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# Recent Progress in Understanding of Medicinal Plants Biotechnology

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**A B S T R A C T**

Medicinal plants are the most important source of life saving drugs for the majority of the world's population. Plant secondary metabolites are economically important as drugs, fragrances, pigments, food additives and pesticides. The biotechnological tools are important to select, multiply, improve and analyze medicinal plants. In-vitro production of secondary metabolites in plant cell suspension cultures has been reported from various medicinal plants and bioreactors are the key step towards commercial production of secondary metabolites by plant biotechnology. Genetic transformation is a powerful tool for enhancing the productivity of novel secondary metabolites; especially by Agrobacterium tumefacians combinatorial biosynthesis is another approach in the generation of novel natural products and for the production of rare and expensive natural products. DNA profiling techniques like DNA microarrays serve as suitable high throughput tools for the simultaneous analysis of multiple genes and analysis of gene expression that becomes necessary for providing clues about regulatory mechanism, biochemical pathways and broader cellular functions.

**Keywords:** Medicinal plants, biotechnology, combinatorial biosynthesis, DNA microarray, transgenic plants

**A R T I C L E I N F O**

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## 1. Introduction

It is estimated that 70-80% of people worldwide rely chiefly on traditional, largely herbal, medicines to meet their primary healthcare needs. The global demand for herbal medicine is not only large, but growing [1, 2].

Various technologies have been adopted for enhancing bioactive molecules in medicinal plants [3].

Biotechnological tools are important for the multiplication and genetic enhancement of the medicinal plants by adopting techniques such as in vitro regeneration and genetic transformation [4]. It could also be harnessed for the production of secondary metabolites using plants as bioreactors. Advances in tissue culture, combined with improvement in genetic engineering techniques specifically transformation technology, have opened new avenues for high volume production of pharmaceuticals, nutraceuticals and other beneficial substances. Recent advances in the molecular biology, enzymology and fermentation technology of plant cell cultures suggest that these systems may become a viable source of important secondary metabolites. DNA manipulation is resulting in relatively large amounts of desired compounds produced by plants infected with an engineered virus, whereas transgenic plants can maintain constant levels of production of proteins without additional intervention. Large-scale use of plant tissue culture is found to be an attractive alternative approach to traditional methods of plantation as it offers a controlled supply of biochemical independent of plant availability [5, 6]. Impact of specific engineering-related factors on cell suspension cultures has also been details [6]. Current developments in tissue culture technology indicate that transcription factors are efficient new molecular tools for plant metabolic engineering to increase the production of valuable compounds [7]. The approach to combine genes from different microorganisms for the production of new and interesting metabolites is known as combinatorial biosynthesis, which has emerged as a new tool in the generation of novel natural products, as well as for the production of rare and expensive natural products.

Combinatorial biosynthesis has been utilized for important classes of natural products, including alkaloids (vinblastine, vincristine), terpenoids, (artemisinin, paclitaxel) and flavonoids. Conventional strategies for expression profiling such as northern blot, reverse northern blot, reverse transcriptase-polymerase chain reaction (RT-PCR), nuclease protection, and enzyme-linked immunosorbent assay (ELISA), western blot, in situ hybridization and immune histochemistry are optimized for single gene analysis. Although, it is possible to modify at least some of these techniques for multiplexing, the procedure becomes increasingly technically cumbersome. For genome wide expression analysis it is necessary to develop technologies having high degree of automation, since in any living organism thousands of genes and their products function in a complicated and orchestrated way. DNA microarrays have been developed in response to the need for a high-throughput, efficient and comprehensive strategy that can simultaneously measure all the genes, or a large defined subset encoded by a genome [8, 9].DNA microarrays are being used to study the transcriptional profile in various physiological and pathological conditions, leading to mining of novel genes and molecular markers for diagnosis, prediction or prognosis of those specific states [10]. This paper reviews the recent achievements and advances in the medicinal plant biotechnology.

## 2. Plant Tissue Culture

Plant tissue culture refers to growing and multiplication of cells, tissues and organs of plants on defined solid or liquid media under aseptic and controlled environment. Plant regeneration through somatic embryogenesis from stem, petiole and leaf explants of Indian chicory (*Cichorium intybus* L.) has been achieved [11, 12]. The commercial technology is primarily based on micropropagation, in which rapid proliferation is achieved from tiny stem cuttings, axillary buds and to a limited extent from somatic embryos, cell clumps in suspension cultures and bioreactors. The cultured cells and tissues can take several pathways. The pathways that lead to the production of true-to-type plants in large numbers are the preferred ones for commercial multiplication. The process of micro propagation is usually divided into several stages such as pre-propagation, initiation of explants, subculture of explants for proliferation, shooting and rooting and hardening. These stages are universally applicable in large-scale multiplication of plants. Micropropagation (in vitro propagation of axillary and/or adventitious buds as well as somatic embryos) is presently used as an advanced biotechnological system for the production of Identical pathogen-free plants for agriculture and forestry [13].

