BIOTECHNOLOGICAL INTERVENTIONS IN UPSCALING OF PLANT SECONDARY METABOLITES

Abstract

Plants are considered as a great hub of secondary metabolites with high value that have uses in a variety of fields. Whenever the natural source is insufficient or chemical synthesis is not viable, plant tissue culture methods are regarded as viable and eco -friendly for the smooth production of secondary metabolites. The main benefits of using plant tissue culture techniques for the production and enrichment of plant secondary metabolites are discussed in this chapter, along with the various biotechnological methods that can be used to upscale their production. The chapter demonstrates that although there are several instances describing the synthesis of differentiated cells and tissues especially hairy roots and undifferentiated cells are the ideal culture method employed for the creation of valuable secondary metabolites under *in vitro* conditions. The potential ways to improve the biosynthesis of valuable compounds produced by any plant *in vitro* systems are outlined in an integrated manner. This includes metabolic engineering, which regulates plant metabolism by overexpressing/repressing a single structural gene or a group of structural genes. The production of secondary metabolites from plant origin at laboratory or industrial scales, various bioreactor system types, their modification, and ideal process parameters are described.

Keywords: Bioreactors, Hairy roots, Metabolic engineering, Secondary

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metabolites

I. INTRODUCTION

The plant kingdom, roughly comprises of around 250,000 species, and it is a source of untold numbers of secondary metabolites [1]. The two kinds of metabolites that are available in plants are namely primary metabolites and secondary metabolites [2]. Primary metabolite plays a crucial role in proper development, growth, and reproduction and typically serves an organism's physiological needs [3]. Primary metabolites include substances which are proteins, lipids, carbohydrates, vitamins, and nucleic acids [3]. Small organic compounds that are not required for the primary functions mainly growth, expansion, or reproduction are known as secondary metabolites [4]. Secondary metabolites are indispensable for the existence and survival of plants since they play a significant protagonist in the interaction between plants with their ecological niche (e.g., defense against predators and diseases [5]. These compounds accumulate in certain tissues and related structures (e.g., vacuoles, glandular and non-glandular trichomes), and their production is affected by a variable number of factors such as genotype, physiological attributes, environmental and climatic circumstances, and pathogens; in other cases, they are only generated during specific developmental phases [6]. According to their elemental composition, plant secondary metabolites are categorized into four main groups: terpenes, phenylpropanoids (phenols), polyketides, and alkaloids [7].Isoprene units make up the broad class of natural compounds known as terpenes. Terpenes generally have the chemical formula $(C_5H_8)_n$, where n is the number of connected isoprene units. Terpenoids are oxygenated hydrocarbons, whereas terpenes are simply hydrocarbons [8].The most prevalent secondary metabolites found in plants are phenols which are distinguished by the occurrence of aromatic ring/rings with one or more hydroxyl groups. It includes both simple molecules like phenolic acid and complex polymerized compounds like tannins [9].Another class of secondary metabolites known as polyketides is created from a precursor molecule that consists of an alternating chain of ketone (or reduced versions of a ketone) and methylene groups: (-CO-CH2-) [10]. The secondary metabolites with nitrogen are referred to as alkaloids and it comprises of one or more nitrogen atoms[11].

Many secondary metabolites from have already been extracted, their structures were elucidated, and their biological action were assessed over the past few decades [12]. The primary source of numerous significant bioactive compounds and pharmacophores continues to be plants [13]. For instance, around 60% of antineoplastic drugs are derived from plants directly or indirectly, and about 25–28% of current treatments are based on modern medicines derived from plants [14]. The British Broadcasting Corporation (BBC) has released a report estimating that the market for plant-derived medicines will increase from $29.3 billion in 2017 to around $39.2 billion in 2022, along with an annual growth rate of 5.9% [15]. Some of the popular secondary metabolite along with its plant sources are tabulated in Table 1.

