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Arunima Vijayan
PG Scholar, Department of
Plant Physiology, College of
Agriculture, Vellayani,
Thiruvananthapuram, Kerala,
India

Dr. Viji MM
Faculty, Department of Plant
Physiology, College of
Agriculture, Vellayani,
Thiruvananthapuram, Kerala,
India

Dr. Smitha Bhasi
Faculty, Department of
Molecular Biology and
Biotechnology, College of
Agriculture, Vellayani,
Thiruvananthapuram, Kerala,
India

Dr. Soumya PR
Faculty, Department of Plant
Physiology, College of
Agriculture, Vellayani,
Thiruvananthapuram, Kerala,
India

Jani Aushna A
PG Scholar, Department of
Plant Physiology, College of
Agriculture, Vellayani,
Thiruvananthapuram, Kerala,
India

Neethu Chandra
PhD Scholar, Department of
Plant Physiology, College of
Agriculture, Vellayani,
Thiruvananthapuram, Kerala,
India

Corresponding Author:
Dr. Viji MM
Faculty, Department of Plant
Physiology, College of
Agriculture, Vellayani,
Thiruvananthapuram, Kerala,
India

Integrating foundational *in vitro* culture systems with advanced biotechnological strategies: A critical review of secondary metabolite enhancement in medicinal plants

Arunima Vijayan, Viji MM, Smitha Bhasi, Soumya PR, Jani Aushna A and Neethu Chandra

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Abstract

Medicinal plants are considered a critical biological source of secondary metabolites (SMs) that are of significant relevance to the pharmaceutical, cosmetic, and nutraceutical industries. Conventional extraction methods are hindered by low yields, environmental dependence, and ecological unsustainability, necessitating the development of reliable alternatives. While previous reviews have addressed individual *in vitro* strategies in isolation, no unified synthesis has integrated classical culture systems with contemporary biotechnological advances under a comparative framework. This review addresses that gap by critically evaluating callus, suspension, organ, and hairy root cultures, along with elicitation and genetic transformation, CRISPR/Cas9 (clustered Regularly Interspaced Short Palindromic Repeats) genome editing, metabolic engineering, multi-omics technologies, and bioreactor optimisation. Among the systems, hairy root culture provides the most consistent yield maintenance, while suspension culture is more suitable for large-scale commercial applications. Integration of CRISPR-based pathway editing and multi-omics-guided biosynthetic mapping represents the most promising frontier for overcoming current bottlenecks. Despite these advances, challenges such as species-specific optimisation requirements, scale-up limitations, and high implementation costs continue to restrict widespread industrial adoption of these technologies. This review provides a research-supported framework for integrating classical and modern strategies to enhance metabolite production, offering guidance for future research aimed at bridging the gap between laboratory-scale innovation and commercially viable, sustainable production systems.

Keywords: CRISPR/Cas, elicitation strategies, Genetic transformation, Hairy root culture, *in vitro* culture, Secondary metabolites

Introduction

Throughout human history, nature has played an irreplaceable role in sustaining human existence by providing essentials such as food, shelter, clothing, medicines, flavourings, and fragrances. The identification and effective application of natural compounds have been deeply intertwined with the progress of human civilisation [1]. In the healthcare field, medicinal plants continue to hold a central position, mainly in developing nations where a large segment of the population relies on traditional herbal remedies for primary healthcare needs [2]. China, India, Japan, Pakistan, Thailand, and Sri Lanka are among the countries where traditional medicinal practices are widespread. In China, 40% of the medicines consumed are based on traditional tribal medicine. In Thailand, herbal medicines frequently use legumes from the families Caesalpiniaceae, Fabaceae, and Mimosaceae. In the mid-90s, it was determined that more than US\$2.5 billion had been generated from the use of herbal medicines [3].

The therapeutic importance of plants is largely attributed to the bioactive compounds that they synthesise. Plants are designed with complex biochemical machineries that are capable of generating a wide spectrum of organic compounds that play more roles than basic physiological requirements. [4]. These compounds are known as secondary metabolites (SM). SMs play pivotal roles in ecological interactions, defence mechanisms, and adaptations to environmental stresses. SMs are considered nature's toolbox for ecological

success, and they also play specialised roles in plants that go beyond basic survival. Defence connections with other organisms. These are just a few of the abundant roles of SMs [5, 6]. The lack of SMs, in contrast to primary metabolites, makes the plant less able to survive and adapt over the long term, rather than die right away [7]. Also, they serve as valuable sources of agrochemicals, flavours, pharmaceuticals, fragrances, pesticides, and food additives [8].

Secondary metabolites and their major significance

In the contemporary scenario, several SMs are used as drugs in the medicinal industry. The discovery of vincristine, an anticancer drug derived from the medicinal plant *Catharanthus roseus* (periwinkle), illustrates the potential of natural products as therapeutic agents [1, 9]. The presence of

diverse varieties of SMs has increased the use of medicinal plants for commercial purposes. The production of SMs from relevant medicinal plants [commonly referred to as Plant-Derived Medicinal Compounds (PDMC)] has increased over the last decade [10, 11]. They are generally present in plants at trace levels and are extensively biosynthesised under unfavourable conditions in their growth environment as an adaptive tolerance mechanism. SMs are by products of several stress responses that plants encounter, including those caused by pathogens (bacteria, fungi, viruses, and insects) and physical factors (temperature, salinity, and drought). Genetic characteristics and nutrient availability also affect the biological production of SMs [6, 12]. Examples of medicinal plants, their secondary metabolites and industrial relevance are provided in Table 1.

Table 1: Medicinal plants, their secondary metabolites, and industrial relevance

Plant Species	Secondary Metabolites (SM)	Industrial Relevance	Reference
<i>Catharanthus roseus</i>	Vinblastine; Vincristine	Antitumour, antihypertensive drugs ^a	[13]
<i>Papaver somniferum</i>	Morphine; Thebaine; Codeine	Analgesics, cough suppressants	[14]
<i>Cinchona officinalis</i>	Quinine; Cinchonine	Antimalarial, antiarrhythmic, antiviral	[15]
<i>Artemisia annua</i>	Artemisinin	Antimalarial, antiviral, anticancer, antischistosomal drugs	[13]
<i>Curcuma longa</i>	Curcumin	Anti-inflammatory, nutraceuticals, cosmetics	[16]
<i>Hypericum perforatum</i>	Hypericin; Pseudohypericin; Paxanthone	Anti-inflammatory, antidepressant, antiviral, antibacterial, anticancer	[17]
<i>Andrographis paniculata</i>	Andrograpanin	Anti-inflammatory, antibacterial, antifungal, anticancer, antiviral	[15]
<i>Digitalis purpurea</i>	Digitoxin; Digoxin	Cardiac glycosides for heart failure ^b	[18]
<i>Glycyrrhiza glabra</i>	Glycyrrhizin	Sweetener, anti-inflammatory, antiviral	[19]
<i>Withania somnifera</i>	Withanolides	Adaptogen, stress-relief supplements ^c	[20]

SM = Secondary metabolite.

