and flavonoids from roots, barks, and rhizomes. After boiling, the mixture is filtered to obtain the liquid extract. Although efficient for robust materials, decoction is not ideal for thermolabile constituents, as prolonged heating may lead to their degradation [4,71–73].

*Percolation*: is a continuous extraction process where solvent—typically ethanol—passes slowly through a bed of moistened, powdered plant material in a percolator. This technique ensures efficient diffusion of phytochemicals and is widely employed for producing tinctures and fluid extracts. Proper particle size, solvent flow rate, and pre-moistening of the material are essential to optimize extraction and prevent turbidity in the final product [4,71,72].

*Hydro distillation*: is one of the oldest traditional methods for extracting essential oils and volatile bioactive compounds from plants. In this process, plant material is boiled with distilled water,

allowing essential oils to diffuse from the tissues and vaporize with the steam. The vapor mixture is then condensed, and the oil separated from water. This solvent-free method is simple and suitable for fresh plant material; however, excessive heat may degrade thermolabile compounds, reduce oil quality, and increase energy consumption and processing time [2].

*Hydro-steam distillation*: was developed to overcome the limitations of hydro distillation. In this method, plant material is placed on a perforated grid above boiling water. The generated steam passes through the material, vaporizing and carrying the essential oils, which are later condensed and separated [2].

*Steam distillation*: Steam distillation is a conventional technique used to extract essential oils, phenolics, and alkaloids from plant materials through controlled heating and evaporation. In this process, steam passes through the plant matrix, vaporizing water-insoluble volatile compounds, which are then condensed and separated. Unlike hydrodistillation, no water is placed at the bottom of the still, resulting in higher efficiency and yield [2].

*Soxhlet extraction*: is a continuous hot extraction technique that enables exhaustive recovery of phytochemicals using organic solvents such as ethanol or methanol. In this method, finely ground plant material is placed in a porous thimble inside the Soxhlet apparatus, which consists of a round-bottom flask, extraction chamber, siphon tube, and condenser. The solvent is heated, vaporized, condensed, and repeatedly percolated through the plant matrix, allowing efficient solubilization of target compounds until complete extraction is achieved. This technique provides higher yields than maceration and is suitable for extracting heat-stable compounds such as fatty acids, phenolics, and triterpenes. However, it is time-consuming and may cause degradation of thermolabile metabolites due to prolonged exposure to high temperatures. Recent automation has optimized Soxhlet systems, reducing solvent use and environmental impact while maintaining extraction efficiency [4,71,72].

*Accelerated solvent extraction (ASE)*: also referred to as pressurized or high-pressure solvent extraction, is a modern technique that enables efficient recovery of bioactive compounds under elevated temperature and pressure. The plant sample, mixed with inert material such as silica or sand and separated by cellulose filters, is packed into a stainless-steel extraction cell. The solvent is maintained in liquid state, improving solubility, diffusion, and penetration by reducing viscosity and surface tension. Extraction typically occurs around 80 °C and 20 MPa for 10 min, yielding high recovery of compounds such as phenolics, carotenoids, lignans, fatty acids, and essential oils. ASE offers several advantages, including minimal solvent consumption, short extraction time, and suitability for both polar and non-polar phytochemicals, making it a credible alternative to conventional and supercritical fluid extraction methods [2,6].

*Microwave-assisted extraction (MAE)*: Microwave-assisted extraction (MAE) is an advanced technique that combines microwave energy with solvent extraction to efficiently recover bioactive compounds from plant materials. It operates within the microwave frequency range of 300 MHz to 300 GHz, using electromagnetic radiation to heat polar molecules through dipole rotation and ionic conduction. This localized heating increases solvent penetration, disrupts plant cell walls, and enhances the diffusion of metabolites into the solvent. The efficiency of extraction depends on the dielectric constant and viscosity of the solvent, with polar, low-viscosity solvents favoring higher yields. MAE significantly reduces extraction time, solvent consumption, and energy demand while maintaining the biological activity of thermally stable compounds such as flavonoids, essential oils, and phenolics. The integration of microwaves with ionic liquids has further expanded its applicability across pharmaceutical, food, and cosmetic industries [2,4,6,69].

*Ultrasound-assisted extraction (UAE)*: is an advanced technique that employs ultrasonic waves (20–2000 kHz) to enhance the recovery of bioactive compounds from plant materials. The process relies on acoustic cavitation—the formation, growth, and collapse of microbubbles—which generates localized shear forces that disrupt cell walls, increase solvent penetration, and accelerate mass transfer. This mechanical action enhances the release of secondary metabolites while reducing solvent use, extraction time, and energy consumption. UAE is particularly suitable for thermolabile compounds, as it operates at lower temperatures than conventional methods. However, excessive

ultrasonic energy may lead to partial degradation of certain phytochemicals due to free radical formation. Overall, UAE offers an efficient, reproducible, and environmentally friendly alternative for extracting phytochemicals such as phenolics, flavonoids, and pigments [4,6,69].