Protocols for the cloning of some medicinal plants such as *Catharanthus roseus* (Apocynaceae), *Chlorophytum borivilianum* (Liliaceae), *Datura metel* (Solanaceae) and *Bacopa monnieri* (Scrophulariaceae) have been developed [14]. Moreover, in vitro flowering, in vitro fruiting and effective micropropagation protocols were studied in *Withania somnifera*, an antitumor medicinal plant using axillary buds implants [15]. A rapid micropropagation protocol was developed for *Hoslundia opposite* using nodal explants derived from mature trees [16]. The integrated approaches of plant culture systems will provide the basis for the future development of novel, safe, effective, and high-quality products for consumers. Micropropagation by conventional techniques is typically a labour-intensive means of clonal propagation. Automation of micropropagation in bioreactors has been advanced by several authors as a possible way of reducing costs of micro propagation [17- 19]. Bioreactors containing liquid media are used for large-scale growth of various tissues. The use of bioreactor for the micropropagation was first reported in 1981 for Begonia [20]. Since then it has proved applicable to many species including shoot, bulbs, microtubers, corms and somatic embryos [21].Three main classes of culture systems in bioreactors can be distinguished: (1) those producing biomass (cells or organogenic or embryogenic propagules, shoots or roots as the final product); (2) those producing metabolites and enzymes; and (3) biotransformation of exogenously added metabolites (which may be precursors in the pathway). Less labour-intensive clonal propagation at lower cost through the use of modified air-lift, bubble column, bioreactors (a balloon-type bubble bioreactor) together with temporary immersion

systems for the propagation of shoots, bud-clusters and somatic embryos have been developed. The bioreactor system has been applied for embryogenic and organogenic cultures of several plant species [22, 23]. Some of the recently produced bioactive secondary metabolites through plant tissue culture are presented in Table 1.

## 3. Combinatorial Biosynthesis

The basic concept of combinatorial biosynthesis is to combine metabolic pathways in different organisms at the genetic level, where genes from different microorganisms are combined for the production of new and interesting plant secondary metabolites [34]. From pharmaceutical point of view, hydroxylations and glycosylations are considered to be particularly useful bioconversions [35, 36]. They can yield new drugs and existing drugs can be improved as to increased activity and decreased toxicity. Recent achievements with the polyketide biosynthesis from microorganisms, especially in Streptomyces, prove the potential of combinatorial biosynthesis. Podophyllotoxin and paclitaxel are clear examples of pharmaceuticals that can only be produced through the isolation from plants. With regard to the production of podophyllotoxin it has been shown that plant cell cultures of *Linum flavumcan* be used to convert deoxypodophyllotoxin, a major lignan of *Anthriscus sylvestris* into 6-methoxypodo-phyllotoxin. The combination of the product of one species and the enzymes of another species to yield a desired product forms a good example of combinatorial biosynthesis.

**Combinatorial Biosynthesis of Terpenoids**

Terpenoids represent a large and important class of natural products with more than 30,000 different structures. From pharmaceutical point of view the sesquiterpenoids are of high relevance. In this group artemisinin, gossypol and zingiberene are of great medicinal and economic interest. Terpenoids are biosynthesized via mevalonate (MVA) pathway or deoxyxylulose phosphate (DOXP) pathway. The MVA pathway has recently been expressed in E. coliharbouring the DOXP pathway, which led to an efficient production of the terpenoids, amorpha-4, 11-diene and taxadiene [38, 39].

**Artemisinin:** Artemisinin is an antimalarial drug isolated from *Artemisia annua* (Asteraceae). The selection of plants yielded varieties containing 0.5-1.16% of artemisinin in the aerial parts based on dry weight. Alternatives could be the production via transgenic plants or engineering the biosynthetic pathway into less complex host cells. This implies that the full elucidation of the biosynthetic pathway is required. Although several biosynthetic pathways have been postulated, until now only the genes encoding the enzymes for the synthesis of the first specific intermediate amorpha-4,11-diene by amorphadiene synthase55,56 and artemisinic acid by the cytochrome P450 enzyme, CYP71AV157 have been isolated and identified. The cDNA encoding amorphadiene synthase has been expressed in E. coli and characterized [40, 41].