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Table 1: List of Some Popular Secondary Metabolites Along with Source and use

Type of secondary metabolite	Name of the secondary metabolite	Name of the plant	Use	References
Terpenes	Azadirachtin	*Azadirachta indica*	Broad-spectrum insecticide	[8]
	Artemisinin	*Artemisia annua*	Treatment of malaria	[8]
	Tetrahydrocannabinol	*Cannabis sativa*	Appetite stimulant	[8]
	Saponins	*Chenopodium quinoa*	Treatment of hypercalciuria	[8]
Phenylpropanoids (phenols)	Resveratrol	*Vaccinium cyanococcus*	Weight loss	[9]
Alkaloids	Hyoscyamine	*Datura stramonium*	Treat stomach and bladder problems	[10]
	Atropine	*Atropa belladonna*	Treatment of bradycardia	[10]
	Codeine	*Papaver somniferum*	Narcotic analgesics	[10]
	Morphine	*Papaver somniferum*	Treat moderate to severe pain	[10]
	Vincristine	*Catharanthus roseus*	Chemotherapy drug	[10]
	Vinblastine	*Catharanthus roseus*	Chemotherapy drug	[10]
Polyketides	Glucoraphanin	*Brassica oleracea var. italica*	Reduction in the risk of carcinogenesis and heart disease	[11]

The existence of several species has been challenged by the extensive and indiscriminate collection of plant materials that produce significant bioactive chemicals. Tissue culture techniques enable rapid multiplication and scaling up of true-to-type plants with minimal dependence on the environment [16, 17]. The need for natural products that are safe is increasing among consumers as synthetic chemicals are seen as possibly harmful. At the same time, industry and research are becoming more interested in plant secondary metabolites [18]. However, certain compounds such as alkaloids, are difficult to synthesize artificially and the cost of synthesis is high and not feasible commercially. Chemosynthesis can only be used to produce a small number of significant plant products having simple chemical structure [19]. Although some compounds can be derived from plants via conventional strategies, there are occasionally geographical and environmental limits that can hinder the marketable production [20]. When equated to the mining of secondary metabolites from *ex vitro* plant populations by traditional approaches, tissue culture techniques offer a dependable and feasible method for doing so quickly and effectively [21]. Furthermore, the ease with which the metabolites may be extracted from *in vitro*-plants make the approach suitable for commercial application [22]. Aside from the benefits outlined above, there are certain metabolites that are not available in the *in vivo* plant but can be extracted from *in vitro* cells [23]. The use of conventional or biotechnological techniques to stimulate the

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aggregation of desirable compounds from *in vitro* cultures is made possible by advances in biotechnology [24].

II. TYPES OF CULTURE SYSTEMS

In vitro raised cultures characteristically produce secondary metabolites in a two-step method that requires independent optimization of the biomass accretion and secondary metabolite synthesis [25]. Friable and compact calli, cell suspension culture, or ordered explants like shoot tips, adventitious roots, or somatic embryos could all be used for production [26] (Figure 1). In some circumstances, a specific level of differentiation might be required for the occurrence of the biosynthesis. Whenever the target metabolite is exclusively produced in specific glands, such as essential oils, the usage of differentiated cell cultures is necessary [27].

Hairy root culture presents fresh prospects for the *in vitro* generation of valuable phytochemicals among differentiated tissues [28]. *Agrobacterium rhizogenes*, a gram-negative bacterium, infects plant and causes hairy roots. A T-DNA fragment from the root-inducing (Ri) plasmid is transferred into the genome of the plant during infection. Some benefits of hairy roots include amplified levels of cellular differentiation, quick growth, biochemical and genetic stability, and high maintenance capability [29]. Additionally, they can build up metabolites in the plant's aerial portions [30]. However, its economic usage to create treasured plant secondary metabolites is restricted due to the challenges of growing hairy roots in any industrial location [31].

A basic and economical technique called cell suspension culture has been widely applied to solve the issues associated with industrial-scale production. Since plant cells are biosynthetically totipotent and have the potentiality create compounds that are indistinguishable to the ones found in the parent plant under the right circumstances [32, 33]. For the ongoing production of plant secondary metabolites with consistent worth and yield, they are regarded as a stable system. The ability to create unique compounds that are not typically produced by native plants is yet another excellent benefit of plant cell cultures [34].

