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Review

Biotechnological techniques in the production of plant-made pharmaceuticals

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Abstract

Plants produce various secondary metabolites, many of which have complex structures. Wild and cultivated plants remain the primary sources of most pharmaceutically significant secondary metabolites. However, the production of these compounds in plants is limited by the capacity of their biosynthetic pathways. This limitation has prompted efforts to develop methods to increase the yield of desired secondary metabolites. Advances in "omic" techniques have accelerated the development of approaches that utilize plants as bio-factories to produce these valuable compounds. This review provides an overview of strategies for producing high-value plant products and using plants as heterologous hosts to produce foreign recombinant proteins for medical applications.

Keywords: Elicitation, Metabolic engineering, Plant-based drugs.

1. Introduction

Plants are rich sources of secondary metabolites, which play an important role in plant defense processes. Secondary metabolites formed from the primary metabolites of plants do not directly contribute to plant growth, development, or reproduction[1]. Although they are not essential for the basal functions in plants, they can be beneficial in altering life conditions. The biosynthesis of secondary metabolites is often restricted to specially differentiated cells that possess a set of enzymes involved in their formation. The large structural variability of plant secondary metabolites allows plants to efficiently eliminate the impact of a wide variety of environmental stress stimuli[2]. The effects of plant secondary metabolites are not only related to the plant organism. They exhibit remarkable biological activities in microorganisms, animals, and humans. Therefore, plant secondary metabolites can be used for therapeutic purposes, including food additives, dietary supplements, and ingredients of cosmetics[3]. The

limited production capacity of desired plant secondary metabolites in medicinal plants has prompted efforts to find novel approaches for increasing their yields.

Plant biotechnology involves various techniques that use tissue culture and genetic engineering to develop genetically modified plants with new or enhanced desirable traits. These traits include an increased production of beneficial secondary metabolites, which facilitate plant resistance to diseases, pests, and environmental challenges. Plant biotechnology enables the production of valuable proteins in plants using genes from viruses, microbes, animals, or humans. The present review article focuses on plant biotechnological approaches and summarizes information regarding their application in pharmaceutical contexts.

2. Plant biotechnological approaches for pharmaceutical applications

2.1 Elicitation techniques

Plants synthesize an extensive range of secondary metabolites, often with highly complex structures. Wild and cultivated plants continue to be the primary sources of most pharmaceutically important secondary metabolites due to the economically unfeasible nature of chemical synthesis. Plant *in vitro* cultures represent an attractive alternative in plant secondary metabolite production, as does the cultivation of large-scale plant cell cultures in bioreactors. However, these approaches are constrained by economic factors and the production capacity of valuable natural compounds in plant tissue cultures. Advances in tissue culture techniques combined with improvements in the genetic engineering of pharmaceuticals, nutraceuticals, and other beneficial substances could eliminate the mentioned limitations[4-6].

Application of several elicitors in growth media of plant tissue cultures modifies natural production ability in quantitative and qualitative senses. A broad array of abiotic and biotic elicitors including heavy metals, physical factors, inorganic salts, yeast extract, hydrolysate from the cell wall of phytopathogenic fungi, and signal molecules have been tested on tissue cultures of various medicinal plants. The advantage of elicitors consists of their ability to induce defense processes and fully utilize the naturalbiosynthetic capacity (including enzymes and metabolic precursors) of plants in the production of precious compounds. Cultivation of plant *in vitro* systems in bioreactors represents a valuable tool in the production of secondary metabolites under controlled conditions making it possible to combine diverse biotechnological strategies[6-9].

Paclitaxel is a bioactive compound derived from *Taxus* spp., and due to its proven anticancer activity, it is utilized in the treatment of various types of cancer. For sustainable commercial production of paclitaxel in *Taxus* cell cultures, the large-scale bioreactor technology has been designed. The development of an effective paclitaxel production system based on cell suspension cultures has included different strategies such as elicitation, immobilization, as well as genetic manipulations of targeted genes involved in the regulation of paclitaxel biosynthesis[10-13]. Although bioreactor cultivation technology **(figure 1)** resulted in the commercial production of paclitaxel, the elicitation technique remains in researchers interest as they seek more efficient strategies for paclitaxel production.