^a Vinblastine and vincristine are WHO essential medicines for cancer treatment.

^b Digitoxin and digoxin are cardiac glycosides with a narrow therapeutic index; dosage must be carefully monitored.

^c Withanolides are classified as adaptogens and are used extensively in Ayurvedic medicine.

Conventional extraction methods for secondary metabolites

SMs are traditionally extracted directly from the plants. The process of extraction involves systematic extraction techniques and selected solvent systems to separate bioactive components from plants [21]. In essence, the products are mixtures of metabolites in liquid, dry powder or semi-solid form. Compounds with a polarity similar to that of the solvent will solubilise when the solvent diffuses into the solid plant parts. Common solvents include methanol, ethanol, and ethyl acetate (for hydrophilic compounds). Dichloromethane (lipophilic compounds). Historically, SMs' isolation relied heavily on classical techniques such as Soxhlet extraction, reflux-based methods, maceration, and percolation, which were widely adopted for their relatively simple procedures. [22]. Eventually, they were modified to more modern strategies, such as microwave-assisted extraction, enzyme-assisted extraction, and supercritical fluid extraction [11]. Among other things, these methods are not highly efficient, leading to incomplete recovery of SMs and to the degradation of thermostable compounds. Furthermore, the lack of standardised protocols in conventional extraction leads to batch-to-batch inconsistencies, resulting in variable compound concentrations and low product quality. The combination of low yields, higher labour requirements, and long-term needs makes these approaches impractical for the industrial-scale production of secondary metabolites.

However, the traditional methods mainly relied on the direct extraction of SMs from the plant. Since most of the medicinal plants are harvested from wild populations rather than the cultivated sources, this over-reliance on natural habitats creates noticeable ecological pressure on forest ecosystems [23]. The predominant problem is that the quantity of secondary metabolites produced is very low, even from the whole plant itself, and destroying the whole plant for a trace quantity is neither economically nor ecologically feasible. [23, 24]. Plant-based production is highly dependent on environmental conditions and the plant growth stage, which determine which parts are suitable for extraction; as a result, this method is very time-consuming. For large-scale industrial production of secondary metabolites, the best approach is to use an *in vitro* culture system for medicinal plants. *In vitro* propagation techniques can serve as an alternative to conventional methods of crop improvement, yielding large, disease-free plants. The year-round production of the crop without depending on environmental conditions will also provide a much stronger rationale for adopting *in vitro* culture [25].

In vitro strategies for production of secondary metabolites

Considering the difficulties in natural production and traditional extraction of secondary metabolites, adopting advanced techniques is the only way to enhance and stabilise production. A substantial number of strategies have been implemented and proven successful, and the most

popular one is the *in vitro* cultivation of medicinal plants. At its core, the *in vitro* culture provides a controlled propagation of plant cells, organs, tissues or callus (an undifferentiated parenchymatic cell) all of which originates by mitotic division and therefore genetically identical to donor plant ^[10]. These cultures are maintained in a controlled environment, which helps ensure optimal growth. Since the conditions are provided externally, the production will not be affected by seasonal variations. Callus culture, suspension culture, micropropagation, and hairy root culture are commonly used *in vitro* techniques (Figure 1). The foundational concept of all *in vitro* propagation is totipotency, which is the inherent ability of a single plant cells to develop in to a complete genetically identical organism under suitable conditions ^[26].

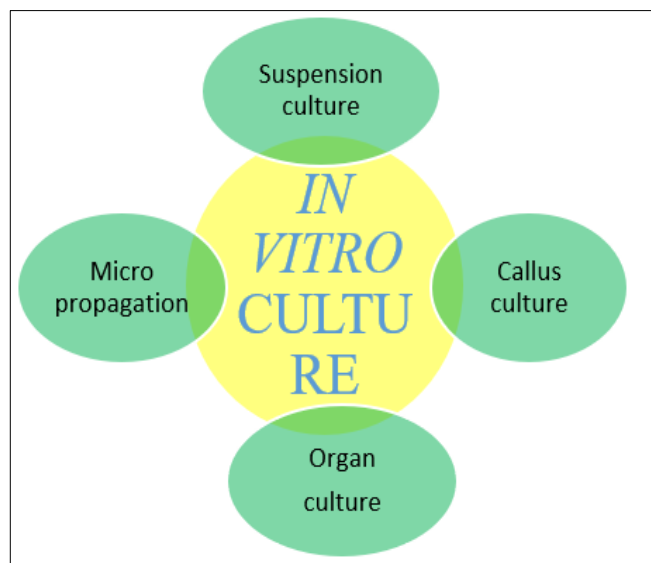


Fig 1: legend Classical *in vitro* culture systems are used to produce secondary metabolites in medicinal plants. The four major systems, suspension culture, callus culture, organ culture, and micropropagation are shown as interconnected components centred on the *in vitro* culture platform.

Classical *in vitro* systems for secondary metabolites production

1. Callus and organ culture

The most common and popular technique, callus culture, can help produce various types of bioactive compounds and can also be used to establish somatic embryogenesis. In some cases, *in vitro*-regenerated plant tissues produce higher levels of phenolic, flavonoid, and antioxidant compounds than their wild counterparts ^[27]. For instance, in a study they demonstrated that adjusting sucrose concentration in the medium could efficiently optimise callus cultures of *Vitis vinifera* for the production of secondary metabolites, and also the callus growth was fastest on 30 g/l sucrose concentration in the medium when compared to other treatments like 20 g/l, 40 g/l, 50 g/l and 60 g/l. Higher concentration reduced proliferation but increased the accumulation of phenolics, flavonoids, and anthocyanins ^[28]. This callus culture provided a season-independent, controlled yield compared with field-grown plants, and it is evident that manipulating culture compounds can enhance SM production. This method is considered more consistent and ensures reliability for pharmaceutical and nutraceutical applications. Likewise, *Catharanthus roseus*, the source of the important alkaloids vinblastine and vincristine, is grown via callus culture. In

this case, the main reason for using callus culture is that its cells can differentiate into two cell types, laticifers and idioblasts, which are the production centres of key biosynthetic enzymes, DAT (deacetylvinidoline 4-O-acetyltransferase) and D4H (desacetoxyvindoline 4-hydroxylase). Ultimately, this enzyme leads to the production of vinblastine and vincristine ^[29].