*Supercritical fluid extraction (SFE)*: is an advanced and eco-friendly technique that uses supercritical carbon dioxide (scCO<sub>2</sub>) as a solvent to isolate mainly nonpolar and thermolabile compounds. By adjusting temperature and pressure, scCO<sub>2</sub> attains both gas-like diffusivity and liquid-like solvating power, enabling selective extraction. The addition of small amounts of polar co-solvents such as ethanol or methanol improves the recovery of phenolic and flavonoid compounds. SFE offers high efficiency, low solvent residue, and preservation of bioactivity, making it suitable for extracting essential oils, lipids, and polyphenols from diverse plant materials [2,4,6].

*Enzyme-assisted extraction (EAE)*: enhances the recovery of bioactive compounds by using hydrolytic enzymes—such as cellulase, pectinase, and amylase—to degrade cell wall components like polysaccharides and lipids, facilitating metabolite release. Two main approaches are enzyme-assisted aqueous extraction (EAAE), mainly applied for oil recovery, and enzyme-assisted cold pressing (EACP), used to hydrolyze seed walls. This eco-friendly technique employs water instead of organic solvents and yields extracts rich in lipophilic and phosphorus-containing compounds. Extraction efficiency depends on parameters such as enzyme concentration, temperature, pH, reaction time, and particle size. EAE has been successfully applied to extract polyphenols, carotenoids, flavonoids, lycopene, and anthocyanins from various plant matrices, showing higher yields and selectivity compared to conventional solvent-based methods [2,6].

*Pressurized hot water extraction (PHWE)*: employs water maintained in a liquid state under elevated pressure (typically around 5 MPa) and temperatures between 100–250 °C, allowing it to act as both a polar and nonpolar solvent. This green technique offers high yields, short extraction times, and high-quality extracts without using organic solvents. PHWE has been successfully applied to recover antioxidants, phenolics, flavonoids, vitamins, and cannabinoids from various plant matrices, demonstrating its efficiency in preserving bioactivity and minimizing degradation of thermolabile compounds [6].

*Pulsed Electric Field Extraction (PEF)*: is a non-thermal method that enhances cell membrane permeability through electroporation, facilitating the release of intracellular phytochemicals. It operates using short, high-voltage pulses in either batch or continuous systems, with extraction efficiency influenced by field strength, energy input, pulse number, and sample characteristics. PEF is especially suitable for heat-sensitive compounds and is considered an environmentally friendly technique due to its low energy consumption, use of green solvents, and ability to yield high-quality extracts [2].

#### 4. In Vitro Culture Techniques Applied to the Production of Secondary Metabolites

Totipotency is an intrinsic feature of plant cells that makes them exceptionally valuable for biotechnological research. *In vitro* culture techniques encompass a set of methods that enable the cultivation of plant cells, tissues, or organs under sterile and controlled conditions [35,74,75]. Among the most widely used techniques are callus culture, suspension cell culture, transformed (hairy) root systems, somatic embryogenesis, organogenesis, and micropropagation [76]. Each of these techniques has specific applications for the production of secondary metabolites. For instance, callus and suspension cultures are particularly suitable for compounds that do not require cell differentiation, whereas hairy root cultures are ideal for metabolites that accumulate in root tissues [77]. Moreover, clonal micropropagation enables the large-scale production of genetically uniform plants with high concentrations of desired metabolites [78]. The selection of the appropriate technique depends on factors such as metabolite type, tissue localization, and plant species. The use of *in vitro* methods not only ensures a constant, climate-independent supply of bioactive compounds but also allows genetic manipulation and the application of elicitors to activate specific biosynthetic pathways [79].

*In vitro* culture offers multiple advantages over the direct extraction of secondary metabolites from field-grown plants. Firstly, it eliminates dependence on climatic, seasonal, and geographic variables that influence metabolite concentrations [80]. Controlled laboratory conditions allow the optimization of production through the addition of biosynthetic precursors, elicitor treatments, or genetic engineering strategies [35,76]. Another major advantage lies in the conservation of endangered species through clonal propagation and tissue culture, contributing to ecological sustainability [75]. The scalability of production in bioreactors also represents a significant industrial advantage, enabling large-scale yields with reduced long-term operational costs [35]. Furthermore, these systems allow the biosynthesis of compounds that are naturally produced only in trace amounts or during specific developmental stages, thus increasing the efficiency of obtaining bioactive products for pharmaceutical and cosmetic industries.

