

VARIOUS APPROACHES FOR SECONDARY METABOLITE PRODUCTION THROUGH PLANT TISSUE CULTURE

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Abstract

There is great interest in developing alternatives to the intact plant for the production of secondary metabolites. This originally had centred on the use of tissue and cell cultures though approaches involve applying molecular biological techniques to enhance or alter the metabolic pathways leading to specific compounds. During the past three decades, research has concentrated on the use of plant cell and tissue cultures. The techniques of plant cell cultures include the following sequential stages or developments: selection among wild plants of a high-producing one, in-vitro culture or callogenesis, which involves the selection and stabilization of producing calli with a view to identifying a high-producing line or strain; maximizing callus or cell suspension, culture conditions and isolation of the best-producing line; industrial scaling-up, mass cultivation in bioreactors; downstream processing, i.e. extraction and purification of the compounds sought.

Key words : Secondary metabolite, Tissue culture, Transformation, elicitor.

Introduction

The potency of plant cell, tissue, and organ cultures to produce many valuable chemical compounds as the parent plant in nature has been identified almost since the inception of in vitro technology. The regular increasing demand in world marketplace for natural, renewable products has refocused attention on in vitro plant materials as potential factories for secondary phytochemical products, and has paved the way for new research exploring secondary product expression in vitro.

The past two decades plant cell biotechnology has evolved as a promising new area within the field of biotechnology, focusing on the production of plant secondary metabolites. The deliberate stimulation of defined chemical products within carefully regulated in vitro

cultures provides an excellent forum for in-depth investigation of biochemical and metabolic pathways, under highly controlled microenvironmental regimes.

Plant-produced secondary compounds have been incorporated into a wide range of commercial and industrial applications, and fortuitously, in many cases, rigorously controlled plant in-vitro cultures can generate valuable natural products. Plants and plant cell cultures have served as resources for flavors, aromas and fragrances, biobased fuels and plastics, enzymes, preservatives, cosmetics (cosmeceuticals), natural pigments, and bioactive compounds.

As a consequence, such a bioreactor is preferable for plant cell cultures, it is the lowest cost process-unit. More recent studies on the shear sensitivity of plant cells, have shown that in fact plant cells

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in general are quite shear-stress tolerant [1,2,3,4]

There is a series of distinct advantages to producing a valuable secondary product in plant cell culture, rather than in vivo in the whole crop plant. These include: a) Production can be more reliable, simpler, and more predictable, b) Isolation of the phytochemical can be rapid and efficient, as compared to extraction from complex whole plants, c) Compounds produced in vitro can directly parallel to the compounds in the whole plant, d) Interfering compounds that occur in the field-grown plant can be avoided in cell cultures, e) Tissue and cell cultures can yield a source of defined standard phytochemicals in large volumes, f) Tissue and cell cultures are a potential model to test elicitation, g) Cell cultures can be radio labeled, such that the accumulated secondary products, when provided as feed to laboratory animals, can be traced metabolically. The culture of differentiated cells, such as (hairy) root or shoot cultures, is an alternative, but is hampered by problems in scaling up of such cultures. Metabolic engineering offers new perspectives for improving the production of compounds of interest. This approach can be used to improve production in the cell culture, in the plant itself or even production in other plant species or organisms. Several strategies are being followed to improve yields of secondary metabolites in plant cell cultures. First of all the screening and selection of high producing cell lines and the optimization of growth and production media can be mentioned as common approaches. In the past years new approaches have been developed: the culturing of differentiated cells (e.g. shoots, roots and hairy roots), induction by elicitors and metabolic engineering.

Hairy root cultures as a source of secondary metabolites

The hairy root system based on inoculation with *Agrobacterium rhizogenes* has become popular

in the two last decades as a method of producing secondary metabolites synthesized in plant roots. Unorganized plant tissue cultures are frequently unable to produce secondary metabolites at the same levels as the intact plant.

The hairy root phenotype is characterized by fast hormone-independent growth, lack of geotropism, lateral branching and genetic stability. The secondary metabolites produced by hairy roots arising from the infection of plant material by *A. rhizogenes* are the same as those usually synthesized in intact parent roots, with similar or higher yields [5]. This feature, together with genetic stability and generally rapid growth in simple media lacking phytohormones, makes them especially suitable for biochemical studies not easily undertaken with root cultures of an intact plant. *A. rhizogenes* mediated transformation of plants may be used in a manner analogous to the well-known procedure employing *A. tumefaciens*. *A. rhizogenes* mediated transformation has also been used to produce transgenic hairy root cultures and plantlets have been regenerated. The *A. rhizogenes* mediated transformation has the advantage of being able to transfer any foreign gene of interest placed in binary vector to the transformed hairy root clone. It is also possible to selectively alter some plant secondary metabolites or to cause them to be secreted by introducing genes encoding enzymes that catalyze certain hydroxylation, methylation, and glycosylation reactions.