This callus induction can also incorporate stress-induced systems, such as drought stress induced by polyethylene glycol (PEG 4000), which can increase SM production ^[29]. Any plant parts cultured in an artificial medium are called organ culture. For example, root, shoot, leaf, flower, ovary, anther, or ovule can be considered as plant organs. Organ culture is highly reliable for studying secondary metabolite production in specific plant parts ^[30].

2. Suspension culture

Suspension culture is another extended version of a systematic *in vitro* culture, which can be established from callus culture. Plant cell suspension culture is usually a sterile, closed system. Usually, an aseptic friable callus fragment is placed in an appropriate liquid medium to initiate the process, providing the necessary culture conditions ^[31]. These cultures should be agitated continuously, which is very important to prevent callus clumping and to provide proper aeration. Establishing a stable cell suspension culture may take 6-9 months ^[32]. A certain amount of agitation also promotes secondary metabolite production. Maintaining a cell suspension culture mostly involves providing the cells with fresh media regularly to keep them alive and prevent browning and viability decline.

3. Micropropagation

Micropropagation is another type of *in vitro* system that has been successfully practised over a long period. The term “micropropagation” originates from the word tiny shoots or plantlets first produced through this *in vitro* multiplication technique. It provides a rapid and reliable means of generating genetically homogeneous and disease-free plantlets, which represent one of the most significant contributions of plant tissue culture to commercial propagation ^[33]. Based on the principle of cellular totipotency, micropropagation engages diverse explants such as leaf segments, roots, internodes, and nodal tissues cultured on nutrient media under aseptic conditions ^[34]. The process is typically divided into four stages: Stage 0, involving explant selection and surface sterilisation with agents such as HgCl₂ or ethanol; Stage I, the establishment of aseptic cultures, because maintaining aseptic conditions is crucial for micropropagation. Stage II, multiplication through shoot proliferation, optimised by specific auxin-cytokinin combinations; Stage III, rooting of regenerated shoots; and Stage IV, acclimatisation and transfer to soil, also known as hardening. Together, these stages provide a structured framework for large-scale clonal propagation and conservation of valuable plant species. The main advantage of micropropagation is a high multiplication rate. Year-round production, uniformity, and genetic fidelity in clones. Ability to eliminate systemic pathogens ^[35]. The phenotypic and biochemical traits of plants often vary due to differences in micropropagation conditions, developmental stages, and seasonal factors. Secondary metabolites vary with the plant's developmental stage and season. Beyond its methodological

and agronomic advantages, plants play a significant role in the pharmaceutical industry's sustainable drug production, which depends on a steady supply of high-quality raw materials [36]. For rapid regeneration and high-quality planting materials for production, it is crucial to develop reliable micropropagation techniques for these commercially significant medicinal plants, as these techniques can enhance the yield and consistency of secondary metabolites, which are vital for pharmaceutical applications [37].

Recent advances in metabolite production

Even though these classical *in vitro* systems provide foundational approaches to metabolite production, (which is summarized in Table 2) recent advances have expanded the possibilities through techniques like elicitation, genetic transformation, metabolic engineering, and culture optimisation. These strategies integrate modern biotechnology with traditional culture systems, enabling scalable, stable and targeted enhancement of SM yields.

Table 2: Comparison of classical *in vitro* culture systems for secondary metabolite production

Parameter	Callus Culture	Organ Culture	Suspension Culture	Micropropagation
Principle / Mechanism	Mitotic division of dedifferentiated parenchymatic cells from explants on hormone-supplemented media	Explant-based growth of specific plant organs with hormonal induction (root, shoot, leaf, anther, ovule, etc.)	Friable callus fragments dispersed in liquid medium with continuous agitation to prevent clumping and ensure aeration	Totipotency-based clonal propagation via shoot/root induction using auxin-cytokinin balance ^a
SM Yield	Moderate; can exceed wild-type in phenolics and flavonoids with optimisation	Organ-specific; higher targeted yields possible in the relevant organ	Variable; agitation promotes SM production ^b ; establishment takes 6-9 months	Moderate; primarily for clonal propagation, not high-yield SM extraction
Growth Rate	Slow to moderate; callus initiation takes weeks	Moderate; dependent on explant type and hormonal regime	Moderate; 6-9 months establishment time (32)	Fast; high multiplication rate achievable year-round
Key Advantages	Simple to establish; adaptable to elicitation; season-independent production; broad species applicability	Targeted organ-specific yields; useful for organ-localised SM production	High scalability; homogeneous cell population; amenable to bioprocessing and bioreactor systems	Disease-free, uniform planting material; year-round production; pathogen elimination
Limitations	Somaclonal variation; lower productivity without optimisation; genetic instability over subculturing	Difficult to scale; limited to specific organs; species-specific protocols needed	Long establishment time; shear sensitivity of cells; clumping tendency in bioreactors	Not optimised for high SM yield; phenotypic variability under changing conditions
Industrial / Commercial Feasibility	Moderate; used in pharmaceutical and nutraceutical research	Low to moderate; limited scalability for industrial use	High; used for commercial taxane production (Phyton Biotech, 75,000 L bioreactor) ^c	High for plant propagation; moderate for SM production
Cost of Establishment	Low to moderate	Moderate	Moderate to high; requires specialised bioreactor equipment ^d	Moderate; requires aseptic facilities
Key References	[27, 28, 29]	[6, 10, 30]	[31, 32, 38]	[33, 35, 37]

SM = Secondary metabolite; PGR = Plant growth regulator

^a Totipotency: the inherent ability of a single plant cell to develop into a complete, genetically identical organism under suitable conditions [26].

^b Agitation at appropriate speed promotes SM production and prevents cell clumping; excessive agitation causes shear damage.

^c cGMP = current Good Manufacturing Practice. Phyton Biotech (Germany) operates the world's largest cGMP plant cell culture facility with 75,000 L bioreactors for commercial taxane production [38].

^d Bioreactor types include modified impellers, bubble columns, airlift reactors, and rotating drum reactors.

Elicitation based strategies

It has been demonstrated that biotic and abiotic stress factors in tissue cultures significantly impact secondary metabolite accumulation. The term "elicitor" can be defined as a substance that, when introduced at a trace concentration into a living system, enhances the biosynthesis of specific compounds. The process of providing an elicitor to a system and enhancing the metabolite production is called elicitation [39]. Elicitors can cause phytoalexins, which facilitate plant cell defence mechanisms.