Exogenous treatment of *Taxus baccata* plant cell cultures by the elicitor methyl jasmonate (MeJA) and ethylene inhibitor silver thiosulfate has resulted in increased paclitaxel production[14]. Another strategy focused on paclitaxel production improvement involves the elicitation of taxadiene synthase transgene-carrying *Taxus baccata* hairy roots cultures with MeJA[11] or a combination of MeJA and gas-carrying compounds like perfluorodecalin[15]. Taura et al., published a strategy for the production of paclitaxel in the bioreactor with foam separation. This technological improvement avoids a paclitaxel´s feedback inhibition on the growth of *Taxus cuspidata* cell cultures and enhances the production five times compared to the control[16].

Another high-valued plant secondary metabolite, artemisinin, is produced in *Artemisia annua.* Its significant antimalarial activity makes artemisinin the key treatment for uncomplicated malaria. Due to the complex chemical synthesis, the maternal plant *Artemisia annua* remains the main source of this compound. Based on this fact, some biotechnological approaches have been developed to enhance artemisinin production. One promising method for artemisinin mass production is hairy root cultivation in bioreactors supported by the application of elicitors[17]. As an alternative to the artemisinin production in the maternal plant, genes involved in its biosynthesis have been inserted in various heterologous hosts such as *Nicotiana tabacum*[18]*, Nicotiana benthamiana*[19]*, Physcomitrella patens*[20]*,* as well as microorganisms *Escherichia coli and Saccharomyces cerevisiae*[21] leading to artemisinin production in variable quantities. The introduction of bioreactor methodologies seems to be a suitable way for the efficient production of natural compounds at the commercial level. Besides paclitaxel and artemisinin production in bioreactors, the production of other valuable plant secondary metabolites namely cichoric acid[22], ginsenosides, as well as tanshinones[23] have been successfully scaled up in corresponding plant cell and organ

cultures supported by elicitation. Currently, the elicitation technique is still an attractive method for the reinforcement of the production capacity, especially in the *in vitro* cultures of medicinal plants.

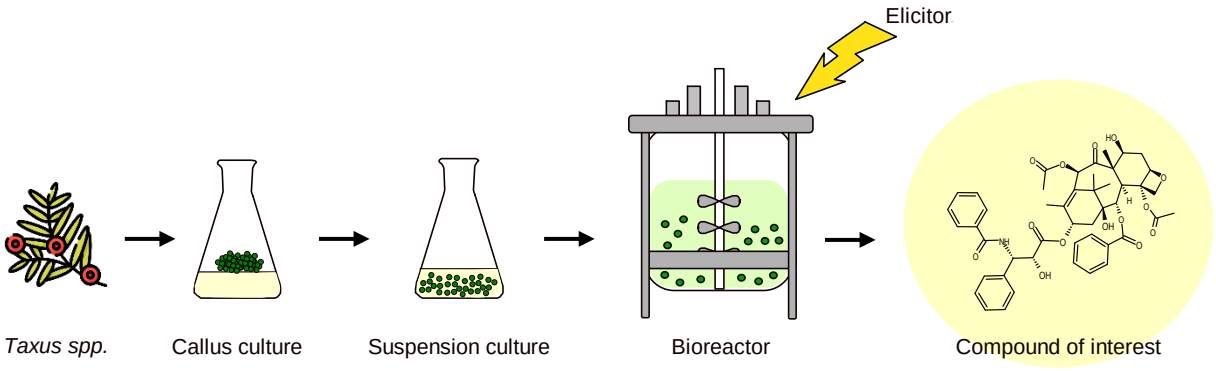

Figure 1: General scheme of key steps for in vitro plant cell cultivation to produce the desired compounds

2.2 Metabolic engineering of plant secondary metabolites

Plant metabolic engineering is based on the modification of existing metabolic pathways allowing either extensive production of the desired metabolite or production of metabolites not naturally present in the plant or cell cultures. Enhanced production of natural compounds through metabolic engineering relies on the discovery, isolation, and purification of enzymes within specific pathways, as well as the identification of their corresponding genes[24-26]. This strategy has some limitations resulting from either the resistance of medicinal plants toward genetic modifications or the inability of cell cultures to produce a particular compound due to the compartmentalization of its biosynthetic pathway. For illustration, dedifferentiated opium poppy cell cultures do not produce opium alkaloids because enzymes that participate in their biosynthesis are localized in sieve elements of an intact plant[27]. Elicitation of opium poppy cell cultures leads to increased formation of sanguinarine – benzo[c]phenanthridine alkaloid that partially shares a biosynthetic pathway with morphinans[9,28]. Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR/Cas9 system represent prominent genome editing tools mediating site-specific gene modifications requiring endogenous DNA repair mechanisms[29,30]. While ZFNs and TALENs belong to the first generation of gene editing tools, CRISPR/Cas9 represents a revolutionary approach that greatly accelerates precise and efficient genome editing[31,32]. CRISPR/Cas9 is a prominent genome-editing tool that stands out among other techniques due to its efficiency, precision, and versatility.