Despite these multiple advantages, *in vitro* culture techniques also face several limitations. High initial setup costs—related to infrastructure, reagents, and specialized labor—remain a key constraint. Additionally, many plant species require species-specific protocols for disinfection, establishment, and regeneration, limiting the universal applicability of these methods [76]. Somaclonal variation represents another challenge, as spontaneous mutations during prolonged culture can alter metabolite profiles [79]. Some secondary metabolites require tissue differentiation or specialized cellular structures for synthesis, which are not always achieved in non-organogenic systems. Large-scale production may also be hindered by the difficulty of maintaining long-term culture stability. Microbial contamination is a recurrent issue that compromises culture viability. Finally, while elicitors can significantly enhance metabolite production, their effectiveness depends on precise control of concentration and exposure time, as improper use can induce cytotoxic effects in cultured cells [81]. These challenges underscore the need for continuous research aimed at optimizing *in vitro* culture protocols and developing integrated biotechnological strategies for improved metabolite production.

#### 4.1. Callus and Suspension Cell Cultures

##### 4.1.1. Callus Culture

Callus culture involves the formation of undifferentiated cell masses derived from plant explants such as leaves, hypocotyls, cotyledons, stems, or roots grown on solid media supplemented with growth regulators, primarily auxins and cytokinins [75,82]. Callus induction depends on multiple factors including explant type, medium composition, hormone concentration, tissue age, and environmental conditions such as light, temperature, and humidity [35,79,83]. Calli are widely used for secondary metabolite production as they maintain several active biosynthetic pathways. Furthermore, they serve as starting material for techniques such as regeneration through organogenesis or somatic embryogenesis, as well as for clonal selection of high-yielding cell lines [76]. This approach is particularly effective for producing bioactive compounds that do not require tissue differentiation for synthesis, such as flavonoids, phenolics, and alkaloids. Hence, callus culture represents a key tool in plant biotechnology and physiological research.

The efficiency of callus culture for secondary metabolite production relies on the optimization of physical, chemical, and genetic parameters within the *in vitro* system. The selection of basal media, carbon source, type and concentration of growth regulators (2,4-D, NAA, BAP, kinetin), and photoperiod are critical variables that must be tailored to each species and target metabolite [80]. In many species, a balanced combination of auxins and cytokinins promotes callogenic proliferation without inducing regeneration. Additionally, the use of biotic (yeast extract, chitin) and abiotic (methyl jasmonate, UV light) elicitors can stimulate secondary metabolite production without affecting cell viability [79]. In certain cases, selecting cell lines from calli with high metabolic activity has enabled the establishment of highly productive cultures. For instance, callus cultures of *Coptis japonica* are used to produce berberine [84], while *Ophiorrhiza mungos* calli are of interest for camptothecin production [85–87]. In *SinoPodophyllum hexandrum*, root callus cultures derived from *in*

*in vitro* seedlings have proven to be a viable source for podophyllotoxin production, yielding up to 0.27 mg/g dry weight in callus from plants from the Kinnaur region [2].

Bioactive compounds such as psoralen, daidzein, and genistein have been extracted from callus cultures of *Cullencorylifolium* cotyledons [88]. Callus culture has also been shown to be effective in inducing phenolic compounds in species such as *Salvia viridis* [89]. Thus, this system is positioned as a versatile platform for physiological studies, industrial production, and biotechnological improvement.

#### 4.1.2. Suspension Cell Cultures: Characteristics and Biotechnological Advantages

Suspension cell culture is an extension of callus culture, in which undifferentiated cells are transferred to a liquid medium and continuously agitated to promote uniform cell division and homogeneous culture formation. Typically, friable callus is inoculated into a liquid medium and maintained under agitation (50–200 rpm), allowing cell dispersion after several passages and resulting in small aggregates of 20–100 cells [35,76]. Mechanical agitation is a key factor driving the transition from callus to suspension cultures, although sterile seedlings or embryos can also be used as inoculum sources [90].

Suspension cultures provide a valuable system for biochemical, physiological, molecular, and genetic engineering studies of plant cells. Due to their scalability in bioreactors, they represent one of the most promising options for large-scale production of secondary metabolites, offering precise monitoring of cell growth, uniform access to nutrients and oxygen, and potential for process automation [35]. *Carthamus tinctorius* cultures have produced complex mixtures of flavonoids, alkaloids, and aromatic glycosides [91]. In addition, cell suspension cultures of *Salvia nemorosa* report the production of phenolic acids, such as rosmarinic acid and salvianolic acid B [92]. Nevertheless, one of the challenges associated with this system is the gradual decline in metabolic stability over prolonged subcultures, making cell line selection and cryopreservation essential to maintain productivity.