1. Biotic and abiotic elicitors

Elicitors are of two types: biotic and abiotic. Biotic elicitors are substances of biological origin that are derived from microorganisms like fungi and bacteria or from plant cell wall fragments, and these will stimulate pathogenic invasion and trigger the plant's innate defence responses. Abiotic elicitors, in contrast, are non-living chemical agents or physical environmental stimuli that provoke stress-associated metabolic responses within the plant system. Some examples of abiotic elicitors are physical (light and

UV), chemical (salts and heavy metals), and hormonal agents (jasmonic acid and salicylic acid) [40]. The quantity and concentration of the substance can affect the overall production of secondary metabolites. Several parameters, such as the concentration and selectivity of elicitors, the duration of elicitor exposure, the age of the culture, and the nutritional composition, also influence the successful production of secondary metabolites [41].

To establish an *in vitro* elicitation system, the first step is to confirm the most suitable system for production, taking into account both the plant and the secondary metabolite to be produced. Also, it is very important to choose the type of elicitor, whether it is biotic or abiotic, followed by the optimization of the quantity of elicitor used. Very low concentrations may be ineffective, and higher ones can trigger hypersensitive responses or cell death. For example, in a study about ajmalicine production in *Catharanthus roseus*, ajmalicine accumulation was higher in cells elicited with higher concentrations (5.0%) of elicitor extracts as compared to lower concentrations (0.5%). However, increasing the concentration further to 10.0% adversely

affected ajmalicine accumulation ^[39]. The duration of application also has to be standardized. After application, proper monitoring of the system and timely harvesting are also very important. The main advantage of the elicitation system is its ability to provide rapid, targeted metabolic enhancement through a cost-effective, scalable approach that can be implemented in novel plant compound production without relying on whole-plant extraction ^[42].

2. Nanotechnology-based elicitors

Nanotechnology bridges the gap between conventional knowledge and contemporary therapeutic needs by providing a strong, sustainable foundation for the scalable manufacturing of high-value plant-derived pharmaceuticals ^[43]. Nanoparticles have been developed into new elicitors. The size range of these nanoparticles is 1-100 nm. Also, they have distinct chemical and physical characteristics that set them apart from the bulk material. They are promising tools that could be used in many different fields, including biotechnology, medicine, electronics, food, agriculture, and cosmetics ^[44]. Still, there is further research required to enhance nanoparticle applications, nanotechnology-based elicitation shows considerable potential to improve the *in vitro* production of SMs. Biotechnological techniques, including *in vitro* culture and targeted elicitation, provide effective means to enhance the production of these bioactive chemicals to meet the growing demand for plant-derived medications and nutraceuticals ^[45]. Studies demonstrated the successful examples of nanoparticles as elicitors in plant *in vitro* cultures. Some examples include silver nanoparticles, which can act as elicitors in *Momordica charantia* cell suspensions, thereby increasing flavonoid accumulation. ^[46], and also when silver nanoparticles were added as an elicitor in *Caralluma tuberculata*, the phenolic and flavonoid content increased. ^[47]. There was a noticeable ninefold increase in gymnemic acid in *Gymnema sylvestre*, and elevated purpurin and alizarin levels were also observed with the use of copper oxide (CuO) nanoparticles ^[48]. Iron oxide (Fe₃O₄) nanoparticles which upregulated SM gene expression in *Dracocephalum kotschy* hairy roots ^[49] is also one among them.

Transformation-based strategies

1. Genetic transformation

Genetic transformation is a useful technique in metabolic engineering and also plays a role in the synthesis of secondary metabolites. Genetic transformation is the process of introducing foreign DNA into the genome of a suitable host organism, thereby allowing the target gene to integrate and be expressed. The two most widely used methods for genetic transformation are direct gene transfer and Agrobacterium-mediated gene transfer ^[50]. Protoplasts and tobacco cells are employed as a system for the direct gene transfer method and in this case, there is no vector acting as a carrier for the DNA uptake. Much more advanced techniques such as particle bombardment/ballistic gun, electroporation, microinjection, and the Polyethylene Glycol methods are also employed for genetic transformation ^[51]. Systems based on *Agrobacterium tumefaciens* are frequently employed because of their high efficiency, stable integration, and notably low transgene copy number in medicinal plants ^[52]. Long-term aseptic hairy root cultures have been established from over 200 species of higher plants, owing to their capacity to produce a wide range of

secondary metabolites and to modify their metabolic pathways for these compounds to various types of stress.

2. Hairy root culture

Among *in vitro* propagation strategies, the hairy root is a highly advanced one, established by infecting with *Agrobacterium rhizogenes*, a gram-negative soil bacterium known to induce abnormal root proliferation in wounded higher plants. To induce the Ri plasmid, this pathogen inserts a DNA fragment from its root into the infected plant's genome, which encodes opines and enzymes that regulate auxin and cytokinin biosynthesis (iaaM, iaaH, and ipt). Additionally, these hormones will cause "hairy roots" [HR], or proliferating roots ^[53]. Hairy roots can be characterised by their morphology, including their growth rate, extensive lateral branching, dense root hair covering, and plagiotropic growth. Mainly, four out of 18 open reading frames (ORFs) of the T-DNA of the bacteria can induce hairy roots, and they are called rol genes (rolA, rolB, rolC, and rolD). These oncogenes can also regulate plant homeostasis, hormonal metabolism, and cellular processes, such as phytochrome signalling pathways. These genes can also accumulate SMs in hairy roots, maintaining the same profile as in the source plants. Secondary metabolite accumulation in hairy root culture is higher compared to both the intact parent plant and conventional callus cultures. A study mentions that the *Hyoscyamus muticus* KB7 hairy root clone, first established in 1998, was maintained by subculturing for almost 16 years, from 1998 to 2016 ^[54]. The system consistently yields tropane alkaloids throughout this period. In another study of *Datura stramonium*, stable, long-term alkaloid production via hairy root culture has been demonstrated ^[55]. These examples confirm the long-term genetic and metabolic stability of hairy root cultures for secondary metabolite production.

The rolB gene is considered the main regulator of secondary metabolism; a 100-fold increase in resveratrol production in *Vitis amurensis* was observed following rolB gene transformation ^[56]. In essence, establishing an SM production system using hairy root cultures is highly recommended due to its high success rate and consistent yield.