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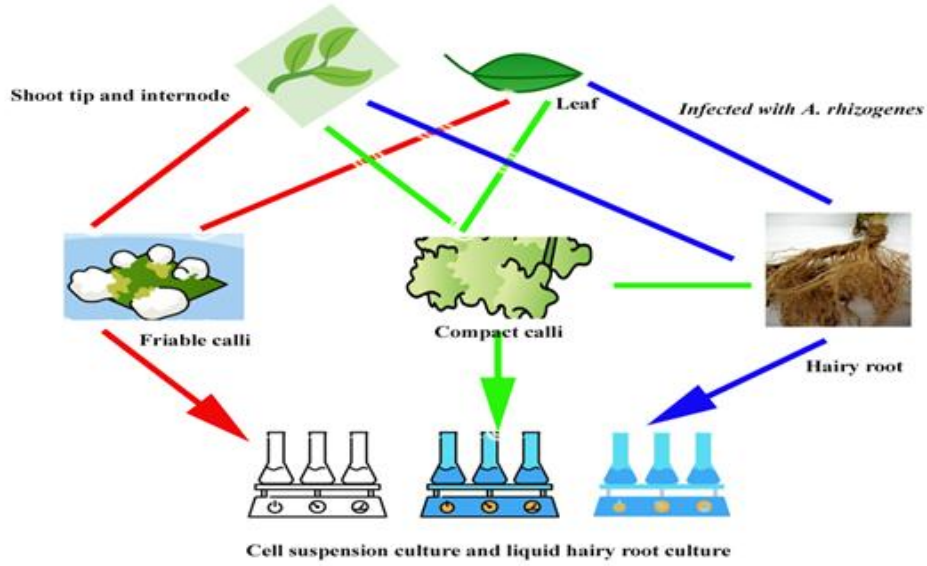

Figure 1: Flowchart Depicting the Different Types of Culture Systems

III. STRATEGIES FOR UPSCALING OF SECONDARY METABOLITES

High yields and reliable outputs are essential for the commercial usage of cell culture for the synthesis of treasured secondary metabolites. Since the production of secondary metabolites in plants is dependent on genotype, choosing the parent plant with the highest concentration of the product of interest for callus induction, as well as choosing high-producing cell/organ lines, is regarded as the initial step in creation of cell or organ cultures [35, 36]. The choice is determined by examining cell/organ growth, and the desired product is then quantified using chromatographic and spectroscopical methods [37]. Even when choosing a line that is extremely prolific, the production yields are occasionally insufficient, and after extensive cultivation, they mislay their production efficacy. Thus, a variety of alternative methods, including conventional and biotechnological methods, can be utilized to increase the smooth production of secondary metabolites and attain effective yield [38, 39].

1. **Traditional strategies:** The growth and metabolite efficiency of *in vitro* cultures can be increased by optimizing a number of variables. The following can be chosen from them: the composition of culture medium, pH, inoculum density, environment of the culture media (such as temperature, light, density and quality), and the agitation speed and aeration [40]. The selection of a suitable culture medium formulation is a crucial step since the culture medium substantially influences the productivity of the biomass and metabolites [41]. It must be chosen in accordance with the functional necessities of the plant species, and there are a number of characteristics that can be tuned, including the type and dosage of plant growth regulators, salt strength, nitrate and phosphate levels, nutrient composition, and carbon supply. For instance, via controlling gene expression and developmental processes, the carbon source has a substantial impact on the signal transduction systems [42, 43].

   Secondary metabolites are produced in plants when exposed to environmental cues or as defences against pests. In this respect, elicitation, a technique for ramping the

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production of secondary metabolites, tries to deceive the cells or tissues into believing they are under biotic or abiotic attack by using substances that set off the body's defence mechanisms [44]. Since they cause the overexpression of genes, elicitors possess the capacity to regulate a variety of cellular functions at the biochemical and molecular level [45]. The elicitors can be of biotic or abiotic origin and can include signalling chemicals such as salicylic acid, methyl jasmonate, microbial cell wall exudates (for example, yeast extract, chitosan), inorganic salts, heavy metals, and physical agents (for example, UV radiation) (Figure 2)[46]. A particle of matter with a diameter of one to one hundred nanometres (nm) is commonly referred to as a nanoparticle [47]. A variety of NPs have been utilized recently to boost secondary metabolites in unique and efficient ways. The most often used types of "nano-elicitors" are carbon, gold, silver, copper, zinc oxide, and titanium dioxide nanoparticles (NPs) [48]. The preliminary rejoinders of plants to NPs may involve calcium ion (Ca^{2+}) and Ca^{2+} flux movements, as well as ROS production by oxidative spurt as important second messengers that regulate the transcriptional echelons of principal regulators of plant secondary metabolite biosynthesis. This is a characteristic seen in many abiotic elicitors [49]. The most popular culture system considered for elicitation treatment and the synthesis of secondary metabolites is cell suspension culture. Hairy root culture has already proven to be a useful culture system for elicitation investigations due to its characteristic traits of hormone less autotrophy, unrestrained growth, biosynthesis, and genetic stability [50]. A less common culture system for producing secondary metabolites is multiple shoots culture, which is especially beneficial when it comes to metabolites found in leaves [51]. Both quantitatively and qualitatively, the elicitors can alter the generation of secondary metabolites [52].