Despite their advantages, suspension cell cultures also face several technical challenges that limit their universal application. The progressive loss of biosynthetic capacity over time, often linked to cell dedifferentiation and culture senescence, remains a significant limitation. Furthermore, not all secondary metabolites are synthesized in undifferentiated cells, restricting the system's applicability to compounds produced in specific tissues such as roots, flowers, or glandular structures [93]. Morphological heterogeneity among cultured cells can also affect production uniformity and create cell aggregates that complicate large-scale bioreactor operation. Additional constraints include the need for adequate aeration, constant pH control, and continuous agitation, all of which increase system complexity [94]. However, advances in genetic engineering, temporary immersion bioreactors, and genome-editing technologies such as CRISPR/Cas9 have significantly mitigated many of these issues [95]. Current trends emphasize the integration of suspension cultures with metabolomic and transcriptomic analyses to optimize cell-specific production pathways. As a result, suspension cell cultures are emerging as a dynamic and adaptable platform for industrial-scale biosynthesis of high-value secondary metabolites.

#### 4.2. Protoplast Culture and Somatic Embryogenesis

Protoplast culture represents an advanced strategy within plant tissue *in vitro* systems, involving the isolation of plant cells devoid of their cell walls through enzymatic or mechanical treatments. These naked cells, which are highly sensitive to osmotic conditions, possess the ability to regenerate their cell wall and redifferentiate into calli, shoots, roots, embryos, or even complete plants under suitable conditions. Cellular totipotency, widely exploited in plant biotechnology, enables the use of protoplasts as platforms for physiological studies, cell selection, somatic hybridization, and secondary metabolite production. For isolation, enzymes such as pectinase and cellulase are commonly used, typically applied to mesophyll tissues or young calli, the latter being an efficient source of viable cells [96]. Protoplast viability is assessed through staining assays and phase-contrast

microscopy, while their culture requires specific isotonic media that promote cell division and differentiation. Protoplast culture is particularly advantageous in species where traditional genetic engineering techniques face limitations, allowing molecular studies of biosynthetic pathways related to bioactive metabolites [35,97].

One of the critical aspects of protoplast culture is the precise control of physicochemical parameters such as osmolarity, pH, and the concentration of sugars and plant hormones. Maintaining isotonic conditions is essential to prevent cell lysis, thus osmotic agents like mannitol and sorbitol are commonly employed. Supplementation with 2,4-D and kinetin has been shown to induce the formation of viable and proliferative microcalli, a prerequisite for complete plant regeneration [98]. In recent studies, the regeneration of *Nicotiana tabacum* plants from protoplasts fused with *Solanum nigrum* demonstrates its usefulness in genetic improvement. More recently, this platform has enabled the investigation of biological pathways through the specific editing of genes in *Nicotiana benthamiana* protoplasts using the CRISPR/Cas system, followed by the regeneration of phenotypically characterized mutant plants [99]. For instance, Huling Wnag [100] monitored the activity of enzymes involved in flavonoid biosynthesis in *Arabidopsis thaliana*, illustrating the relevance of protoplast systems for dynamic metabolic studies. These methodological advances are transforming protoplast culture into a key tool for both basic and applied research in plant physiology and secondary metabolite biotechnology.

The use of protoplasts for the production of secondary metabolites, while historically less exploited than callus or hairy root cultures, has proven to be a viable strategy with unique advantages. Pioneering studies in model species laid the foundation for this approach. For example, in *Hyoscyamus muticus*, protoplast-derived cell culture clones producing scopolamine were established, with significant somaclonal variation observed in the content of this alkaloid [101]. A more advanced application was demonstrated in *Catharanthus roseus*, where protoplasts, when immobilized in a guluronic acid-rich alginate gel, produced ajmalicin levels far exceeding those of immobilized whole cells. This “artificial cell wall” system not only prevented protoplast lysis but also functioned as an elicitor and, crucially, when supplemented with CaCl<sub>2</sub>, suppressed cell wall regeneration, maintaining the protoplasts in a productive state for up to 15 days [102]. These studies not only confirm the feasibility of the technique but also identify and propose solutions to key challenges such as osmotic fragility and cell wall regeneration, positioning protoplast systems as a tool with great potential for bioproduction and the manipulation of metabolic pathways [103].

Somatic embryogenesis, in contrast, involves the generation of embryos from somatic cells under specific hormonal conditions, enabling the regeneration of whole plants for cloning elite genotypes. This approach has been successfully optimized in pomegranate, achieving high embryo induction rates and vigorous plant establishment [104]. One of its main advantages is the potential for automation in bioreactor systems and the generation of artificial seeds, facilitating large-scale propagation of genotypes of interest. However, both techniques require strict control of physical and chemical conditions to ensure efficient regeneration. Success depends on the species, type of explant, culture medium, and hormonal balance, with 2,4-D being one of the most commonly used auxins during the induction phase [35].