The preparation of knockout mutant plants using CRISPR/Cas9 genome-editing tools enhances traits such as disease resistance and adaptability to abiotic stress[33]. Production of specific secondary metabolites in medicinal plants can be increased/decreased by CRISPR/Cas9 technology. This tool has been successfully used to selectively alter the production of phenolic compounds, flavonoids, alkaloids, terpenes, carotenoids, and oils in parental plants[34]. In *Salvia miltiorrhiza*, targeted *SmCPS1* gene to manipulate tanshinone biosynthesis demonstrating the gene editing to elucidate metabolic pathways[35]. Similarly, in *Dioscorea zingiberensis* target gene editing aims to increase diosgenine content and in *Papaver somniferum* a gene *4´-OMT2* in alkaloid biosynthesis was knocked-out[36].

In addition to metabolic and genetic engineering, the gene transfer and reconstruction of the desired biosynthetic pathway in heterologous host organisms is a promising way to diversify sources of plant secondary metabolites. The advantage of heterologous host systems lies in their amenability to genetic modifications and stable expression potential[37]. Different heterologous hosts have been used for the production of a wide variety of plant metabolites and proteins[38]. For instance, *Escherichia coli* (*E. coli*) has been used as a host organism for the total biosynthesis of thebaine – an opiate alkaloid obtained from opium poppy straw. Modifying the *E. coli* genome by two additional genes encoding enzymes thebaine-6-*O*-demethylase and morphinone reductase allowed the production of hydrocodone in thebaine producer[39].

The *Nicotiana benthamiana* transient expression system is the preferred platform for functionally characterizing non-native plant enzymes and reconstituting multistep pathways for complex plant secondary metabolites[40]. Recently, *Nicotiana* technology was used for strictosidine production, the last common intermediate in the biosynthesis of monoterpene indole alkaloids[41]. On the other hand, a variety of recombinant proteins have been produced in plants, leading to a partial shift in the production of protein-based pharmaceuticals from bacterial, fungal, and mammalian cell cultures to plants and plant cell cultures. Human-type I collagen, which self-assembles into fine, homogeneous fibrils, is produced in tobacco plants and has been utilized in tissue engineering and regenerative medicine[42]. Bovine trypsin, sold under the name TrypZean (Sigma-Aldrich), is derived from maize. This product is particularly valuable in animal cell cultures because it is free from contaminants of animal origin. Additionally, rice has been used to manufacture human lysozyme and lactoferrin. Taliglucerase alpha is a recombinant protein used in enzyme replacement therapy for Gaucher's disease. It is the first plant cell-based recombinant therapeutic protein

produced by Protalix company and approved by the FDA[43]. **Table 1** summarizes various bioactive compounds produced in plants and other heterologous organisms using biotechnological tools.

Bioactive compound	Biotechnological techniques	Plant/heterologous host
Ajmalicine	Elicitation	*Catharanthus roseus*[44]
Artemisinin	Heterologous expression	*Nicotiana tabacum,*[18] *Nicotiana benthamiana,*[19] *Physcomitrella patens,*[20] *E. coli, S. cerevisiae*[21]
Cichoric acid	Elicitation	*Echinacea purpurea*[22]
Ginsenosides	Organ and cell cultures	*Panax ginseng, Panax quinquefolium*[23]
Paclitaxel	Metabolic engineering Heterologous expression Elicitation	*Taxus mairei* *E. coli, S. cerevisiae* *Taxus baccata, Taxus x*[45]
Podophyllotoxin	Heterologous expression Elicitation Metabolic engineering	*Linum album* *Linum album* *Podophyllum hexandrum* Royle[45]
Sanguinarine	Elicitation	*Eschscholzia californica*[46]
Strictosidine	Heterologous expression	*Nicotiana benthamiana*[41]
Tanshinones	Metabolic engineering Elicitation	*Salvia miltiorrhiza*[23,47]
Taxadiene	Heterologous expression	*E.coli, S. cerevisiae, Artemisia annua*, *Panax ginseng*, *Nicotiana benthamiana*[45]
Thebaine	Heterologous expression	*E. colli*[39]
Bovine trypsin (TrypZean)	Heterologous expression	*Zea mays*[43]
Human type I collagen	Heterologous expression	*Nicotiana spp.*[42]
Taliglucerase alpha	Heterologous expression	*Daucus carota*[43,48]

