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FUTURE APPROACHES TO THE FORMATION OF SECONDARY NATURAL PRODUCTS IN PLANT CELL SUSPENSION CULTURES[1]

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ABSTRACT.—Based on the inherent slow growth of plant tissue cultures and the problem that many plant cell suspension cultures do not produce naturally occurring metabolites, this paper deals with areas of research that could lead to selective induction of the synthesis of the secondary natural products. This approach is supported by the introduction of morphinan alkaloid formation in *Papaver somniferum* cell suspension cultures, harringtonia alkaloids in *Cephalotaxus harringtonia*, sesquiterpene aldehydes in *Gossypium arboreum*, and shikonin formation in *Lithopermum erythrorhizon*. The isolation and immobilization of key enzymes in secondary metabolite formation is discussed and documented with examples. This approach, as well as the formation of secondary natural compounds in fast-growing bacterial or fungal cells through recombinant DNA methodology, would greatly facilitate economic feasibility and commercial application of the formation of secondary metabolites in plant cell suspension cultures.

Considerable advances have been made recently in the production of secondary natural products in plant cell suspension cultures. In general, these advances were realized through the manipulation of media ingredients, especially plant growth hormones, through the development of an efficient cloning procedure (1) and through the use of sensitive analytical methods, such as radioimmunoassay (2) and Mikes (3). The use of medium manipulations is generally a random approach and is, therefore, time-consuming, but it has resulted in plant cell culture systems (4,5) that produce specific chemicals at concentrations higher than those produced by the mother plant.

From these considerations, the following step-wise strategy was developed by Zenk and co-workers (6):

1. selection of high yielding plants,
2. establishment of cell cultures from the selected plants,
3. clonal selection of desired cells (may have to be repeated),
4. establishment of a stable cell line with a high production capacity, and
5. optimization of a production medium.

This strategy has been used with excellent results, e.g., in the development of high alkaloid-producing *Catharanthus roseus* cell lines. However, a number of plant cell suspension cultures fail to produce the expected secondary natural products, or if they do produce small quantities of the desired compounds, they lose this capacity after a few transfers. Because the intact plant obviously can synthesize the desired compound and because, usually, in suspension cultures, only a few (if any) transfers are involved, it appears reasonable to assume that during the initiation or initial transfers of the plant cell cultures, the gene(s), which code(s) for a specific enzyme or a number of enzymes in the pathway leading to the desired secondary natural product, became dormant or repressed. To correct this situation, i.e., to de-repress the synthesis of the desired secondary natural product, means have to be found somehow to induce the synthesis of secondary natural products. Therefore, the strategy of Zenk and co-workers (6) should perhaps be expanded to include an induction step:

1. selection of a high-yielding plant,

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2. establishment of cell cultures from the selected plant,
3. development of an optimum growth medium, without consideration of secondary natural product production,
4. development of methods to induce secondary natural product formation,
5. clonal selection of highly inducible cell strains, and
6. development of optimum production medium.

In accordance with this strategy, methods need to be developed to induce secondary natural product formation. Looking at the literature of phytopathogen-host interaction, it becomes clear that a large number of secondary natural products are phytoalexins, i.e., secondary metabolites synthesized in plants upon physical, chemical, microbiological, or fungal damage of the plant (7). The interaction of microorganisms and plants has been investigated in a few phytopathogen-host systems and resulted in a partial elucidation of the biochemical mechanism by which phytoalexin synthesis is induced (8). The signals that specify synthesis of secondary products in plant cells are elicitors, are associated with the invading microorganism, and can induce the synthesis of the enzymes that catalyze the reactions in the pathway that lead to the secondary metabolite (9-11). It, therefore, should be possible to add conidia of a phytopathogenic fungus to plant suspension cultures and obtain induction of secondary products. The choice of the particular fungus appears to be important inasmuch as specific races of pathogenic fungi have only an inducing effect on certain cultivars (12). However, certain fungi are wilt-producing pathogens and infect a great variety of plants. A good example is the wilt-producing fungus *Verticillium dahliae*, which was found to be pathogenic to more than 20 nonrelated plant varieties (13,14).