**Paclitaxel:** Paclitaxel, mostly described by the tradename Taxol, is a diterpenoid that can be found in the bark and needles of different species of Taxus. The biosynthesis of paclitaxel starts with the cyclization step from geranylgeranyl diphosphate (GGDP) to taxadiene. Most of the 19 known enzymatic steps in the biosynthesis are related to hydroxylation and other oxygenation reactions of the taxadiene skeleton. Several genes from different Taxus species that are responsible for steps in the biosynthesis and builing a basis for today's combinatorial biosynthesis in a heterologous microorganism have been isolated and identified. Today, all the genes have been cloned into E. coli and activity screening confirmed the function of isolated enzymes.

**Combinatorial Biosynthesis of Alkaloids:** Combinatorial biosynthesis has been reported for the alkaloids, vincristine, vinblastine, ajmaline and morphine from plants and for rebeccamycin and staurosporine from *Streptomyces albus*. The morphine biosynthesis consists of 17 steps in *Papaver somniferum*. In the biosynthesis, a key intermediate, (S)-norcoclaurine is biosynthesized by condensation of dopamine and 4-hydroxyphenylacetaldehyde (4-HPAA). The catalyzing enzyme (S)-norcoclaurine synthase has recently been identified from *Thalictrum flavum* and cloned in E. coli86, 87. Further, key enzymatic steps towards (S)-reticuline include three NADPH oxidoreductases and cytochrome P450 and an acetyl-CoA dependent acetyltransferase. Recently, the last step reducing codeine to morphine by codeinone reductase has been elucidated and the gene expressed in E. coli and insect cells [42, 43].

**Genetic Transformation Technology and Production of Transgenic Plants:** Genetic transformation technology has been proved to be a powerful tool for the production of plants with desired traits in many crops97, 98. It promises to overcome some of the substantial agronomic and environmental problems that have not been solved using conventional plant breeding programmes [44].

**Agrobacterium and non-Agrobacterium Mediated Gene Transfer:** Plant transformation mediated by *Agrobacterium tumefaciens*, a soil plant pathogenic bacterium, has become the most commonly used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants. This soil bacterium possesses the natural ability to transform its host by delivering a well-defined DNA fragment, the transferred (T) DNA, of its tumour-inducing (Ti) plasmid into the host cell. The rapid progress in the area of crop biotechnology is mainly because of the development of efficient regeneration and suitable Agrobacterium-mediated transformation protocols for different crop species. Transformation systems based on *A.tumefaciens* are well established for Taxus (yew), Echinacea, Scrophularia (figwort), Digitalis (foxglove), Thalictrum (meadowrues) and Artemisia. An *A. tumefaciens*- mediated transformation system was developed for *Artemisia annua*.

**Direct Gene Transfer**

**Generation of Transgenic Medicinal Plants by Particle Bombardment:** Particle bombardment procedure was introduced in 1987, which involves the use of a modified shotgun to accelerate small (1-4μm) diameter metal particles into plant cells at a velocity sufficient to penetrate the cell wall. There is no intrinsic limitation to the potential of particle bombardment since DNA is governed entirely by physical parameters. Different types of plant materials have

been used as transformation targets including callus, cell suspension cultures and organized tissues such as immature embryos and meristems. Cell suspension cultures were established from leaf explants of gentian (*Gentiana triflora*, *G scabra* for generation of transgenic plants by particle bombardment. Efficient transformation of the tropane alkaloid-producing medicinal plant, *Hyoscyamus muticus*, was also achieved by particle bombardment [45]. An efficient and stable transformation has been achieved in garlic plants (*Alium sativum*). The results indicate that boilistic transformation can lead to the transfer, expression and stable integration of a DNA fragment into chromosomal DNA. The relative simplicity of this system is a good recommendation for its future use in the production of genetically modified plants.

**Generation of Transgenic Medicinal Plants by Chloroplast Transformation:** Stable transformation of the chloroplast by inserting foreign genes into the chloroplast genome - was first achieved in the single cell green alga, *Chlamydomonas reinhardtii* in 1988, soon to be followed by tobacco plant, and more recently, *Arabidopsis thaliana*. More than 40 transgenes have been stably integrated and expressed using the tobacco chloroplast genome to confer desired agronomic traits or express high levels of vaccine antigens and biopharmaceuticals. Leaf discs are bombarded with plasmid constructs containing a selectable antibiotic resistance marker physically linked to the gene of interest, flanked by DNA for inserting into the correct site of the chloroplast genome. The antibiotic resistance marker most frequently used is the aad A gene encoding resistance for spectinomycin and streptomycin, driven by the promoter of the chloroplast encoded 16S rRNA.