3. CRISPR/Cas genome editing

CRISPR/Cas9 gene editing is a newly emerging tool with a promising role in plant genetic engineering, enabling precise genome modifications to enhance SM production because the majority of the secondary metabolites biosynthesis can be triggered by the activation of some specific genes. The CRISPR-Cas system is part of the prokaryotic adaptive immune response. CRISPR systems usually help defend against viral infections. This is done by storing the vital DNA fragments of bacteria and archaea and using them to detect and resist future invasions. This mechanism is exploited in genetic engineering to modify and target DNA sequences in plants. This CRISPR-mediated gene editing begins with the design of a single-guide RNA (sgRNA) complementary to the targeted DNA sequence. This sgRNA directs Cas protein to the specific locus. The most commonly used Cas proteins are Cas9 and Cas12a. A double-strand break (DSB) is generated at the targeted site after the Cas protein attaches to the corresponding DNA. The DSB is then repaired via one of two pathways. These are HDR (homology-directed repair) and NHEJ (non-

homologous end joining). HDR allows precise modifications with the help of a donor template, and gene function can be disrupted by small insertions or deletions, leading to NHEJ^[21]. Incorporation of this novel technology for pathway analysis and targeted gene transformation can enhance the SMs production.

Engineering-based strategies

1. Biotransformation

Biotransformation involves the structural modification of a chemical molecule by biological agents and offers a valuable means of generating important bioactive compounds, revealing metabolic bottlenecks, and sustaining productivity under precursor-limited conditions^[57]. Also, this can be defined as the potential of plant cell culture to catalyse the transformation of the current precursor into a much more important final product^[58]. This process involves altering a chemical molecule in a biological system and producing new compounds. The metabolites produced can reach higher concentrations in the system than the parental compound^[21, 59].

In biotransformation, the substrate has a structural relationship with the natural biosynthetic intermediates. A diverse range of biological systems that are suitable for biotransformation processes, such as fungi, yeast, bacteria, algae, actinomycetes, and various animal and plant cell systems. A volume of substrates can be transformed in a living system through various activities, such as oxidation, reduction, methylation, demethylation, aminoacylation, glycosylation-acylation, and esterification^[21]. Comparing conventional chemical synthesis with biotransformation offers notable advantages, such as improved efficiency, reduced generation of unwanted byproducts, and a lower environmental footprint. The number of parameters should be optimised to assess biomass accumulation and SM synthesis in cultures. The process is typically approached in two steps: the initial phase focuses on optimising cellular biomass accumulation through control of medium and environmental parameter control, while the second phase targets secondary metabolite production through strategies such as precursor feeding, elicitation, and immobilisation. The selection of cells or organs with high productivity and optimisation of the medium parameters, such as stable medium, salt, and plant growth regulator levels and other external parameters like temperature, illumination, light quality, medium pH, agitation, aeration, and environmental gas, are controlled in the first step of the culture process. Replenishment of nutrients, precursor feeding and elicitation, permeabilization, and immobilisation strategies help accumulate metabolites and can be applied in the second step of the process^[21]. Besides cell cultures, organ cultures, and genetically transformed hairy root cultures, these systems offer significant advantages, largely because of their genetic or biochemical stability^[60]. Also, these systems retain multi-enzymatic biosynthetic potential comparable to the similar source plant, while operating at lower cost obligations^[61]. Their effective utilisation across diverse medicinal plants highlights their growing importance in biotransformation systems.

2. Precursor feeding

Precursor feeding is a process in which the major substrates are transformed into a different substrate with new characteristics through the action of specific enzymes or

microorganisms^[6]. Some specific biosynthetic intermediates or metabolic precursors are added to the culture medium to accelerate the production of desired secondary metabolites.^[5]

This approach emphasises the principle that supplying key precursors can bypass rate-limiting steps in biosynthetic pathways, thereby increasing metabolic yields by maintaining a controlled culture environment^[39]. Precursor feeding is an approach that involves selecting species having well-characterised SMs, establishing a suitable *in vitro* culture system, and supplying key precursors that can bypass rate-limiting steps in biosynthetic pathways, thereby increasing metabolic yields. Supplying optimal concentrations of specific metabolite intermediates at standardised time points can further facilitate targeted accumulation of desired compounds, improving both yield and specificity. The accumulated metabolites can be monitored using analytical techniques such as HPLC or TLC. This method is well-suited to large-scale industrial production of SMs.

Despite the advantages, notable challenges include a high concentration of precursors, which can induce a cytotoxic imbalance and reduce cell viability and overall metabolite productivity. Uptake and transport limitations can hinder efficient assimilation, especially when the precursor requires specialised transporters or membrane permeability enhancement^[60]. Metabolic diversion, whereby precursors are rerouted into unintended pathways, can compromise both yield and specificity, ultimately reducing the efficiency of secondary metabolite production^[62]. Successful precursor-feeding implementation requires a profound understanding of biosynthetic routes, enzymatic bottlenecks, and regulatory checkpoints, which is the best way to avoid imbalances and inefficiencies in SM accumulation.

3. Metabolic engineering

The increased understanding of biosynthetic pathways and the engineering of targeted compounds has made the engineering of secondary metabolites much more accessible. Metabolic engineering is a unique technique that involves improving cellular activities by manipulating the enzymatic, transport, and regulatory functions of the plant cell using recombinant DNA technology^[63]. In secondary metabolites, regulation primarily occurs at the subcellular or enzymatic levels. Regulatory genes are usually targets for regulating the biosynthetic pathway or transporter genes. Metabolic engineering to enhance secondary metabolite biosynthesis, either *in vitro* or by introducing a foreign gene, has been reported using *Agrobacterium*-mediated transformation^[64]. Sometimes this can lead to nonspecific mutations, which, in turn, accumulate toxic intermediates. Metabolic engineering follows a well-structured framework that starts by selecting the targeted compound and continues with pathway mapping, which helps interpret biosynthetic routes. This is followed by gene identification and cloning techniques, which help in insertion into a chosen host, and then genetic transformation. The pathway will then be refined to create ideal viability conditions. There are some core strategies in metabolic engineering that outline the biosynthesis of increased products and the redirection of metabolic flux. Gene overexpression, Transcription factor (TF) Pathway redirection, Transporter engineering, and Compartmentalisation are the important ones. There are some disadvantages to this method of gene modification,

including a relatively low success rate and the potential for unintended, nonspecific mutations that can lead to toxic compound accumulation ^[65]. So, to attain the full potential of metabolic engineering the need of further studies is evident.