In order to test this hypothesis, we initially investigated the possibility that *V. dahliae* can induce the synthesis of gossypol and its derivatives in *Gossypium arboreum* cell suspension cultures. Although these cultures originally produced gossypol and derivatives, a rapid transfer schedule selected for fast-growing cell lines that lost their capacity to synthesize the secondary metabolites in question. Addition of 4×10^5 heat-denatured conidia of *V. dahlia* 277 (a strain isolated from sugarbeets) to a 20-ml *G. arboreum* cell culture caused a hundredfold increase in concentration of gossypol and derivatives after a 120 h incubation (Figure 1). Concomitant with the induction of the synthesis of gossypol and derivatives, a decrease in growth rate (Figure 2) was observed (15). This would indicate that by the addition of nonviable *V. dahliae* conidia, a stress condition was initiated causing induction of secondary natural product formation and a channeling of metabolic efforts into gossypol and derivative formation, and away from primary metabolism.

Although these results clearly show that gossypol and derivatives are phytoalexins (16) and can be induced by elicitor molecules from phytopathogenic fungi, it is of interest if this induction method is feasible for other plant systems, especially in cell suspension cultures where production of secondary natural products has been very difficult. One such system, *Cephalotaxus harringtonia*, produced small amounts of cephalotaxine and the esterified antitumor alkaloid homoharringtonine (Figure 3) in callus cultures immediately upon initiation (17,18). However, synthetic activity was lost upon subsequent transfers (Delfel, personal communication). In our laboratory, initiation of callus and suspension cultures of *C. harringtonia* gave the same results with a slow doubling time of the cells and no production of free or esterified alkaloids (Zhou and Heinstein, unpublished results). However, addition of autoclaved *V. dahliae* 277 conidia (3 ml) to 100 ml *C. harringtonia* cultures caused a drastic increase in alkaoid formation (Figure 4) after 6-8 days of incubation. To quantitate and identify the alkaloid unequivocally, the culture extracts were analyzed by gc and characterized by gc/ms as their trimethylsilyl derivatives (19). The results clearly show (Table 1) that alkaloid concen-

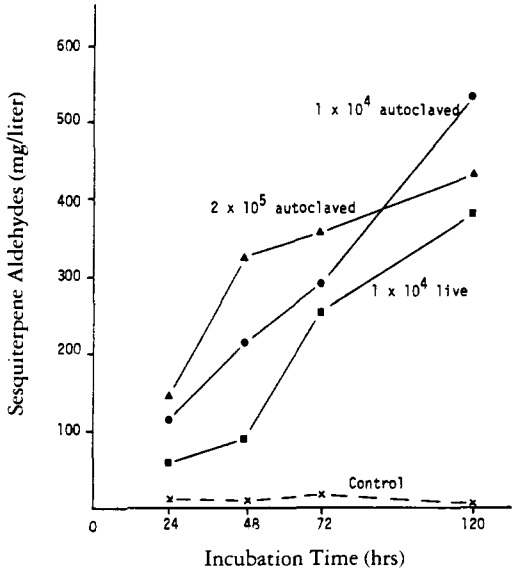


FIGURE 1. Induction of sesquiterpene aldehyde formation in *Gossypium arboreum* cell suspension cultures by autoclaved or viable *Verticillium dahliae* 277. One ccm of 9-day-old *G. arboreum* was suspended in 20 ml of fresh medium 36 h before an experiment was started (0 h) with the addition of 1×10^4 viable conidia (■____■), 1×10^4 autoclaved (●____●), or 2×10^5 autoclaved (▲____▲) conidia. Duplicate cultures were harvested at the times indicated, extracted with MeOH, and the sesquiterpene aldehydes quantitated with phloroglucinol. Each point is an average of six incubations.

tration, as well as alkaloid formation, can be increased considerably by this induction method.

