SECONDARY PRODUCTS FROM PLANT TISSUE CULTURE

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Summary

The information presented in this article is organized to relate the diversity and potential of plant cell culture (see also *Plant Cell Culture*) in production of secondary metabolites, means of regulation of production through elicitation and induction, some preliminary economics of using plant cell culture for secondary metabolite production and limitations/opportunities for marketing plant cell culture products as pharmaceuticals and/or nutraceuticals.

1. Diversity and potential of plant cell culture

In addition to food and fiber, plants are exploited for a large variety of commercial chemicals, including agricultural chemicals (pyrethins, rotenone, azadiachtin, neriifolin, salannin, alleopathic chemicals), pharmaceuticals (codeine, morphine, scopolamine, atropine, vinblastin, L-dopa, serpinine, hyoscyamine, diosgenin, digoxin, quinine, vincristine, shikonin, ajmalacine, limblasin) (see also - *Pharmaceuticals from medicinal plants*), food colors (anthocyanins, beta-cyinins, saffron), flavors (strawberry, grape, vanillin, tomato, celery, asparagus, fatty acids, waxes), fragrances (mint, rose, vetiver, jasmine, patchoaly, sandlewood, lemon, onion, garlic cinnamon, capaicin) and

sweeteners (stevoside, thaumatin, miraculin, monellin) For example, pharmaceuticals and intermediates that are derived from higher plants represent about 25 percent of all prescription drugs. This is compared to the antibiotics that are derived from lower eukaryotic life forms such as fungi and actinomycetes and represent about 12 percent of the marketed drugs in the modern world (see also - *Production of antibiotics*). A few examples of plant derived pharmaceuticals are digitalis (produced from *Digitalis purpurea*, prescribed for heart disorders), codeine (*Papaver somniferum*, sedative), vinblastine and vincristine (*Catharanthus roseus*, leukemia treatment), artemisinin (*Artemisia annua*, malaria), quinine (*Cinchona officinalis*, malaria) and paclitaxel (*Taxus brevifolia*).

Plant tissue culture production methods (called "phytoproduction") can be developed to profitably manufacture some of these chemicals. Routian and Nickell obtained the first patent for the production of substances by plant tissue culture in 1956. Numerous investigators have reported production of useful compounds in both callus and suspension cultures. For example, suspension cultures of *Thalictrum minus* produced the stomachic and antibacterial berberine. Callus cultures of *Catharanthus roseus* produced the antihypertensive ajmalicine. Callus cultures of *Stizolobium hassjo* produced the antiparkinsonian drug, 1,2-dihydroxybenzen that is also known as dioxyphenylalanine or L-dopa. Suspension cultures of *H. niger* L. produced a derivative of the anticholinergic hyoscyamine. Some secondary metabolites have been observed in much higher concentrations in cultured cells than in whole plants of the same species. These include ginsengoside from *Panax ginseng* (27 percent of cell dry weight in culture, 4.5 percent in whole plants), anthraquinones from *Morinda citrafolia* (18 percent in culture, 2.2 percent in plants) and shikonin from *Lithospernum erythrorhizon* (12 percent in culture, 1.5 percent in plants). Additional examples of substances synthesized by cell culture are discussed below.

Plants produce many of these compounds as inducible chemical defense systems to deter pathogenic microorganisms and herbivores. In many cases, these are known as phytoalexins. Other plant responses are the reinforcement of plant cell walls, formation of callose, biosynthesis of antimicrobial hydrolytic enzymes and biosynthesis of pathogen related (PR) proteins. Phytoalexins, which are low molecular weight compounds that serve a role in plants that is analogous to antibodies in the defense system of mammals, are also synthesized as a result of exposing cultured cells to elicitors. The two major classes of phytoalexins are terpenoids and isoflavonoids. Terpenoids may come as products of either mevalonate or non-mevalonate metabolism and the biosynthesis of the isoflavonoids is via the phenylpropanoid pathway. Paclitaxel (Taxol®), the biosynthesis of which contains elements of both pathways, will conveniently be used as an example to demonstrate differential elicitation in this article (see *Section 2.1 - ß-glucans, and Section 2.3-methyl jasmonate*).