Nutrient and precursor feeding are employed to boost secondary metabolite outputs. Precursor feeding uses cell cultures to transform native precursors into products by leveraging already-existing enzyme system, while nutrient feeding includes replenishing the nutritional media [53, 54]. Another method for addressing the issues of poor shear resistance and cell aggregation is to immobilize plant cells. The most popular techniques for performing this treatment are namely surface immobilization and gel entrapment. In this method, the cells are contained in one gel or a group of gels. The most common matrix is calcium alginate, which is also known as agarose, gelatin, carrageenan, or polyacrylamide [55, 56]. The high cell density inside the small bioreactors reduces costs and lowers the jeopardy of contamination, increases product accretion, and minimizes fluid viscosity. This approach also simplifies downstream processing and extends the viability of cells trapped in the stationary stage [57].

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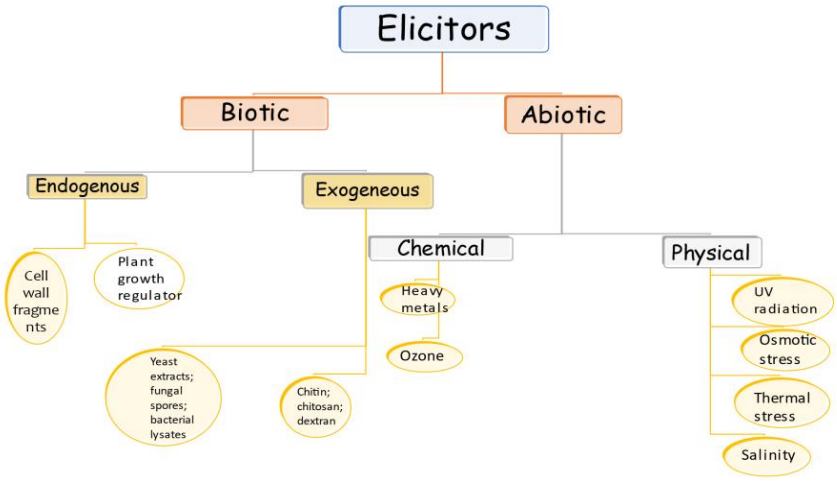

Figure 2: Schematic Diagram Representing the Types of Elicitors

In order to enable the elimination of secondary metabolites from plant cell vacuoles and membrane structures easily, electric and magnetic field stress, ultrasound techniques, and other techniques are used. This facilitates the discharge of products in the culture medium, which streamlines the purification procedure [58].

2. **Metabolic engineering:** Metabolic engineering involves changing endogenous pathways to direct greater flux toward specific desirable molecules or rerouting one or more enzyme reactions to either synthesize new compounds or facilitate the breakdown of existing ones [59] (Figure 3). By altering biosynthetic pathways through investigations of gene over expression, metabolic engineering provides a fresh viewpoint on how genes involved in the manufacture of secondary metabolites express themselves [60]. This involves manipulating the genes that encrypt the crucial and rate-limiting enzymes present in the biosynthetic pathways as well as studying enzymatic reactions as well as biosynthetic processes at the genetic, transcriptomic, and proteomic levels [61]. The metabolic engineering method also makes advantage of the suppression of rival pathways to boost the metabolic flux of specific intermediates in the biosynthetic pathway for greater production. The accumulation of early intermediates can be induced by inhibiting specific metabolic stages [62].