To succeed in establishing a hairy root culture system for a certain plant species, several essential conditions should be taken into consideration. These conditions include the bacterial strain of *A. rhizogenes*, an appropriate explant, a proper antibiotic to eliminate redundant bacteria after cocultivation, and a suitable culture medium. Based on the types of opines produced, the strains of *A. rhizogenes* can

be separated into five lines: octopine, agropine, nopaline, mannopine, and cucumopine [6]. Agropine strains are the most often used strains owing to their strongest induction ability. Most plant materials, such as hypocotyl, leaf, stem, stalk, petiole, shoot tip, cotyledon, protoplast, storage root, or tuber, can be used to induce hairy roots [7, 8, 9, 10, 11]. However, for different species, the proper explant material may vary and the age of the material is most critical, with juvenile material being optimal. To induce hairy root, explants are separately wounded and cocultivated or inoculated with *A. rhizogenes*. Usually two or three days later, the explant Optimizing the composition of nutrients for hairy root cultures is critical to gain a high production of secondary metabolites. Factors such as the carbon source and its concentration, the ionic concentration of the medium, the pH of the medium, light, phytohormones, temperature, and inoculum are known to influence growth and secondary metabolism [12,13,14,15]. Heavy metal ions and the concentrations of phosphate, nitrate, and ammonia have also been well studied [12,13,16]. The addition of auxin and elicitors often increases the levels of secondary metabolites [17,18].

Plant Cell Immobilization

Immobilization of plant cells is considered to be of importance in research and development in plant cell cultures, because of the potential benefits that could be provided [19, 20]. It is the confinement of plant cells in a suitable support matrix which holds the cells and allow the flux of substrate and product from the matrix. This technique has extends the viability of cells in the stationary (and producing) stage and enable maintenance of biomass over a prolonged time period. It is a simplified downstream processing (if products are secreted). Higher cell density enabling a reduced bioreactor size, thereby

reducing costs and the risk of contamination.

An immobilization system which could maintain viable cells over an extended period of time and release the bulk of the product into the extracellular medium in a stable form, could dramatically reduce the costs of phytochemicals production in plant cell culture.

However, an immobilized system also has the problems described below:

- a) Immobilization is normally limited to cases where production is decoupled from cell growth;
- b) The initial biomass must be grown in suspension;
- c) Secretion of product into the extracellularly medium is imperative;
- d) Where secretion occurs there may be problems of extracellular degradation of the products;
- e) When gel entrapment is used, the gel matrix introduces an additional diffusion barrier.

Elicitor Treatment

The accumulation of secondary metabolites in plants is part of the defense response against pathogenic attack, which is triggered and activated by elicitors, the signal compounds of plant defense responses [21]. Therefore, the treatment of plant cells with biotic and/or abiotic elicitors has been a useful strategy to enhance secondary metabolite production in cell cultures. Understanding signal transduction paths underlying elicitor-induced production of secondary metabolites is important for optimizing their commercial production. The most frequently used elicitors in previous studies were fungal carbohydrates, yeast extract, methyl jasmonate (MJ) and chitosan. MJ, a proven signal compound, is the most effective elicitor of taxol production in *Taxus chinensis* Roxb. [22] and

ginsenoside production in *Panax ginseng* [23, 24, 25, 26] cell/organ culture. In the bioreactor cultures, eleutheroside content increased significantly by elicitation of methyl jasmonate (MJ) when the *Eleutherococcus senticosus* embryo was cultured in Liquid MS with suspension. Microbial infections of intact plants often elicit the synthesis of specific secondary metabolites. Effects of elicitors on secondary metabolism have been investigated at the enzymatic levels to determine their mode of action. A yeast polysaccharide preparation induced L-tyrosine decarboxylase in suspension cultures of *Thalictrum rugosum* and *Eschscholtzia californica*; the enzyme was induced after 5 hours after addition of the elicitor at 30 to 40 $\mu\text{g/g-cell}$ fresh wt. Recent developments in phytochemical elicitation have shown that simple inorganic and organic molecules can induce product accumulation. Sodium orthovanadate and vanadyl sulphate induced the accumulation of isoflavone glucosides in *Vigna angularis* cultures and indole alkaloid accumulation in *Catharanthus roseus* cultures, respectively. Other substances found to stimulate alkaloid accumulation in *C. roseus* include sodium chloride, potassium chloride and sorbitol as well as abscisic acid. Processes such as

these, employing simple and cheap elicitors have much promise in industrial scale plant cell cultures.