Another system investigated thoroughly with only partial success is the formation of morphinan alkaloids in *Papaver somniferum* cell suspension cultures. The yields of codeine and morphine in these cultures were about 20-60 μg/liter, too low to warrant commercial utilization for large-scale cultures (20,21). Similar results were obtained in our laboratory (Martin, Kim, and Heinstein, unpublished results). However, addition of autoclaved *V. dahliae* conidia or autoclaved *Fusarium moniliforme* var. *subglutinans* caused a considerable increase in codeine and morphine formation in *P. somniferum* cell suspensions (Table 2). It appears, therefore, that stress conditions in plant cell suspension cultures, such as the interaction with a pathogen, can induce the formation of secondary natural products in cases where the cell suspensions do not produce the desired compounds under normal or usual conditions. The rationale, therefore, would be to develop optimal growth conditions to obtain maximum cell mass accumulation, followed by the induction of the secondary natural product formation. Indeed, this approach has been used previously in commercial application. Fujita *et al.* (22) have developed a modified Lismaier-Skoog medium for optimal cell mass accumulation of

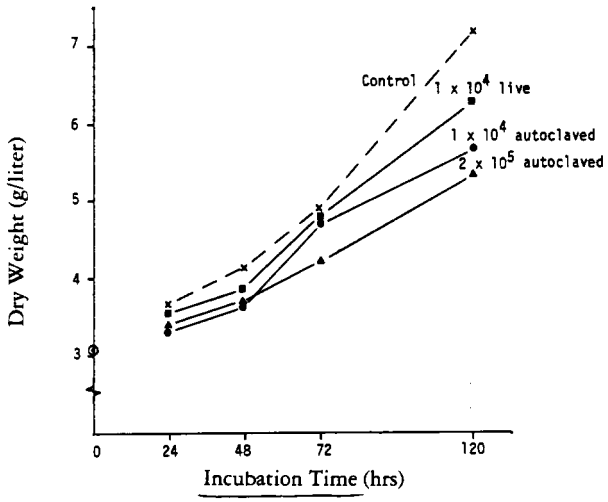
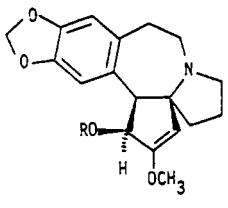
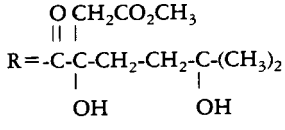


FIGURE 2. Effect of viable and autoclaved *Verticillium dahliae* 277 on growth of *Gossypium arboreum* cell suspension cultures. Experimental conditions are as in Figure 1. The MeOH extracted residue was dried for 16 h at 60° and used for dry weight determination. The concentration at zero time=ca. 3 g/liter.

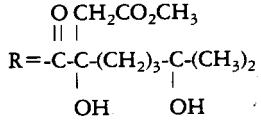
Lithospermum erythrorhizon in large-scale cell suspension cultures. The cells were then shifted to a production medium that contained a 30-fold higher $CuSO_4$ concentration, and as a result, in the production medium, the cells were able to synthesize much higher shikonin concentrations (Table 3). It appears that, as with biotic stress conditions, the high $CuSO_4$ concentrations caused an apparent abiotic stress condition and induced the formation of the secondary natural product. It remains to be seen if this ap-



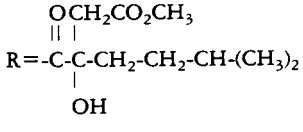
R=H, Cephalotaxine



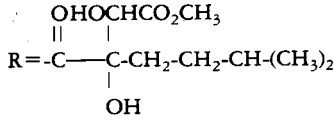
Harringtonine



Homoharringtonine



Deoxyharringtonine



Isoharringtonine

FIGURE 3. *Cephalotaxus harringtonia* alkaloids (26).