The detailed biosynthetic pathway of taxanes is under intense investigation. These tricyclic diterpenoids arise from geranylgeranyl pyrophosphate by sequential intramolecular cyclizations of double bonds to form a simple diterpene skeleton. The functional taxanes require nine enzymatic oxidations, acylations and attachment of the N-benzoyl-3-phenylisoserine ester to the -OH at C-13 of 10-deacetylbaccatin III. Recent advancements in understanding of the final steps of biosynthesis of

unfunctionalized taxanes containing the 4, 5, 20 oxetane ring has been found to be non-enzymatic. Other taxanes also appear to be promising as drug candidates. Synthesis and activity of 9-dihydrotaxol, a promising new taxane analog with greater potency, from 13-acetyl-9-dihydrobaccatin III obtained from leaves of *Taxus canadensis,* has been reported.
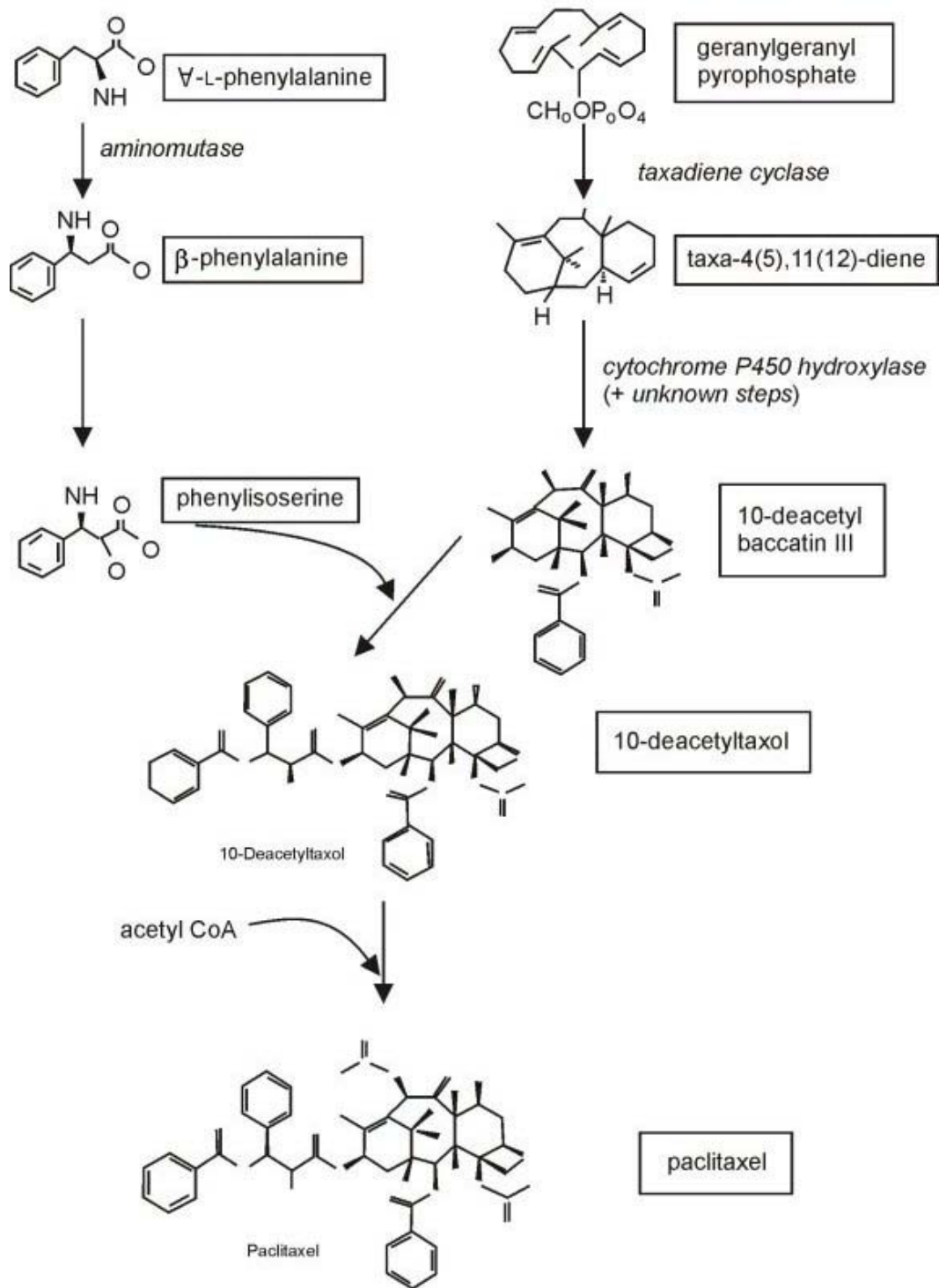