Whenever the phenylpropanoid pathway is expressed in yeast, flavonoids are created. By successfully cloning genes from several plant and microbial species, flavonoid compounds of many different types can be synthesized in yeast. Through enhanced expression of the genes for cinnamate-4-hydroxylase (C4H), phenyl ammonia lyase (PAL), 4-coumarate-CoA (4CL), and chalcone synthase (CHS), flavanone has been successfully synthesized in yeast. By expressing the genes for flavone synthase, I (FSI) and flavone synthase II (FSII), flavones have also been generated in flavanone-producing recombinant yeast [63, 64]. Methylerythritol 4-phosphate pathway and mevalonic acid pathway (MVA) and are two completely distinct enzymatic processes that are used in the production of terpenes in higher plant cells. Ergosterol is the main end product of the production of ergosterol in yeast, and only the MVA route is involved [65]. The

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noncarotenogenic yeast *Schizosaccharomyces pombe* can't make any carotenoids, however it can make ergosterol from FPP from sterol biosynthetic pathway. Finally, the heterological expression of the carotenoid biosynthetic gene in a noncarotenogenic yeast, *S. pombe*, the geranyl geranyl pyrophosphate synthase gene from the bell pepper (*C. annum*) successfully readdressed carbon flow from the terpenoid pathway that forms ergosterol and finally the production of carotenoid [66]. *S. cerevisiae* effectively synthesized cathenamine from tryptamine and secologanin by functional expression of strictosidine synthase and strictosidine glucosidase genes from *C. roseus* [67].

Future research on the uses of various yeast species for the effective microbiological production of such substances should be done in conjunction with the enhanced understanding of the biosynthetic routes of numerous plant secondary metabolites. Through metabolic engineering, rate-limiting stages can be bypassed, flux can be reduced through competitive pathways, catabolism can be decreased, and regulatory genes can be overexprssed, among other tactics [68].

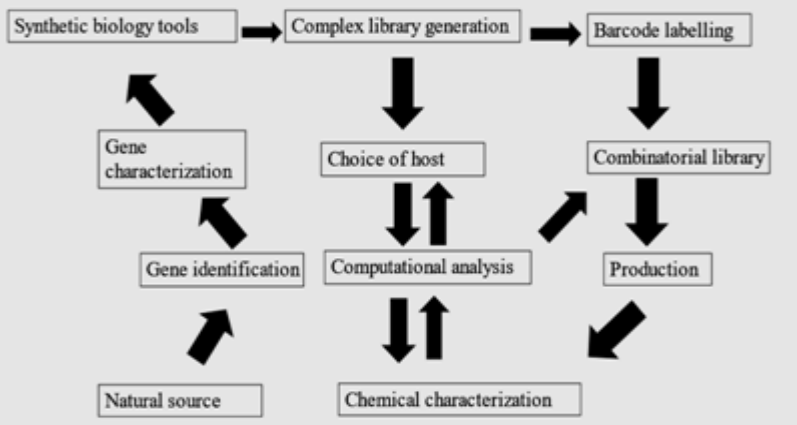

Figure 3: Schematic description of the metabolic engineering

3. **Bioreactors: A rapid approach for secondary metabolite production:** Mechanical vessels known as "bioreactors" are used to culture organisms or tissues in liquid nutrition medium in a regulated environment (Figure 4). A bioreactor is a defined as a system where a biological conversion takes place. Any conversion that involves enzymes, microbes, or cells from plants or animals falls under this classification [69]. In contrast to typical chemical reactors, bioreactors support and regulate living things. Since organisms are subtle and unstable when compared to chemicals, bioreactor systems must be built to give a better leverage of control over process and lesser contaminations [70]. A large portion of antibiotics and other pharmaceuticals have been produced using bioreactors and bacterial fermentation. The creation of penicillin during the World War II marked the beginning of antibiotics being produced on a massive scale [71].

A bioreactor can operate in batch, fed-batch, continuous perfusion, chemostat or by combination of these modes. In a batch system, the required nutrients are given to the culture at the outset [72]. The fed-batch is initiated at a modest volume, and the culture is then given a concentrated provender solution filled up to its maximum volume without having the medium withdrawn [73]. In a chemostat, used medium and cells are concurrently removed while fresh medium is continuously provided to the culture [74].

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Fresh medium is provided in perfusion culture at the same rate as used medium is removed [75]. Reactors used to upscale plant secondary metabolites can be broadly categorized as liquid-phase and gas-phase, based on the continuous phase [76]. The majority of large-scale biosynthetic progressions based on cell suspension cultures as well as hairy root cultures are carried out in stirred tank reactor, at various volumes, and according to their precise engineering specifications, including heat and mass transmission using impeller or turbine blades.