4. Multi-omics technologies

The use of multi-omics technologies, including genomics, proteomics, transcriptomics, and metabolomics, has significantly changed how secondary metabolites are produced *in vitro* in medicinal plants. Decoding the complex biosynthetic pathways was a major challenge in optimising *in vitro* culture systems, as these pathways were largely unknown before. With the development of high-throughput sequencing and advanced analytical platforms, researchers were able to overcome this barrier, laying the groundwork for improving *in vitro* systems ^[66, 67]. Genomic resources from whole-genome sequencing of medicinal plants have enabled the identification of key biosynthetic genes and metabolic clusters, making it more practical to manipulate secondary metabolite pathways in cell and tissue culture systems. Characterising differentially expressed genes that control the accumulation of pharmaceutically important substances, including alkaloids, flavonoids, phenolic acids, and terpenoids, under *in vitro* conditions has been made possible by transcriptomic techniques, especially RNA-seq-based profiling. For example, in *Dendrobium officinale* across different developmental stages, a comparative transcriptome analysis revealed that the key flavanol biosynthetic genes CHS and FLS are highly expressed during the biennial stage. This information provided critical guidance on the processes for optimising the harvest period of *in vitro* systems ^[67]. Also, the study concluded that proteomics complemented transcriptomic data, revealing the roles of protein abundance and post-translational modifications in influencing SM biosynthesis under different culture circumstances. Metabolomics, using platforms such as GC-MS and LC-MS/MS, provides the most direct means to profile the chemical diversity of *in vitro*-produced metabolites, capturing tissue-specific and condition-dependent variations in metabolite composition that guide the selection of optimal culture parameters. The integration of these omics layers with the strategies of bioinformatics tools opened the system-level understanding of biosynthetic networks. This simplified the identification of bottleneck enzymes and regulatory transcription factors, thereby enabling targeted engineering in plant cell culture ^[66].

Moreover, multi-omics approaches have been integrated with elicitation strategies, yielding effective results in *in vitro* systems. This significantly influences the biosynthetic pathway and triggers SM production. For instance, using integrated transcriptomic and metabolomic analysis, treatment with methyl jasmonate has been shown to upregulate genes encoding key biosynthetic enzymes in the terpenoid and phenylpropanoid pathways ^[68]. The development of comprehensive databases that collect omics data from medicinal plants, such as the Global Pharmacopoeia Genome Database (GPGD) and the 1K Medicinal Plant Genome Database, has accelerated the identification of candidate genes and important bioactive compounds for *in vitro* production.

Culture optimization based strategies

To attain stable productivity in the *in vitro* system, culture optimisation is an effective strategy. For the production of secondary metabolites, culture optimisation can be a critical determinant. This usually encompasses media formulation, immobilisation, permeabilization, and bioreactor scale-up. Considering medium optimisation, the selection and fine-tuning of media components such as carbon sources, nitrogen ratios, pH, plant growth regulators, and inoculum density will significantly influence biomass accumulation and secondary metabolite biosynthesis. Even if the conditions are favourable for cell growth, they will not simultaneously influence product formulation ^[38]. There are statistical approaches, such as Plackett-Burman designs and response surface methodology, which have proven especially effective in identifying key variables and their interactions, thereby overcoming the drawbacks of classical one-factor-at-a-time optimisation ^[69].

To achieve cell immobilisation, entrapment in calcium alginate matrices is the most common method, offering several bioprocess advantages, such as elevated cell density during growth even with smaller bioreactor volumes, reduced hydrodynamic shear, and maintenance in the stationary phase, where more secondary metabolite synthesis occurs. This approach has been very successfully applied many times; enhancing the production of paclitaxel, vanillin, and capsaicin is one of them ^[38, 70].

In permeabilization strategies, recruiting agents like chitosan, dimethyl sulfoxide (DMSO), Triton X-100, and organic solvents, including decanol and hexadecanol, facilitate the controlled release of intracellularly stored metabolites from vacuoles into the culture medium. Hence, it prevents product inhibition, simplifies downstream purification, and enables continuous production cycles ^[70].

Scaling up from laboratory shake flasks to industrial bioreactors will provide multifaceted engineering challenges, comprising non-Newtonian rheology, shear sensitivity of plant cell aggregates, inadequate mixing, and limited oxygen transfer. These have been addressed through the use of modified impellers, bubble columns, airlifts, and rotating drum reactors, along with advanced operating modes such as fed-batch, two-stage, perfusion, and semi-continuous cultivation systems ^[6]. In particular, Phyton Biotech (Germany) currently operates the world's largest cGMP plant cell culture facility with 75000 L bioreactors for commercial taxane production, underscoring the commercial viability of optimised scale-up strategies ^[38].

Comparative evaluation of *in vitro* systems: advantages, limitations, and commercial feasibility

A critical evaluation of *in vitro* systems shows that none of the strategies is universally superior, but the optimal choice is finalised by the nature of the target secondary metabolites, the plant species under study and the intended scale of production. Coordination of these systems will help maintain a scalable, reproducible, and environmentally friendly, sustainable replacement for whole-plant extraction, and also overcome the major drawbacks of conventional extraction methods, such as seasonal variability, ecological impact, and inconsistent yield. The integration of advanced strategies, such as elicitation, metabolic engineering, precursor feeding, biotransformation, genetic engineering, and multi-omics technologies, has strengthened the productivity and sustainability of these platforms ^[71, 72].

Nevertheless, the practical implementation of each system involves distinct challenges related to yield potential, biological stability, technical complexity, and cost, which must be carefully considered in the context of the intended application. A comparison of advanced biotechnological strategies is presented in Table 3.

Among the systems reviewed, hairy root cultures stand out as the most reasonable for stable, consistently high SM yields. By inducing *Agrobacterium rhizogenes* hairy roots, which maintain a biochemical profile similar to the parent plant, exhibit high proliferation rates, and sustain extended periods of SM production without genetic drift. All these qualities make them the most reliable strategy for targeted compound biosynthesis [73, 74]. However, suspension cultures offer superior scalability and industrial feasibility, making them more suitable for large-scale secondary metabolite production. By coordinating strategies such as elicitation, it is possible to monitor and stimulate biosynthetic pathways [23, 75]. Callus culture, while foundational to an *in vitro* system and broadly applicable, is primarily used for SM production in early-stage research or in species where other systems are less common [10]. Micropropagation plays a distinct role, though it is not optimal for high-yield metabolite extraction. Micropropagation provides genetically uniform, pathogen-free plant material that supports the consistency and reliability of downstream production systems [10, 76]. So, it is evident that these systems are not mutually exclusive, but the integration of multiple strategies like hairy root culture with elicitation, or combining suspension culture with precursor feeding,

frequently provides superior metabolite yield compared to any single approach alone [23]