Figure 1: Biosynthesis of paclitaxel and related taxanes

Isoprenoids are all derived from a common precursor, isoprenepyrophosphate (IPP), the synthesis of which involves conversion of 3-hydroxy-3-methylglutaryl CoA into mevalonic acid by 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR); this is a key regulatory step in isoprenoid biosynthesis. The discovery of an alternative "Rohmer" nonmevalonic acid (NMVA) pathway for isoprenoid production in plants condenses pyruvate and glyceraldehyde-3-phosphate to IPP, which is then converted to first to geranylpyrophosphate (GPP) and then to monoterpenes or farnasylpyrophosphate (FPP) that condenses with an isopentyl unit to geranylgeranylpyrophosphate (GGPP) for synthesis of diterpenes. A number of the enzymes in the NMVA pathway have recently been cloned and sequenced in the *Arabidopsis thaliana*. The list includes a transketolase which converts pyruvate and glyceraldehyde-3-P to 1-deoxyxylulose-5-phosphate, which ultimately becomes isoprene, the common C5 precursor of all isoprenoids; isopentenyl pyrophosphate isomerases, which convert IPP to dimethylallyl pyrophosphate which join to form geranyl pyrophosphate (C10- basic monoterpene unit); and farnesyl pyrophosphate synthase, which joins a isopentyl unit to geranyl pyrophosphate to form the C15 unit (basic sesquiterpene unit). The geranylgeranyl pyrophosphate synthase, which joins an isopentyl unit to farnesyl pyrophosphate to form geranylgeranyl pyrophosphate, the C20 basic unit for diterpenoids has recently been cloned from *Taxus* by use of a complementary cDNA from yeast. Additionally, hydroxymethyl glutaryl CoA reductases, which catalyze the formation of isopentyl pyrophosphate from acetyl CoA in the classical MVA pathway, have also been cloned and sequenced from *A. thaliana*.

The phenylpropionate pathway is initiated by reactions catalyzed by phenylalanine lyase (PAL), cinnamate 4-hydroxylase (CHS) and 4-coumarate:CoA ligase (CCL). Flavonoid and lignin biosynthesis branches at this point with the conversion of 4-coumaroyl-CoA to chalcone, from which flavonones are derived by the enzyme chalcone isomerase (CHI). Elicitation rapidly and coordinately induces mRNA for PAL, CHS and CHI. Elicitation of some phenylalanine-derived secondary metabolites (isoflavonoids) is mediated by *de novo* mRNA transcription and enzyme synthesis in cell cultures of different species. Pharmacological evidence implicates trans-cinnamic acid as a feedback modulator of the expression and enzymatic activity of the first enzyme in the phenylpropanoid pathway, L-phenylalanine ammonia-lyase (PAL). There is evidence for a feedback loop at the entry point into the phenylpropanoid pathway that had previously been inferred from potentially artifactual pharmacological experiments.