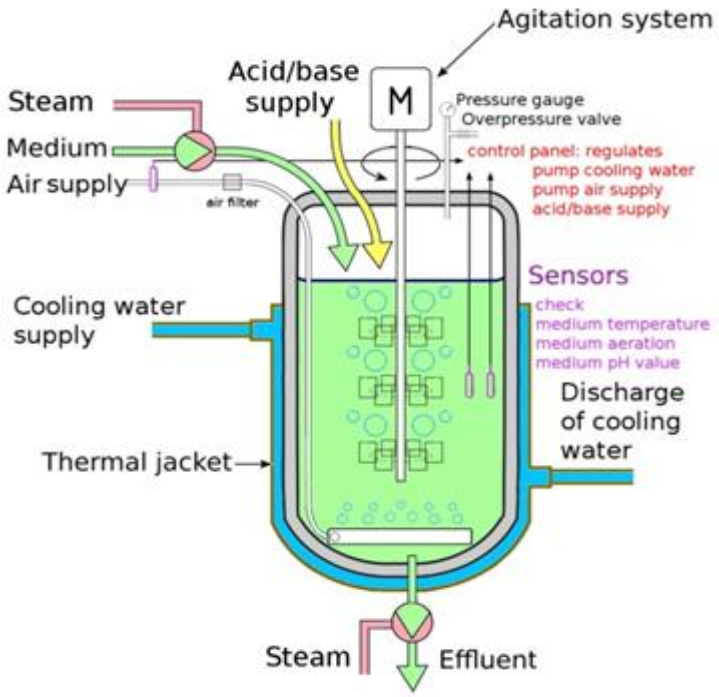

Figure 4: Basic Structure of A Bioreactor (Adapted from [])

Stirred Tank Reactor (STR) are typically more suitable for the development of plant cells than animal cells [77]. In Ahrensburg, Germany's largest plant cell growth facility, which includes a stirred tank reactor battery with a capacity of up to 75,000 l, serves as evidence [78]. Air-Lift Reactor (ALR) often demand less power for a given performance than STR. ALR is beneficial where there is a need for mild agitation and inexpensive oxygen transfer, as well as with fluids that are substantially less viscous [79]. A column-shaped reactor called a bubble column reactor (BCR) has its cells immersed in the liquid. The up flow of air bubbles produced by air purveyor at the column base is used to mix the liquid [80]. For the mass proliferation of numerous plant species, a novel type flood reactor system (periodic immersion arrangement) was designed. In this kind of bioreactor, the plant material was held in place by a supporting net in order to prevent the explants from completely submerging in the liquid media [81]. A stirred tank, peristaltic pump, and tubular culture chamber together form the Convective Flow Reactor (CFR). A displacement pump positively recirculated the liquid between the tubular reactor and stirred tank, while the medium in the stirred tank was oxygenated. Even if CFR performed better than a BCR, it might not be a practical large-scale [82]. Turbine Blade Reactor is a hybrid of ALR and STR. Air is given from the bottom chamber and disseminated by an eight-blade impeller that further stirs the medium, and the cultivation

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area is disconnected from the agitation space with the help of a stainless-steel mesh, so that the hairy roots/plant cells are not in connection with impeller [83].

Nutrient Mist Reactors (NMRs) are a type of gas-phase reactors where the liquid medium is supplied into the bioreactor in a mist stage by the usage of ultrasonic transducers which produces very small droplets of a few micrometres (0.5-30.0 µm). Plant cell culture is dispersed in air phase on a mesh supported by immobilization [84]. For hairy root cultures, NMR offers definite benefits including simple operation, minimal shear stress, which allows for rapid nutrient replenishment and toxic metabolite elimination, as well as simplicity of scaling up [85].

"Disposable bioreactors" are one time-use, sterile plastic bags which are often combined by swinging them back and forth while being inoculated and aerated through plastic vents. Disposable cultivation containers are typically made of biocompatible plastics that have been authorized by the FDA (Food and Drug Administration) (for example, polyethylene, polystyrene, polyetrafluorethylene, and polypropylene) [86]. There are various reports outlining the appropriateness of disposable bioreactors for the culture of plant cells and hairy roots. Disposable bioreactors are mostly employed for cell expansions, glycoprotein exudations, and cell lines [87].

Due to the significance of certain plant secondary metabolites, studies have been conducted to determine whether or not their production is feasible on an industrial scale. Due to plant cells relatively unpredictable productivity, sophisticated shear sensitivity, moderate growth rate, and lower oxygen requirements, this process is not always straightforward [88]. The scale-up entails the usage of bioreactors of various sizes and features. Among these advantages are the ease, likelihood, and high effectiveness with which the metabolites may be separated from biomass media [89]. A list of scaling up of some of the secondary metabolites is summarized below in Table 2.