Despite the considerable promise of *in vitro* culture systems, technical limitations continue to constrain their broader application. Low secondary metabolite yields remain the foremost challenge at the biological level, compounded by an incomplete understanding of the biosynthetic pathways leading to SM accumulation [38]. Physiological heterogeneity in cultured cells, instability in genetic traits due to somoclonal variation from prolonged subculture, and poor extracellular secretion of the targeted compounds all together reduce production efficiency and complicate downstream processing [77, 78]. From a technical perspective, these demanding conditions are very important for maintaining the *in vitro* system. The shear sensitivity of plant cells and their tendency to adhere in bioreactor systems pose a significant engineering challenge during industrial-scale-up [38]. Trained personnel, specialised infrastructure, and high-efficiency, high-cost analytical tools such as HPLC for compound verification and quality control also limit the accessibility of these systems in low-storage systems [10]. Additionally, access to high PDMC-yielding genotypes is restricted as plant lines are often held by private institutions or protected under intellectual property rights agreements, which limit the availability of independent research and commercial development [72, 76]. A balanced action between *in vitro* productivity and real-world commercial feasibility remains the fundamental challenge in medicinal plant biotechnology.

Table 3: Comparison of advanced biotechnological strategies for secondary metabolite production in medicinal plants

Strategy	Mechanism	Yield Impact	Advantages	Limitations
Biotic & Abiotic Elicitation	Introduction of biotic (fungal/bacterial extracts) or abiotic (heavy metals, UV, JA, SA ^a) stimuli to activate plant defence and SM biosynthesis	Significant, concentration-dependent, rapid targeted metabolic boost	Cost-effective; rapid SM enhancement; scalable; no whole-plant extraction needed	Concentration-sensitive (excess causes cell death); requires optimisation of timing and duration
Nanotechnology-Based Elicitation	Nanoparticles (1-100 nm) act as novel elicitors; unique physicochemical properties upregulate SM biosynthesis gene expression	High; up to 9-fold increase in specific SMs (e.g., gymnemic acid with CuO NPs ^b)	Highly targeted and stable; bridges conventional and modern production; multidisciplinary applications	Nanoparticle toxicity concerns; regulatory/safety issues; further optimisation research needed
Genetic Transformation	Introduction of foreign DNA via <i>Agrobacterium tumefaciens</i> or direct gene transfer (particle bombardment, electroporation, microinjection, PEG ^c method)	Moderate to high; depends on species and transformation efficiency	Stable integration; low transgene copy number with <i>Agrobacterium</i> ; heritable, long-term expression	Low success rate in some species; risk of nonspecific mutation; toxic intermediate accumulation
Hairy Root Culture	<i>Agrobacterium rhizogenes</i> -mediated Ri plasmid insertion activating rol genes (rolA, rolB, rolC, rolD) to induce proliferating roots	High and stable; SMs exceed parent plant and callus cultures; consistent yield for up to 16 years ^d	Genetic/biochemical stability; hormone-independent growth; multi-enzyme biosynthetic potential; lower culture cost	Requires <i>Agrobacterium</i> infection; species-specific efficiency; difficult to scale industrially
CRISPR/Cas9 Genome Editing	sgRNA-directed Cas9 to a specific DNA locus, inducing DSB ^e repaired via HDR (precise edit) or NHEJ (gene disruption)	High precision; bypasses rate-limiting biosynthetic steps without nonspecific mutations	Highly precise; avoids nonspecific mutations; compatible with <i>in vitro</i> platforms; targeted pathway editing	Off-target effects possible; delivery challenges in plants; species-specific optimisation required
Biotransformation	Biological systems (bacteria, fungi, yeast, plant cells) convert substrate molecules to new high-value products via oxidation, reduction, methylation, glycosylation, esterification, etc.	Can exceed parental compound concentration; efficient even under precursor-limited conditions	Higher efficiency than chemical synthesis; fewer byproducts; less waste; uses renewable biological systems	Requires optimised two-step culture conditions; complex downstream processing; species-specific enzyme activity
Precursor Feeding	Addition of biosynthetic intermediates or metabolic precursors to culture medium to bypass rate-limiting steps and accelerate SM accumulation	Improved yield and specificity; bypasses bottleneck enzymes in the biosynthetic pathway	Targeted accumulation; bypasses rate-limiting steps; compatible with large-scale industrial production	High concentrations cause cytotoxicity ^f ; metabolic diversion risk; uptake/transport limitations
Metabolic Engineering	Manipulation of enzymatic, transport, and regulatory functions using recombinant DNA technology; gene	Significant when successful; increases biosynthetic flux; targets	Redirects entire metabolic pathways; stable genetic modification; applicable to	Low success rate; risk of nonspecific mutations; toxic intermediate

	overexpression, TF ^g engineering, transporter engineering, compartmentalisation	regulatory bottlenecks	diverse SM classes	accumulation; complex regulatory requirements
Multi-omics Technology	Integration of genomics, transcriptomics, proteomics, and metabolomics with bioinformatics to decode biosynthetic networks and identify bottleneck enzymes and regulatory TFs	Accelerates pathway mapping; identifies candidate genes; guides optimal culture parameter selection	System-level understanding; accelerates gene discovery; integrates with elicitation; enables data-driven engineering	High data complexity; requires bioinformatics expertise; expensive platforms (GC-MS ^h , LC-MS/MS, RNA-seq)
Culture optimisation	Fine-tuning of media components (carbon, nitrogen, pH, PGRs ⁱ), immobilisation (calcium alginate), permeabilisation (DMSO, chitosan), and bioreactor scale-up (fed-batch, perfusion, two-stage systems)	Critical determinant of productivity; immobilisation elevates cell density; permeabilisation enables continuous production	Maximises existing system productivity; statistical tools (Plackett-Burman, RSM ^j) identify key variables; compatible with all <i>in vitro</i> systems	High bioreactor engineering complexity; non-Newtonian rheology; shear sensitivity; high establishment cost

SM = Secondary metabolite.

^a UV= Ultra violet; JA = Jasmonic acid; SA = Salicylic acid.

^b CuO NPs = Copper oxide nanoparticles. A ninefold increase in gymnemic acid was observed in *Gymnema sylvestre* [48].

^c PEG = Polyethylene glycol.

^d The *Hyoscyamus muticus* KB7 hairy root clone, established in 1998, consistently yielded tropane alkaloids over 16 years of subculturing [54].

^e DSB = Double-strand break; HDR = Homology-directed repair; NHEJ = non-homologous end joining.

^f Cytotoxicity: cell viability declines at high precursor concentrations, reducing overall metabolite productivity [62].

^g TF = Transcription factor.

^h GC-MS = Gas chromatography-mass spectrometry; LC-MS/MS = Liquid chromatography-tandem mass spectrometry.