The ability of plants to exude a complex array of micro- and macromolecules into the rhizosphere, with the potential to affect the inter-relationships between plants and beneficial or deleterious soil-borne organisms extends to other compounds. **Paclitaxel**, a natural diterpenoid that is currently in use as therapy for various refractory cancers, has been obtained commercially from roots and bark of *T. brevifolia*. In an effort to explore the biological role of paclitaxel, the effect of paclitaxel and related compounds on the growth of plant pathogenic fungal hyphae were investigated. Paclitaxel and cephalomannine (the complete paclitaxel structure that has an isopentene substitution for the phenylpropene on the sidechain) inhibited the growth of several fungi, especially *Phylophthora* and *Pythium* species (Oomycetes) and *Rhizoctonia solani* (Basidiomycetes). Baccatin III (the complete tricyclic structure without any sidechain

on position 13) had no effect on the growth of any fungus tested. Strains of *Aspergillus* (Deuteromycetes) and *Fusarium* (Ascomycetes) were not inhibited by any taxane.

Mauka (*Mirabilis expansa*) have been studied as well for anti-fungal properties against *T. reesei, T. harzianum, Pythium, Phytophthora, Bacillus, Pseudomonas, Agrobacterium, Erwinia, Xanthmonas, Rhizobium* and *Serracia marcesens*. Roots of *L. erythrorhizon* produce naphthaquinones from phenylalanine and geranyl pyrophosphate, which accumulate to levels of 3 to 5 percent of their dry weight in five year old tap roots and in hairy root culture. In addition to skin medicinal uses, the natural function of these root exudates appears to be antimicrobial in affecting growth of many soil bacteria and fungi, but not microrrhizal fungi.

Cultured plant cells derived from roots of higher plants synthesize secondary metabolites that have been used for centuries in human health care. *Agrobacterium*-transformed root cultures produce a wide variety of phytoceuticals and other bioactive chemicals. The production of a Chinese folk medicine, artemisinin (qinghaosu), has been studied extensively in *A. rhizogenes* transformed root culture of *A. annua*, as an alternative to chloroquine treatment of malaria. Solanaceae species that produce tropane alkaloids such as scopolamine and hyoscyamine, which bind to acetyl choline receptors, include *Mandragora* (mandrake) that produces hallucinogens and poisons. *Datura* sp. and *Atropa belladonna* are used as cosmetics and medicine, respectively. Asteraceae species produce polyacetylenes, such as thiarubrine and terthienyl which are used as nematicides, phototoxins, antifungals and antibacterials from *Calendula officinalis* (marigold) roots. Boraginaceae produce naphthaquinones such as shikonins that are antimicrobial. *Cephaelis ipacacuanaha* produces the alkaloid emetine that is used to induce vomiting. A sedative triterpene called galphimine B has been produced in *Galphimia glauca* tissue culture. *Ginkgo biloba* (maidenhair tree) produce diterpene ginkgolides and *Coleus forskolii* (an Indian herb) produces a labdane diterpenoid forskolin that activates adenylate cyclase and is used to treat bronchial asthma. *Camptotheca acuminata* (Chinese herb) produces the alkaloid camptothecin, the most recent of anticancer drugs. *H. muticus* (Egyptian henbane) produces hyoscyamine in hairy root culture after transformation with *Agrobacterium rhizogenes;* similarly, *Datura stramonium* and *C. roseus* have also been stable in hairy root culture for years. Hairy root culture of carrot (*Daucus carota*) has been used in phytoremediation studies.

Cocultures of hairy roots and other microorganisms have been found to produce unique secondary metabolites. For instance, *Hordeum vulgare* roots infected with a vesicular-arbuscular mycorrhizal (VAM) fungi, *Glomus intraradices,* induces accumulation of a terpenoid glycoside. Similarly, *C. roseus* roots co-cultured with *Acaulospora scrobiculata* changed regulation of indole alkaloid biosynthesis. Cocultures of *D. carota* and *Trifolium repens* f. *lodigense* (Ladino clover) roots and the wound nematode *Pratylenchus* spp. have been studied. Cocultures of aphids (*Rhopalosiphum padi*) and safflower (*Carthamus tinctorius*) lead to production of defense response polyacetylenes.