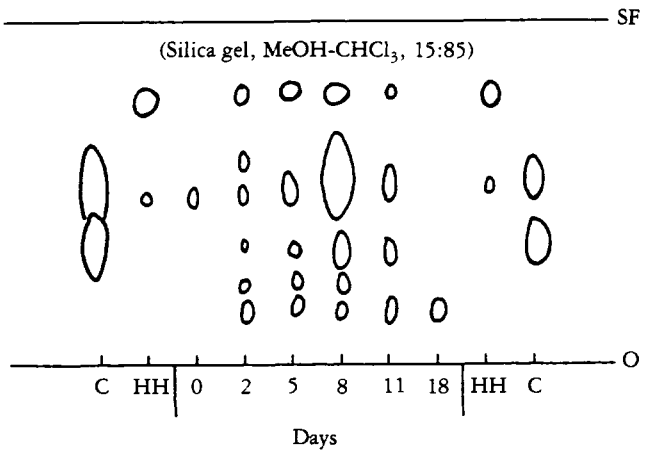


FIGURE 4. Induction of alkaloid formation in *Cephalotaxus harringtonia* cell suspension cultures by *Verticillium dahliae* 277 conidia. Experimenal conditions are as in Table 1. HH=homoharringtonine, C=cephalotaxine.

TABLE 1. Stimulation by *Verticillium dahliae* Autoclaved Conidia of Alkaloid Ester Formation in *Cephalotaxus harringtonia* Cell Suspension Cultures[a]

Days (after addition of conidia)	Alkaloid Esters	
	Cells (mg/g dry weight)	Medium (mg/g dry weight)
0	0.01	0.003
2	0.075	0.103
5	0.09	0.415
8	0.11	0.245
11	0.068	0.051
18	0.019	0.013

[a]Ten-day-old cultures (50 ml) of *C. harringtonia* were stimulated with 3 ml of autoclaved (5 min, 115°, 18 psi) conidia of a 7-day-old *V. dahliae* 277 culture. Alkaloid esters were extracted, quantitated by gc, and authenticated by gc/ms. Each determination is the average of two experiments in duplicates.

TABLE 2. Formation of Morphinan Alkaloids in *Papaver somniferum* Cell Suspension Cultures[a]

Addition	Yield mg/liter)		Yield (mg/g DW)	
	Morphine	Codeine	Morphine	Codeine
None	0.25	0.46	0.07	0.08
Verticillium dahlia[b]	4.64	4.10	1.25	1.11
Fusarium moniliforme[b]	3.91	4.04	1.40	1.44

[a]Ten-day-old *P. somniferum* cultures (250 ml) were inoculated with 3 ml autoclaved conidia from a 9-day-old *V. dahlia* 277 culture or a 4-day-old *Fusarium moniliforme* culture. Morphinan alkaloids were determined by tlc and gc, and authenticated by ms. Each determination is the average of two experiments in duplicates.

[b]Autoclaved (10 min, 115°, 18 psi).

TABLE 3. Shikonin Production in *Lithospermum erythrorhizon* Cell Suspension Cultures (Fujita *et al.*, Plant Tissue & Cell Culture, 1982)

Medium	Shikonin (mg/g DW Inoculum)
LS[a]	0
White	38
LS→White	290
M-5 (Modified LS)→M-9 (30×$CuSO_4$)	3670

[a]Linsmayer-Skoog.

proach is uniformly applicable, and much more research is required to explore this possibility for the formation of secondary natural products in cell suspension cultures.