Table 1: Enhanced production of some bioactive compounds using different techniques

The use of biotechnological tools significantly influences various aspects of human life. The most notable impact has been seen in agriculture and food production, where significant cost reductions have resulted from the adoption of genetically modified crops with high yields. Utilization of plant-based biotechnological approaches has transformed drug preparation in the pharmaceutical industry. Plant-based production of bioactive compounds including vaccines and therapeutic proteins enables sustained and cost-effective production regarding primary investment and profit. The global plant-based biologics market size was $116 million in the year 2021 and is predicted to achieve $182 million in the year 2031[49].

2.3 Plants-derived vaccines

As genetic engineering technologies advance, plants are increasingly being used as bio-factories to produce antibodies and foreign antigens. Plants as bio-factories offer several advantages; they can be grown inexpensively on a large scale in greenhouses,

bioreactors, or open fields. Additionally, plants can express complex antigens while eliminating the risk of carrying human pathogens or endotoxins, which are potential contaminants in viral, bacterial, insect, or mammalian cell expression systems. Recently, the use of plants has become a more attractive and acceptable platform for vaccine production. Several plants, including tobacco, rice, maize, potato, alfalfa, lettuce, tomato, carrot, peanut, and soybean, serve as hosts for gene introduction. This process is achieved *in vitro* using protoplasts, cell cultures, or hairy root cultures[43]. Plant expression platforms provide several advantages for producing recombinant subunit vaccines. These platforms can be divided into three main categories:

1. Transient expression systems.
2. Stable expression in transgenic plants or cell cultures.
3. Stable expression in the plastid genome of transplastomic plants.

Vaccines against viruses have been successfully produced using all three platforms, each possessing specific advantages and limitations. Numerous attempts have been made to produce vaccines against SARS-CoV-2, HBV, HCV, Ebola, and Zika viruses in plant systems, with some currently in various phases of clinical trials[50].

Biotechnological approaches, mainly genome editing methods, allow the preparation of transgenic plants to produce plant bioactive compounds, nutraceuticals, or plant-based vaccines. The use of transgenic plants is associated with several risks that may have potential impacts on the environment, biosafety, and human health. Therefore, their responsible usage requires careful consideration of ethical, environmental, and regulatory issues in accordance with GMO regulation rules including EU legislation on GMOs and the Cartagena Protocol on Biosafety[51,52].

3. Conclusion

Plants have developed adaptive mechanisms to protect themselves from environmental stressors. Plant secondary metabolites play a crucial role in these defense strategies. The effect of plant secondary metabolites is not only related to the plant organism. They possess remarkable biological activities toward microorganisms, animals, and humans. *In vitro* techniques such as callus, hairy roots, and suspension culture cultivation in conjunction with elicitation, have been used to enhance the levels of pharmaceutically important secondary metabolites in plants.

Research challenges in plant-based biotechnology include a wide range of scientific issues that need to be addressed to fully realize the potential of this field. The utilization of biocompatible nanoparticles in combination with elicitation and metabolic

engineering to enhance plant secondary metabolism is an emerging area with significant implications for phytopharmaceuticals. Future research should focus on understanding the precise interactions between nanoparticles and plants and refine this method for commercial application.

CRISPR/Cas9-based genome editing has revolutionized plant genetic modification, allowing precise and targeted changes to plant genomes. This emerging technology enables the introduction of specific mutations improving plant traits. While advancements in genome editing tools have improved the ability to modify plant genomes, achieving precise editing without off-target effects remains a challenge.

The implementation of plant biotechnology in different areas of life requires the establishment of harmonized regulatory rules to ensure the safety of final products (both agricultural and pharmaceutical).

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