#### 4.3. Clonal Micropropagation

Micropropagation is a widely used *in vitro* technique for the asexual reproduction of plants under sterile conditions, enabling the generation of genetically identical individuals from meristematic explants or somatic tissues [74,105]. This method provides an efficient and scalable means of producing homogeneous plant material that can serve as a reliable source of secondary metabolites under controlled conditions [74,106]). Unlike traditional propagation methods, micropropagation allows for year-round plant production, independent of climatic factors, while reducing the required cultivation space and accelerating the multiplication of medicinal and endangered species [35]. This methodology typically employs Murashige and Skoog (MS) medium

supplemented with plant growth regulators, primarily auxins (IAA, IBA, NAA) and cytokinins (BAP, kinetin), in specific ratios that promote shoot and root formation [105].

Micropropagation strategies can be divided into several critical phases: culture establishment, shoot proliferation, elongation, rooting, and ex vitro acclimatization. Each stage requires precise conditions that directly affect the yield and quality of the culture. Factors such as explant selection, system sterility, type and concentration of growth regulators, medium composition, and environmental conditions (photoperiod, temperature, and humidity) are essential for successful outcomes [107]. Multiplication rates can be optimized through repeated subculturing and the application of biotic or abiotic elicitors that stimulate secondary metabolite synthesis, including salicylic acid, methyl jasmonate, or chitosan [108]. Moreover, the use of temporary immersion bioreactors has significantly improved system efficiency by reducing production costs, space requirements, and cultivation time [109,110]. In species such as *Centella asiatica* and *Rehmannia elata*, micropropagation has led to enhanced production of triterpenoids and iridoid glycosides, respectively, compared with field-grown plants [111,112].

The use of cloned microplants not only ensures genetic uniformity but also preserves the biosynthetic capacity of the parental plants to produce secondary metabolites. Several studies have demonstrated that metabolites such as podophyllotoxin, rosmarinic acid, crocin, and various flavonoid compounds are efficiently synthesized by micropropagated plants [113]. For instance, *Linum flavum* has shown high levels of podophyllotoxin accumulation; *Crocus sativus* microplants have produced significant quantities of crocin, picrocrocin, and safranal; and *Psoralea drupacea* cultures have yielded bakuchiol concentrations exceeding those of mother plants [114]. These outcomes are largely attributed to the conservation of biosynthetic patterns in specialized tissues and the capacity to induce specific metabolic pathways through precisely modulated culture conditions.

In recent years, micropropagation has been strategically integrated with multi-omics approaches to elucidate the genetic regulation of specialized metabolic pathways in medicinal plants [115]. This integrated strategy has opened new avenues for deciphering withanolide metabolism, as demonstrated in *Withania somnifera*, where in vitro systems combined with omics technologies have facilitated both large-scale production and the identification of genes involved in withanolide biosynthesis [116]. Complementing this approach, comprehensive transcriptomic analyses of different *Withania* chemotypes have identified differentially expressed genes encoding cytochrome P450s, glycosyltransferases, and transcription factors potentially responsible for tissue-specific withanolide biosynthesis (Singh et al., 2020). Similarly, in *Artemisia annua*, the characterization of key regulatory genes from the WRKY, MYB, and bHLH families has enabled the development of micropropagated lines with enhanced expression of artemisinin biosynthetic genes [117]. These genetically characterized lines represent a viable biotechnological alternative to more costly and less sustainable methods such as chemical synthesis or large-scale field cultivation.

In summary, clonal micropropagation not only preserves but also amplifies the biosynthetic potential of donor plants under controlled conditions, establishing itself as an indispensable tool for the sustainable and large-scale production of high-value secondary metabolites.

#### 4.4. Hairy Root Culture: An Innovative Platform for Secondary Metabolite Production

The cultivation of transformed roots, also known as hairy roots, is induced by infection with *Agrobacterium rhizogenes*, which transfers root-inducing (Ri) genes that promote rapid and stable root growth. This technique enables the continuous and stable production of secondary metabolites under *in vitro* conditions without the need for full tissue differentiation (Giri & Narasu, 2000). Transformed roots retain most of the biosynthetic capacity of the original plant tissue and, in many cases, produce higher concentrations of metabolites than natural roots. Moreover, they exhibit rapid growth in liquid media without requiring exogenous growth hormones, significantly reducing production costs. This approach has been successfully employed to produce ajmalicine in *Catharanthus roseus*, rosmarinic acid in *Salvia miltiorrhiza*, and podophyllotoxin in *Linum flavum* [35]. Among its limitations is the need to generate specific transformed lines for each plant species and target metabolite, which can be

laborious and technically demanding. Nevertheless, the high productivity and genetic stability of hairy roots make them an ideal model system for industrial-scale production [118].