**DNA Microarray in Pharmacognosy:**

Use of an authentic herbal material is the first step of ensuring quality, safety and efficacy of herbal medicines. DNA polymorphism-based assays have been developed for the identification of herbal medicines. Recently, microarrays have been applied for the DNA sequence-based identification of medicinal plants. This includes quality control and standardization of the herbal drugs, identification and validation of new targets, the profiling of on-target and off-target effects during the optimization of new therapeutic agents, understanding molecular mechanisms of action, structure–activity relationships and the prediction of side-effects, and the discovery of diagnostic, prognostic, and pharmacodynamic biomarkers. Oligonucleotide probes specific for polymorphisms in the D2 and D3 regions of 26S rDNA gene of several Fritillaria species were designed and printed on the poly-lysine coated slides to prepare a DNA chip. Differentiation of the various Fritillaria species was accomplished based on hybridization of fluorescent labeled PCR products with the DNA chip. The results demonstrated the reliability of using DNA chips to identify different species of Fritillaria, and that the DNA chip technology can provide a rapid, high throughput tool for genotyping and plant species authentication [46].

**Expressed Sequence Tags (ESTs) of Medicinal Plants**

Monoterpene indole alkaloid (MIA) pathway genes were identified from random sequencing of C. roseus cDNA library which revealed 3655 unique ESTs, composed of 1142 clusters and 2513 singletons. Several novel MIA pathway candidate genes were identified by the cloning and functional characterization of loganic acid O-methyltransferase involved in secologanin biosynthesis. Biochemical pathways such as triterpene biosynthesis were also identified and its metabolite analysis revealed localization of oleanane-type triterpenes exclusively to the cuticular wax layer. The results illuminated biochemical specialization of Catharanthus leaf epidermis for the production of multiple classes of metabolites. Also, jasmonate-induced changes on the transcript and alkaloid profiles of tobacco BY-2 and *C. roseus* cell cultures have been monitored through the similar approach. ESTs corresponding to 40 enzymes involved in the conversion of sucrose to sanguinarine were identified from elicitor induced cell culture of *Papaver somniferum*. Substantial increase in the level of RNA was observed in case of elicited cell culture as compared to control and the identified metabolites were sanguinarine, dihydrosanguinarine, methoxylated derivatives dihydrochelirubine and chelirubine, and the alkaloid pathway intermediates N methylcoclaurine, N-methylstylopine, and protopine [47]. Similarly, cDNA library of *Artimisia annua* glandular trichome revealed the presence of many ESTs involved in isoprenoid biosynthesis such as enzymes from the methylerythritol phosphate pathway and the mevalonate pathway, amorpha-4,11-diene synthase and other sesquiterpene synthases, monoterpene synthases and two cDNAs showing high similarity to germacrene A synthases [48]. An inventory of hundreds of genes, potentially involved not only in alkaloid biosynthesis but also possibly in plant secondary metabolism in general, has been built.

**Table 1:** Recently produced bioactive secondary metabolites from plant tissue cultures

| Plant name | Active ingredient | Culture type |
|---|---|---|
| *Cassia acutifolia* [24] | Anthraquinones | Suspension |
| *Catharanthus roseus* [25] | Catharanthines | Suspension |
| *Mentha arvensis* [26] | Terpenoids | Shoot |
| *Nothapodytes foetida* [27] | Camptothecin | Callus |
| *Podophyllum hexandrum* [28] | Podophyllotoxin | Suspension |
| *Rhus javanica* [29] | Gallotanins | Root |
| *Salvia fruticosa* [30] | Rosmarinic acid | Callus and Suspension |
| *Silybum marianum* [31] | Flavonolignan | Root |
| *Taxus species* [32] | Taxol | Suspension |
| *Withania somnifera* [33] | Withafarin | Shoot |

## 4. Conclusion

The improved in vitro plant cell culture systems have the potential for commercial exploitation of secondary metabolites. Micropropagation, combined with Agrobacterium transformation, provides a method for routine genetic transformation of many important medicinal species. The production of secondary metabolites could be enhanced using bioreactors and has a tremendous potential for the large-scale synthesis of therapeutically active compounds in medicinal plants. During the past decade, remarkable progress in plant genetic-transformation technology has been witnessed. This rapid progress has resulted in constant flow of new and improved transformation protocols for many medicinal plant species. Genetic transformation may provide increased and efficient system for in vitro production of secondary metabolites. This review also highlights the possibilities for the use of bioconversion and combinatorial biosynthesis strategies for the production and development of plant natural products at different levels in biosynthetic pathways. There are several benefits of using microorganisms instead of plants or plant cell cultures, including their fast replication, low costs of cultivation, and the possibility for bioprocessing on an industrial scale. Microarray analysis of gene expression could be useful for elucidating the molecular mechanisms and networks underlying the complex pharmacological function of herbal extracts and mixtures. DNA microarrays have the potential for applications in different phases of herbal drug discovery and development. The tools described in this review would certainly be of increasing importance in the field of medicinal plant biotechnology research in near future.

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