ⁱ PGR = Plant growth regulator.

^j RSM = Response surface methodology

Future outlook

In vitro secondary metabolite production is at the edge of the transformation era; novel intersections of biotechnological tools and the growing demand for plant-derived medicinal compounds are catalysing this growth. Classical *in vitro* systems, such as callus cultures, suspension cultures, and micropropagation, have made significant progress. However, they also have limitations in yield, compound specificity, stability, and scalability, underscoring the need for further advancement [53, 73].

One of the most promising strategies is the integration of CRISPR/Cas9 genome editing into *in vitro* platforms. Targeted, precise modification of rate-limiting biosynthetic genes, targeted transcription factor engineering, and inhibition of competing metabolic pathways increase metabolic input without triggering product mutations, a major concern in traditional metabolic engineering approaches [65, 79]. The expanding application of multi-omics technologies, including transcriptomics, proteomics, and metabolomics, will further accelerate the mapping of pathways for desired compounds. Extensive databases, such as the Global Pharmacopoeia Genome Database (GPGD) and the IK Medicinal Plant Genome Database, have proven their potential to empower researchers to elucidate the novel biosynthetic gene clusters and regulatory sequences of underexplored medicinal plants with significant potential in the medicinal industry [23, 79].

In the range of biotechnology, nanotechnology-based elicitation is an emerging strategy. Unique characteristics of nanoparticles equip them to act as elicitors that upregulate the SMs biosynthesis. Bioreactor technologies will need to evolve beyond the current commercial benchmark set by Phyton Biotech for taxane production. The key future direction should be sensor-integrated systems capable of real-time metabolic detection and automatic parameter adjustment [6, 38]. Perfusion-based two-stage bioreactor systems, which eliminate the biomass accumulation from metabolic synthesis, offer promise for the selective accumulation of compounds during the stationary phase [6].

With the integration of biotransformation and precursor feeding, these systems can result in cost-effective bioconversion of common substrates into high-value products with minimal waste [21, 57]. Ultimately, the future of *in vitro* SMs production lies in the combined effect of genome editing, multi-omics, nanotechnology and advanced bioprocessing. These approaches collectively make medicinal plant biotechnology a sustainable, commercially relevant factor for drug discovery and nutraceutical development [71, 80].

Conclusion

The *in vitro* cultivation of medicinal plants has emerged as a scientifically promising and ecologically reasonable alternative to conventional extraction approaches, addressing challenges such as inconsistent yields, seasonal growth issues, and unsustainable wild harvesting. Among the systems, hairy root culture stands out for its genetic stability and consistently high metabolic yields, whereas suspension culture offers superior scalability for industrial applications. Micropropagation, which is not primarily aimed at improving metabolic yield, ensures a consistent supply of uniform, pathogen-free plant material, which is essential for downstream production.

Integration of recent advances like CRISPR/Cas9-based pathway editing, multiple omics-guided biosynthetic mapping, nanotechnology-based elicitation, and optimised bioreactor systems will hold significant potential to overcome bottlenecks in yield and compound specificity. There is no universally superior strategy; a combination of tailored approaches for targeted compounds and species often yields the best results. As demand for plant-derived bioactive compounds increases globally, the integration of plant biotechnology, genomics, and bioprocess engineering is an efficient approach for producing SMs at the industrial scale. Future research should focus on expanding these integrated techniques to increase production and exploring more understudied medicinal plants with high potential.

Abbreviation	Full Form
Cas9	CRISPR-associated protein 9
Cas12a	CRISPR-associated protein 12a
cGMP	Current Good Manufacturing Practice
CHS	Chalcone synthase
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR-associated protein 9
CuO NPs	Copper oxide nanoparticles
D4H	Desacetoxyvindoline 4-hydroxylase
DAT	Deacetylvindoline 4-O-acetyltransferase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSB	Double-strand break
Fe ₃ O ₄ NPs	Iron oxide nanoparticles
FLS	Flavonol synthase
GC-MS	Gas chromatography-mass spectrometry
GPGD	Global Pharmacopoeia Genome Database
HDR	Homology-directed repair
HgCl ₂	Mercuric chloride (surface sterilisation agent in micropropagation)
HPLC	High-performance liquid chromatography
HR	Hairy roots
iaaH	Indole-3-acetamide hydrolase gene (T-DNA of <i>Agrobacterium rhizogenes</i>)
iaaM	Tryptophan monooxygenase gene (T-DNA of <i>Agrobacterium rhizogenes</i>)
ipt	Isopentenyl transferase gene (T-DNA of <i>Agrobacterium rhizogenes</i>)
JA	Jasmonic acid
KB7	Hyoscyamus muticus hairy root clone designation
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
Mpro	Main protease (of SARS-CoV-2)
NHEJ	Non-homologous end joining
NPs	Nanoparticles
ORCID	Open Researcher and Contributor ID
ORFs	Open reading frames
P450 2E1	Cytochrome P450 enzyme isoform 2E1
PDMC	Plant-derived medicinal compounds
PEG 4000	Polyethylene glycol 4000
PGR	Plant growth regulator
Ri plasmid	Root-inducing plasmid (of <i>Agrobacterium rhizogenes</i>)
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
rolA	Root locus gene A (T-DNA oncogene of <i>Agrobacterium rhizogenes</i>)
rolB	Root locus gene B (T-DNA oncogene of <i>Agrobacterium rhizogenes</i>)
rolC	Root locus gene C (T-DNA oncogene of <i>Agrobacterium rhizogenes</i>)
rolD	Root locus gene D (T-DNA oncogene of <i>Agrobacterium rhizogenes</i>)
RSM	Response surface methodology
SA	Salicylic acid
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
sgRNA	Single-guide RNA
SM / SMs	Secondary metabolite / Secondary metabolites
T-DNA	Transfer DNA
TF	Transcription factor
TLC	Thin-layer chromatography
Triton X-100	Polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether (permeabilising agent)
UV	Ultraviolet
WHO	World Health Organization

Declarations

Competing interests

The authors declare that they have no competing interests.

Author's contributions

- AV: Conducted literature search, drafted the manuscript, and prepared tables and figures.
- VMM: Supervised the work, provided critical revisions, and served as the corresponding author.
- SB: Proofreading and contributed to editing and refinement of content.
- SPR: Assisted in structuring the manuscript and technical editing.
- JAA: Contributed to reference formatting, proofreading, and technical editing.
- NC: Verified citations, assisted in data organization, and contributed to manuscript polishing.

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Not applicable. (This review does not involve human participants, human data, or animal studies.)

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