Hairy root culture represents an innovative and highly efficient strategy within *in vitro* plant culture technologies for the production of pharmacologically and agriculturally relevant secondary metabolites. This system relies on the genetic transformation of plants by *Agrobacterium rhizogenes*, which induces vigorous, stable, and highly differentiated root proliferation through the integration of rol genes from the Ri plasmid. Unlike other *in vitro* systems such as callus or suspension cultures, hairy roots maintain a metabolically active biosynthetic profile characteristic of root tissues, favoring the accumulation of alkaloids, phenolics, and terpenoids [2,35]. Their genetic stability, rapid growth rate, and continuous biosynthetic capacity in the absence of phytohormones position this system as a promising platform in plant biotechnology [119].

Growth parameters in hairy root cultures vary according to the plant species and target metabolite but commonly involve the use of Murashige and Skoog (MS) medium supplemented with a carbon source such as sucrose (3–5%), maintained under agitation and darkness to promote root development. Induced roots can be cultivated in flasks or bioreactor systems, where variables such as aeration, pH, temperature, and dissolved oxygen concentration influence productivity [35]. Modern strategies, including elicitation with methyl jasmonate, salicylic acid, or metallic nanoparticles, as well as the addition of biosynthetic precursors and co-cultivation with mycorrhizal fungi, have proven effective in increasing the accumulation of metabolites such as vincristine, triterpenoid glycosides, and phenolic acids [2,115].

The main advantages of hairy root culture include its high productivity in root-specific metabolites, long-term genetic stability, and the absence of somaclonal variation—factors that often limit callus or suspension systems. Additionally, the ability of hairy roots to grow in defined media without phytohormones allows for precise control over biosynthetic processes. However, certain drawbacks persist: genetic transformation efficiency varies among plant species, and industrial scaling requires specialized bioreactors capable of maintaining root integrity, particularly in long-term cultures where hypoxia or toxic metabolite accumulation may induce necrosis [35].

Notable examples of secondary metabolite production via hairy root cultures include the biosynthesis of rosmarinic acid in *Agastache rugosa*, rutin in *Fagopyrum esculentum*, glycyrrhizin in *Glycyrrhiza glabra*, and indole alkaloids such as vinblastine and scopolamine in *Catharanthus roseus* and *Atropa belladonna*, respectively [120]. Additionally, *Artemisia annua* hairy roots have been engineered for enhanced artemisinin production through the introduction of genes from the mevalonate pathway and cultivation in bioreactors, achieving yields of up to 0.32 mg/g dry weight in 25 days [115]. These strategies enable the optimized use of slow-growing or endangered medicinal species without harvesting large quantities of biomass from natural ecosystems, thereby promoting sustainable and biodiversity-friendly production practices.

#### 4.5. Elicitation as a Strategy to Enhance Secondary Metabolite Production

The use of elicitors in *in vitro* culture has become an effective strategy to enhance the biosynthesis of secondary metabolites in plant tissues grown under controlled conditions. Elicitors are biotic or abiotic agents capable of inducing physiological defense responses in plants by simulating pathogen attack or environmental stress. This induction activates specialized metabolic pathways—such as those of phenylpropanoids, alkaloids, terpenoids, or flavonoids—thereby increasing the production of compounds of pharmaceutical, cosmetic, or agro-industrial interest [35]. Under *in vitro* conditions, where environmental control is optimal and cells remain metabolically active, elicitor application enables the manipulation of gene and enzyme expression associated with these biosynthetic routes. The response depends on the elicitor type, concentration, exposure time, culture system (callus, cell suspension, or hairy roots), and the plant species involved [2,121]. This technique offers significant advantages over conventional production systems by reducing environmental dependency and facilitating process scalability through bioreactor integration.

Elicitors are generally classified as biotic (derived from living organisms) or abiotic (physical or chemical factors). Biotic elicitors include polysaccharides such as chitosan, pectin, and yeast extracts, while abiotic elicitors comprise metallic compounds ( $\text{AgNO}_3$ ,  $\text{CdCl}_2$ ), hormonal regulators such as salicylic acid (SA) and methyl jasmonate (MeJA), and nanoparticles ( $\text{ZnO}$ ,  $\text{TiO}_2$ ) [35,121]. These compounds act as molecular signals that trigger intracellular signaling cascades, including the activation of receptor kinases, ion channels, and G-protein-coupled receptors. Such signaling promotes the transcription of key biosynthetic genes—such as PAL, CHS, and HMGR—responsible for flavonoid, phenolic, and terpenoid synthesis, respectively [115]. For example, in *Taxus chinensis*, methyl jasmonate (MeJA) treatment significantly enhanced paclitaxel production through transcriptional activation of taxane biosynthetic genes [122]. Similarly, synergistic elicitation approaches have demonstrated efficacy across diverse medicinal species, as evidenced by the combination of sodium fluoride with MeJA to boost alkaloid yields in *Cephalotaxus* [121]. Concurrently, in *Salvia miltiorrhiza*, salicylic acid promoted tanshinone accumulation by activating a key transcriptional regulatory module involving SmbHLH transcription factors [123]. These findings demonstrate the ability of elicitors to redirect secondary metabolism in both differentiated and undifferentiated plant tissues.