Exposure of *C. roseus* hairy roots cultures to pectinase or jasmonic acid increase secondary metabolite accumulation that includes tabersonine, ajmalicine, serpentine, lochnericine and ho(e)rhammericine. Elicitor dosage and exposure time criteria have

also been reported for the indole alkaloid biosynthesis by *C. roseus* hairy roots. Hyoscyamine and proline accumulate in water-stressed *H. muticus* hairy roots; exposure of these cultures to elicitor preparation from *R. solani* do not increase tropane alkaloid production but induce sesquiterpene phytoalexin formation, for which an HMGR gene may be responsible, based upon transgenic experimentation using *A. rhizogenes*. A fungal elicitor is effective for the latter system at 1 and 10 mmol of glucose equivalent L^{-1} medium (see *Section 2.2- ethylene*), however the elicitor dosage is dependent on the amount of tissue in the reactor. Calcium may also play a role in *R. solani* elicitation as well as abiotic elicitors in *H. muticus* hairy root cultures. Betalain and pigment production by hairy root cultures of *Beta vulgaris* have been studied in bubble column reactors. Scalability of transformed hairy root cultures has been questioned, however considerable attention has been given to engineering parameters in liquid and gas-dispersed bioreactors.

Large scale suspension plant cell culture has also been successful (see *Section 3*). In Japan three submerged fermentation processes using plant cell cultures were developed by Mitsui Petrochemical Industries to produce berberine, ginseng and shikonin in scales from 4000 to 20,000 liters. Regulatory approval for use of these products as medicinals has impeded commercialization (see Section 4). In North America commercial production of sanguinarine and vanilla flavor were attempted but failed. However, Phyton (Ithaca, NY) utilized a 75,000 liter reactor reportably fitted with interference-flow impellers (Intermig®, at a facility near Hamburg, Germany to develop commercial production of paclitaxel. The extraction and purification of paclitaxel was initially from Pacific yew trees *T. brevifolia* and shrubs and trees of other *Taxus* species: *T. baccata*, *T. cuspidata*, *T. sumatrana*, *T. chinensis*, *T. yunnanensis* and *T. hicksii*. Bristol-Meyer Squibb manufactured paclitaxel using semi-synthesis of from 10-deacetylbaccatin III, which was isolated from needles of the Himalayan yew, *T. wallinchina.* The cell culture process was licensed in May 1995 by Bristol-Meyer Squibb, which in 1998 designated $25 million for development of an FDA-approved commercial process.

2. Regulation of production through elicitation and induction

From the examples presented above, it is clear that plants synthesize a remarkable diversity of secondary metabolites and adjust their metabolic activities in response to biotic and abiotic stress. Also, examples have already been given wherein elicitors stimulate synthesis of phytoalexins in cultured plant cells. Biotic elicitors include glucan polymers, glycoproteins, low molecular weight organic acids and fungal cell wall materials; abiotic elicitors include ultraviolet or far-red radiation, salts of heavy metals, other chemicals, shear stress and osmotic stress. Various cellular responses, which relate expression of genes to pathogen-related reactions, include early membrane responses such as the changes in membrane potential, ion flux, oxidative burst, protein phosphorylation and induction of jasmonic acid formation, are induced by oligosaccharide elicitors.

2.1 ß-glucans

High affinity binding sites for oligosaccharins have been characterized: oligo-ß-

glucosides, oligochitins, yeast N-glycan and ß-1,4-linked galacturonate oligomers of degree of polymerization greater than ten, which form egg-box complexes with millimolar concentrations of calcium ions. These change tobacco culture morphology, external alkalinization, oxidative burst phenomena and phytoalexin accumulation in submicromolar concentrations. Differential elicitation of PAL and peroxidase activities by chitin/chitosan preparations of systematically variations in chain-length and degree of acetylation in non-wounded wheat leaves.