The above proposal is based on the premise that cultures of plant cells, which accumulate large cell masses in a relatively short time, can be obtained. This is not always the case. For example, *C. harringtonia* exhibits a very slow growth rate in callus (17) and suspension cultures (Zhou and Heinstein, unpublished results). This appears to be a major disadvantage in the commercial use of cell suspensions for the formation of the expensive, antileukemic, alkaloid ester, homoharringtonine. Therefore, alternate routes need to be developed for the formation of homoharringtonine in cell suspension cultures. One alternate route would be the isolation and immobilization of a key enzyme in the pathway or immobilization of intact plant cells. In order to accomplish this efficiently, situations need to be identified in which a physiologically inactive precursor is produced in the plants in relatively large amounts, but the physiologically active compound is present in rather small quantities. For example, thebaine is the main morphinan alkaloid in *Papaver bracteatum*, yet codeine and morphine are the desired pharmaceutically important alkaloids. Therefore, Furuya *et al.* (23) have immobilized *P. somniferum* cells and have shown that the conversion of codeinone to codeine can be accomplished at a bioreactor conversion of 20-30%, which was maintained over a period of 27 days (Figure 5). More significantly, perhaps, was the fact that 88% of codeine was excreted into the medium (personal communication). Although codeinone is not the desired starting material, but rather thebaine, it can be assumed

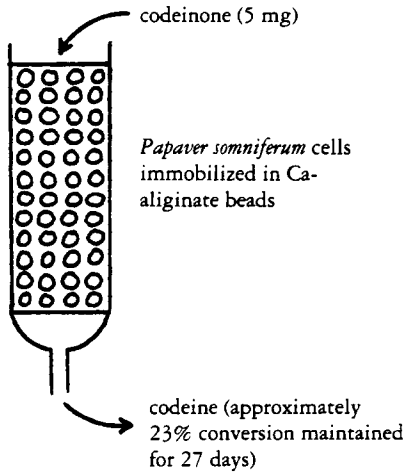


FIGURE 5. Biotransformation of codeinone to codeine. (Furuya, *et al.*, *Phytochemistry*, **23**: 999, 1984).

that it is just a matter of time until the conversion of thebaine to codeine can be accomplished (23).

Others have used the concept of an immobilized enzyme to produce important substrates for biosynthetic studies (24) and the biotransformation of other important secondary natural products in immobilized plant cells, in a most elegant way (25).

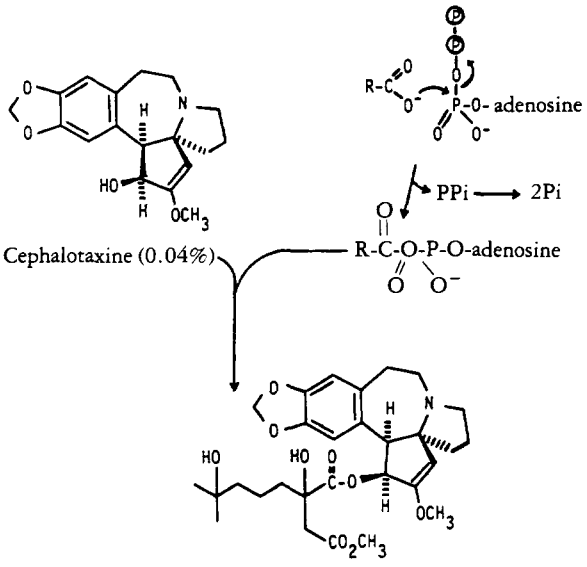
An alternate approach to circumvent the relatively slow growth rate of plant cell suspension cultures is the transfer of the plant genes, which code for the enzymes catalyzing the biosynthetic reactions required for the formation of the secondary natural product, into a bacterial or fungal cell. The fermentation time could then be lowered to 24-48 h rather than the 6-8 weeks that are necessary for a fermentation with a typical plant cell suspension. For the complete *de novo* synthesis of rather complicated compounds such as the *Cephalotaxus* alkaloids, many gene products will be required. At present, the technology to accomplish this is simply not available. For example, the transfer into a prokaryote of ten to twenty genes, in the right sequence and with the proper promoters to insure correct reading, is extremely difficult and not possible with the present recombinant DNA technology. However, a one- or two-step enzyme catalyzed conversion should be possible. In order to succeed with this recombinant DNA technology approach, the right example needs to be selected and must meet, at least, the following requirements:

1. Initially, a reaction needs to be selected which is catalyzed by one enzyme.
2. The substrate for this reaction should be available in relatively large quantities, in vivo or through chemical synthesis.
3. The substrate for the reaction should have little or no physiological-pharmacological activity, yet the product of the one-step reaction should have the desired physiological-pharmacological activity.
4. The one-step conversion should be difficult to accomplish chemically.
5. The end product should have considerable commercial value due, perhaps, to scarcity of plant material or geographical difficulties in obtaining plant material for extraction.