The effectiveness of elicitor application depends on the careful optimization of parameters to maximize productivity without causing cytotoxic effects. Elicitor concentration, cell growth stage, and exposure time are critical determinants for elicitation success. In cell suspension cultures, the exponential growth phase is typically the optimal time for elicitor application due to high metabolic activity. Conversely, in transformed hairy root cultures, prolonged exposure to MeJA or chitosan has maintained elevated metabolite levels over extended periods [121]. Plant species exhibit distinct elicitor responsiveness patterns—for instance, *Carthamus tinctorius* accumulates flavonoids, flavones, and anthocyanins under salinity stress, while *Panax ginseng* shows enhanced triterpenoid production in response to salicylic acid elicitation. Similarly, *Centella asiatica* demonstrates increased triterpenoid accumulation when treated with salicylic acid [121]. These observations highlight the necessity of tailoring elicitation strategies to each biological system and target metabolite.

The advantages of elicitor use in *in vitro* cultures include the enhancement of metabolite productivity without genetic modification, shorter cultivation periods, reduced consumption of natural resources, and independence from environmental or seasonal fluctuations [124]. Furthermore, elicitor combinations or sequential treatments can induce synergistic biosynthetic responses [125]. However, limitations exist, such as variability among species and genotypes, possible cytotoxicity, and the requirement for extensive optimization studies [35,77,115]. Another challenge lies in scaling up elicitation systems, particularly when elicitor effects are transient or dependent on specific culture conditions [126]. Despite these constraints, elicitation remains a cornerstone technique in plant biotechnology, especially when integrated with omics tools, metabolic engineering, and gene editing.

The integration of emerging technologies has further strengthened the role of elicitors in industrial bioprocesses. For instance, combining MeJA elicitation with transcriptomic analysis has enabled the identification of key regulatory genes involved in artemisinin biosynthesis in *Artemisia annua* [127]. Similarly, the use of functionalized nanoparticles as elicitors has shown promising results for modulating gene expression without cytotoxicity—as demonstrated in *Camptotheca acuminata*, where camptothecin production was significantly enhanced [128]. These approaches reflect the current trend toward combining elicitation with advanced genomic and systems biology tools, allowing for rational design of strategies to maximize specific metabolite production [121].

In conclusion, the use of elicitors in *in vitro* plant culture represents a powerful, sustainable, and adaptable tool for the production of bioactive compounds, with expanding prospects in industrial, pharmaceutical, and agricultural applications.

#### 4.6. Model Plants and Bioreactor-Based Production

The use of model plants in *in vitro* culture has enabled the standardization of secondary metabolite production with pharmaceutical and industrial relevance. This biotechnological approach has been key to overcoming the limitations of conventional cultivation, particularly when bioactive compounds accumulate in specific organs or are produced only in trace amounts under natural conditions. Several plant species have been selected as models due to their ability to synthesize high levels of target compounds under controlled environments [129,130].

For instance, *Catharanthus roseus* has been extensively studied for its capacity to produce indole alkaloids such as vincristine and vinblastine, which are essential in chemotherapy. In cell suspension cultures, this species has achieved vinblastine production levels of up to 0.002% of the cell dry weight [49]. Another well-established model is *Panax ginseng*, whose root cultures have proven highly efficient for the production of ginsenosides, saponins with adaptogenic activity. These metabolites preferentially accumulate in roots; therefore, *in vitro* propagation has been directed toward organogenic or hairy root cultures to enhance compound accumulation. Reported total ginsenoside concentrations can reach up to 5% of dry weight in *in vitro* cultivated roots [131].

Similarly, *Hypericum perforatum* (St. John's wort), rich in hypericins and hyperforins, requires the development of differentiated glandular structures, as these metabolites are synthesized and stored in specialized foliar glands absent in undifferentiated cell cultures [132,133]. Other model plants such as *Withania somnifera* and *Bacopa monnieri* have also been implemented due to their production of withanolides and bacosides, respectively—metabolites with neuroprotective and adaptogenic properties. In the case of *Withania coagulans*, withanolide A concentrations of up to 1.9 mg/g dry weight have been achieved in optimized callus cultures [134,135]. In the case of *Bacopa monnieri*, optimized shoot cultures have achieved remarkable bacoside yields of up to 37.3 mg/g dry weight through strategic nutrient supplementation with serine and magnesium [136]. These systems offer advantages by eliminating genetic and environmental variability and ensuring a consistent and sustainable supply of plant material with a defined chemical profile.