Tissue Engineering

This technique involves introducing and expressing foreign genes in plants. However, while it has been relatively simple to obtain high levels of transient gene expression in plants, it has been considerably more difficult to obtain stably transformed plants. Stable transformation is dependent on several factors, the most important being the plant species to be transformed and the transformation protocol used. An attenuated soil-borne pathogen, *Agrobacterium tumefaciens*, is the most commonly used vector to transform numerous dicotyledonous (broad leaf) plants, including familiar fruits and vegetables such as tomatoes, mustards and beans [27].

Electroporation of whole tissues is another transformation method that has been used for the transformation of monocot plants [28]. Particle gun-mediated transformation, often called biolistic transformation, is a commonly used procedure that has its advantages. This is the most frequently used procedure for transient transformation of tissues and is based on bombarding tissues with microscopic, DNA-coated tungsten or gold particles. As with

Table 1: Comparison of different transformation processes.

Transformation procedure	Advantage	Disadvantage
<i>Agrobacterium</i> mediated	Very effective, cheap and simple to use and can be used in germ -line transformation. The copy number of DNA insertions is often low.	Requires the use of a tissue culture regeneration procedure. Host range may be limited by the plant hypersensitive response.
Electroporation	Very effective for transient expression. No plant host range problems. High DNA delivery rate.	Requires the use of a tissue culture regeneration procedure. Copy number of DNA insertions can be high and lead to gene silencing/cosuppression.
Particle bombardment	Very effective especially for transient expression. Has been used to produce transplastomic plants. No plant host range problems.	Requires the use of a tissue culture regeneration procedure. Copy number of DNA insertions can be high and lead to gene silencing/cosuppression.

There are numerous types of promoters that regulate different types of expression ranging from constitutive to inducible. Each of the inducible promoter systems has characteristic features that take advantage of either agricultural practices (e.g., safener inducible) or cell culture conditions (e.g., pristnamycin-responsive promoter) to facilitate controlled gene

expression. These inducible systems are especially useful when examining the consequences of transgene expression in complex biochemical pathways. The most commonly used constitutive promoters are of viral origin such as the cauliflower mosaic virus 35S promoter that drives very high levels of transcription in most tissues of the plant.

Table 2 : Promoters used in transformation

Name	Comments
35S.	Viral origin, very well described and commonly used. High expression in vascular tissue but significantly lower expression in meristem tissue (30)
Ubiquitin	Plant origin promoter that drives high-level constitutive expression but expression level during development may vary. (31)
b-Conglycinin promoter	A well-characterized promoter that directs embryo-specific expression. (32)
APase promoter	This promoter drives phosphate-inducible expression in roots. However, the rate of induction is slow. (33)
2A11	is a fruit-specific promoter derived from tomato. (34)
n/StMCPI	The patatin promoter drives high-level expression in tubers and in sucrose-treated leaves, StMCPI is a tuber-specific promoter that is regulated independent of environmental or hormonal signals. (35)

Conclusion

Many laboratories carry out their research and development programmes because the possibilities exist to increase the yields of their production processes. Furthermore, even if certain compounds are not worth developing up to a commercial stage, some biosynthetic compounds could be used as valuable precursors for organic synthesis, or could themselves constitute entirely new products. The market size for the end product, the cost benefit ratio of the production technology, the

competition with substitutes and the existence of other sources of supply are major factors which influence the choice of the appropriate manufacturing technique, especially when deciding in favour of plant cell or tissue cultures. Thus, it is probable that metabolites, synthesized through simple enzymatic reactions under the control of a single gene, could be more efficiently produced by genetically-engineered microbial cells rather than by plant cell or tissue cultures. This is a valuable technique for human welfare and more importantly for developing countries. The phytopharmaceutical companies need more

focus to improve the natural compounds for reduce therapeutic cost and to increase production to cater the needs of this area.

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