One reaction that meets all the above requirements is the hypothetical conversion of cephalotaxine to homoharringtonine (Figure 6).

Extraction of *C. harringtonia* yields 0.04% of dry weight cephalotaxine (26); however, homoharringtonine concentration is considerably lower (0.0036%). Plant material is hard to obtain because *C. harringtonia* is native to the Republic of China and difficult to propagate through seeds. Chemical synthesis of cephalotaxine has been accomplished in an overall yield of 55% (27). However, the esterification of cephalotaxine to homoharringtonine is difficult. Direct esterification is not possible, but an indirect route has been accomplished with an approximate 8% yield (28). Homoharringtonine has the potential of being as valuable as vinblastine or vincristine as a plant-derived antitumor agent (29), whereas cephalotaxine is inactive.

To be successful in carrying out the conversion of cephalotaxine to homoharringtonine in bacteria, a number of other problems need first to be solved. For example, in order to screen cDNA libraries cloned in appropriate bacterial expression vectors (30), antibodies, specific for the enzyme that catalyzes the reaction, or mRNA coding specifically for this enzyme, are required. In both cases, induction of the formation of homoharringtonine in *C. harringtonia* cell suspension cultures with elicitor compounds (as above) would ensure not only an increased mRNA population coding for the enzymes in the pathway leading to homoharringtonine, but also the enzymes themselves. This would facilitate isolation of the desired mRNA (31), as well as the enzyme that catalyzes the conversion of cephalotaxine to homoharringtonine for antibody for-



Homoharringtonine (0.0036%)
(Chemical esterification: 8% yield)

FIGURE 6. Proposed enzymatic conversion of cephalotaxine to homoharringtonine.

mation. In case problems arise in maintaining expression of the clone's gene(s) or in order to eliminate problems associated with the production of an eukaryotic protein in a bacterial system, the cloned gene(s) can be transferred to a yeast plasmid.

Additional problems—as, for example, the impermeability of the engineered bacteria to the substrates for the reaction—may arise. Especially the activated acid (acyl adenylate) will be difficult to be absorbed by the bacterium. Since, in eukaryotic systems, a single enzyme catalyzes the formation of the acyl adenylate and its condensation with the alcohol, it is conceivable that the enzyme catalyzing the formation of homoharringtonine from cephalotaxine and the appropriate acid, can also activate the acid to the acyl adenylate in the bacterial cell using ATP available *in situ*. Similarly, inability to excrete the synthesized homoharringtonine may poison the engineered bacterium. However, the overall approach is attractive enough, in terms of commercial application of plant secondary natural product formation, to warrant considerable future interest in this strategy that would permit the formation of useful, although expensive, drugs from plant cells in a large-scale fermentation.

The future of the commercial production of secondary natural products in cell suspension cultures, therefore, depends primarily on strategies that increase yield and shorten fermentation periods. In order to develop these strategies, as outlined above, emphasis will need to be placed on a number of areas of basic research. These areas include:

1. Growth and production media, controlled-abiotic stress.
2. Induction—gene activation using biotic stress or elicitors.
3. Identification, isolation, and characterization of key biosynthetic enzymes.
4. Immobilization of intact plant cells or enzymes identified above.
5. Regulation of key biosynthetic enzymes, including mRNA systhesis and isolation.
6. Plant genes—structure, function, and expression.
7. Isolation and transfer of plant genes to prokaryots, especially genes coding for enzymes identified above.

The manipulation of plant cells, plant genes, and plant enzymes, rather than the plants themselves, should, in the future, provide us with a host of useful secondary metabolites.

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