Using purified oligosaccharides, elicitation occurred only with oligomers with a degree of polymerization greater than five. Similar phenomena are reported using suspension cultured tomato cells, except that effects from chitin oligomers of varying degrees of polymerization DP5 = DP4 >>DP3 >> DP2 >DP1. While deacetylated oligomers were not active in rice, both chitin and chitosan derivatives function in the presence of MJ act as modifiers of secondary metabolite production in the *Taxus* system. This fact has been apparent as claims in recent patents.

Two types of oligosaccharides, both potentially derived from the chitin cell walls of pathogenic fungi, act as potent elicitors in suspension-cultured plant cells. The first of these, *N*-acetylchitooligosaccharides induce phytoalexin (momilactones and oryzalexins) formation in the rice cells even at nanomolar ranges. Inhibition studies with various other oligosaccharides show specificity of the binding site for oligosaccharides with chain lengths greater than *N*-acetylchitohexaose.

Using alkalinization of extracellular medium as means to measure rapid responses to elicitation, time- and concentration-dependent saturation of chitin oligosaccharide binding sites in tomato suspension-culture cells has suggested that the first reactions of elicitation with a biotic elicitor are binding to a specific receptor protein on the plasma membrane.

N-acetylchitooligosaccharide elicitor effects on transient ion fluxes through the plasma membrane have been studied in suspension rice cell cultures in conjunction with phytoalexin production. Desensitization of primary defense responses by repeated treatments with chitin oligosaccharides as related to surface binding phenomena has been documented.

The second type of ß-glucan elicitor is chitosan, the deacetylated form of chitin. In actuality, the difference between chitin and chitosan is a continuum of the degree of N-acetylation of the glucosamine residues in the polymer. The degree of acetylation was found to be important in inducing defense responses. Chitosan elicitors induce formation of phytoalexins in legumes (soybean, chickpea, bean, alfalfa, pea) and solanaceous sp. (potato, sweet pepper). However, anthraquininone biosynthesis was stimulated in *M. citrifolia* by chitin and chitosan. During the first few days of incubation after adding elicitor, production of chitinase enzyme activity increased and then declined when anthraquinone biosynthetic enzymes became active. Autoclaved components from the yeast *Rhodotorula rubra* induce the enzymes of the phenylpropanoid pathway in cell cultures of *Ruta graveolens* L., which leads to accumulation of acridone epoxides, furoquinolines and furanocuomarins.

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Biographical Sketch

James Carl Linden is a Professor at Colorado State University with a joint appointment in the departments of Microbiology, Immunology and Pathology and of Chemical and Biological Engineering. He holds a Ph.D. in biochemistry with minor in plant physiology from Iowa State University in 1969 and a B.S. from Colorado State University in 1964 in chemistry. He did post-doctoral work in plant biochemistry at the Botanisches Institut der Universität München as an Alexander von Humboldt Stipendiate from 1969-1971 and St. Louis University School of Medicine from 1971-1972 in mammalian cell culture research. After five years of industrial experience in the use of immobilized enzymes, Dr. Linden returned to academia at Colorado State University in the Department of Agricultural and Chemical Engineering and began studies on the enzymatic hydrolysis of cellulose in plant material, alcohol toxicity of Clostridium acetobutylicum fermentations, plant cell culture, thermophilic cellulase production and mechanisms of plant cell wall hydrolysis. He has in this time spent short periods as visiting scientist at the ETH, Zurich (1980), the Technical University, Budapest (1991) and the Universität Regensburg (1994). He is a member of the American Chemical Society, the American Society of Plant Physiology, and the Society of Industrial Microbiology. During the past twenty years, Dr. Linden has published 54 refereed and 71 non-refereed papers and supervised 16 Master's Thesies and 13 Ph.D. Dissertations.