Table 2: List of some Secondary Metabolites and their Enhancement

Compound	Source	Culture system	Elicitor/ Precursor	Enhancement	Reference
camptothecin	*Nothapodytes foetida*	Cell culture	gamma rays	-2-fold	[90]
	Ophiorrhiza alata	Hairy root	polystyrene resin (Diaion HP-20)	-7-fold	[91]
podophyllotoxin	*Linum album*	Cell culture	10 µM salicylic acid	-3-fold	[92]
taxanes	*Corylus avellana*	Cell culture	silver nanoparticles (5 ppm)	-3.7-fold	[93]
vinblastine	*Catharanthus roseus*	Hairy root	0.1-mM silver nitroprusside	-2-fold	[94]
vincristine	*Catharanthus roseus*	Hairy root	0.1-mM silver	-2-fold	[95]

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			nitroprusside		
rosmarinic acid	*Coleus blumei*	Hairy root	20 µM methyl jasmonate	-2.8-fold	[96]
rosmarinic acid	*Coleus forshohlii*	Hairy root	0.1-mM salicylic acid	-3.4-fold	[97]
rosmarinic acid	*Lavandula officinalis*	Cell culture	1mM jasmonic acid	-1-fold	[98]
rosmarinic acid	*Lavandula vera*	Cell culture	50 µM methyl jasmonate	-2.4-fold	[99]
Withanolides	*Withania somnifera*	Cell culture (inbioreactor)	NA	-5.7-fold	[100]

Shikonin and ginsenoside production are two significant turning points in the production of secondary metabolites from cell cultures/hairy roots, and the promising successful instance of the scale-up process is the production of taxol by Phyton Biotech Company (Germany) to meet a portion of the demands of Bristol-Meyers Squibb Company in 2002 [101]. The largest cGMP plant cell culture facility in the world is run by Phyton Biotech and features 75,000 L-size bioreactors that can produce up to 880,000 L of taxanes annually [102].

IV. CONCLUSION AND FUTURE SCOPE

Techniques for cultivating plant cells and tissues are appealing for producing a variety of secondary metabolites, such as significant alkaloids with anticancer activities. Despite the significant advancements made in this field over the past few decades, production in some instances is at very low yields, there are numerous challenges faced when scaling up the production, and only modest marketable success is attained. The ability to increase production yields was constrained by incomplete knowledge of the biosynthetic processes that produce bioactive compounds. Engineering the biosynthetic pathway(s) of the metabolites in plant cells has emerged as a potential alternative which needs to be used to increase production efficiency. New elicitors and permeabilizing substances like cyclodextrins or coronatin are also promising. Metabolic engineering and biotechnological technologies may be utilized in the future to solve the scarcity of physiologically active, financially useful, and medicinally significant plant secondary metabolite molecules. Building on advancements in plant science, *in vitro* plant cell culture has made enormous achievements in the manufacture of chemicals and medications. The basis of the creation of products with commercially acceptable standards of quality will be provided by the expanded use of genetic systems and a developing understanding of the structure and regulation of secondary metabolism pathways. An enhanced knowledge of secondary metabolite pathways in economically significant plants may be the cause of the recent rise in the usage of plant cell culture methods. New methods for the affordable, commercial cultivation of rare or critically endangered plants, their cells, and the compounds they will produce may be made possible by advancements in plant cell cultures. Understanding the plant biochemical pathways that result in the synthesis of secondary metabolites and ultimately figuring out

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how to modify those processes depend on the integration of omics skills, including genomics, transcriptomics, proteomics, and metabolomics. Importantly, the analysis of gene-to-metabolite systems for secondary metabolite synthesis in plants at regulatory and at catalytic level necessitates the use of transcriptome and metabolome data. In order to identify potential gene candidates connected to their production, this would be helpful in revealing the tight association between genes and their targeted molecules. In the last few years, significant advancements have already been made in the secondary metabolite generation from plant cell cultures. The incessant efficacy of plants as renewable sources of chemicals, notably therapeutic compounds, will be protracted and boosted by these new tools. Continued and increased efforts in this area will result in the successful biotechnological production of particular, beneficial, and as of yet unidentified plant compounds.

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