Overall, *in vitro* culture systems enable the controlled production of secondary metabolites, avoiding the seasonal and environmental degradation factors that affect natural biosynthesis. Other species such as *Ocimum basilicum* for triterpenoids [137], *Camellia sinensis* for catechins [138], *Plumbago zeylanica* for plumbagin [139], have also been successfully used in cell or callus cultures to produce triterpenoids, catechins, plumbagin, and gymnemic acids, respectively. These species have been investigated for their potential to produce metabolites with antioxidant, hypoglycemic, antibacterial, and anticancer activities. Consequently, the establishment of production systems based on model plants has been crucial to advancing toward sustainable bioproduction of plant-derived bioactive compounds for pharmaceutical, nutraceutical, and cosmetic applications.

The use of bioreactors in plant tissue culture has revolutionized plant biotechnology by enabling scalable and controlled production of high-value secondary metabolites. Unlike conventional field cultivation, bioreactor systems provide constant microenvironmental conditions, reducing variability and risks associated with natural environments. Various types of bioreactors are currently employed, including stirred-tank, bubble-column, temporary-immersion, and mist bioreactors, each adaptable to different culture types such as hairy roots, cell suspensions, calli, or somatic embryos. These platforms have resulted not only in increased biomass accumulation but also in enhanced synthesis of compounds such as taxol, shikonin, ginsenosides, and caffeoyl derivative [35,115].

From a technical standpoint, the critical parameters for efficient bioreactor operation include control of pH, temperature, dissolved oxygen, inoculum density, and agitation rate. In the case of hairy root cultures, minimizing shear stress through low-energy impellers or the use of support meshes is essential, given their sensitivity to mechanical damage and their tendency to form entangled matrices that hinder mass transfer. Successful scale-up depends on selecting high-yield cell lines, optimizing the culture medium, and designing efficient operational processes. These factors combine to achieve consistent and economically viable production, with yields exceeding 60% metabolite release into the medium under two-phase configurations [140].

Several strategies have been developed to enhance metabolite production in bioreactors, including the addition of metabolic precursors, the use of elicitors (such as methyl jasmonate or salicylic acid), genetic modification to activate specific biosynthetic pathways, and combined tissue cultures. For instance, co-cultivation of *Panax ginseng* with *Echinacea purpurea* in 5 L bioreactors supplemented with 200  $\mu$ M methyl jasmonate significantly increased the production of ginsenosides and phenolic compounds. Likewise, perfusion and temporary-immersion systems have overcome limitations related to oxygenation and the accumulation of toxic exudates, particularly in dense root or cell cultures [94].

The yield of secondary metabolites in bioreactor systems varies depending on the plant species, culture type, and system configuration. In *Taxus chinensis* cell cultures, a combined strategy of methyl jasmonate elicitation (100  $\mu$ M), sucrose feeding (20 g/L), and ethylene incorporation (18 ppm) in airlift bioreactors significantly enhanced taxuyunnanine C production to 344 mg/L with a productivity of 20.2 mg/L/day [141].

#### 4.7. Current Trends and Future Perspectives

The integration of in vitro culture with modern biotechnological tools—such as CRISPR/Cas9 genome editing, transcriptomics, metabolomics, and nanotechnology—is shaping the next generation of plant-based biofactories [35]. These technologies enable fine-tuned control over metabolic fluxes, building upon foundational metabolic flux analysis (MFA) approaches that provide critical insights into the theoretical capabilities of plant metabolic networks [142]. Furthermore, plant-based expression systems are emerging as promising platforms for biopharmaceutical production, offering inherent biosafety, cost-efficiency, and scalability for sustainable manufacture of therapeutic proteins [143]. Smart bioreactor systems capable of real-time monitoring and dynamic adjustment of culture parameters are emerging as transformative solutions for industrial phytochemical production [144,145]. Overall, in vitro culture systems represent a cornerstone of green biotechnology, combining precision, sustainability, and scalability for the future of natural product biosynthesis.

## 5. Conclusions

This review emphasizes the relevance of in vitro plant culture systems as efficient, sustainable, and reproducible tools for producing secondary metabolites of high biotechnological and pharmaceutical value. Techniques such as callus and suspension cultures, hairy roots, micropropagation, and somatic embryogenesis provide controlled environments that overcome the limitations of traditional cultivation, ensuring stable yields and enabling genetic and metabolic optimization.

Beyond their scientific and industrial applications, these technologies offer an opportunity to explore and preserve the vast plant biodiversity found in regions such as Ecuador and across the world, unlocking new bioactive compounds with therapeutic, nutritional, and cosmetic potential. By promoting biotechnological innovation accessible to different contexts and communities, in vitro culture methods can contribute to both scientific advancement and social development through sustainable resource use and the valorization of native flora.

In this way, in vitro biotechnology serves not only as a bridge between plant science and industry, but also as a pathway toward inclusive and environmentally responsible progress.

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