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Further Approaches in the Production of Secondary Metabolites by Plant Tissue Cultures

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The production of useful compounds via plant tissue and organ culture systems has received increasing attention during recent years. Often applied tehniques are cell culture, organ culture and culture of genetically transformed cells and tissues. In most of the cases only low yields of the desired secondary metabolites were obtained by cell culture systems. Many efforts have been made to improve the productivity of the plant tissue cultures, such as studies on hormone-dependency, media composition or light effects. Examples of recent work in this field which may lead to a wider use of plant tissue culture are discussed.

Introduction

Until today, plants remain a main source of a large range of products such as flavors, insecticides and many pharmaceuticals. Most of the compounds used are so called 'secondary metabolites, which are believed to play a minor role in the basic life process of the plants, but are important for their fitness in plant-plant and plant-environment interactions. Since it is possible to cultivate plant cells in large quantities, plant cell suspension cultures were regarded as a potentially suitable system for producing phytochemicals at an industrial level[1]. Even while several plant cell suspension cultures have been shown to produce secondary metabolites in large number (e. g. *Berberis willsoniae, Coleus blumei, Coptis japonica and Lithospermum erythrorhizon*), most of the plant species fail to accumulate significant amounts of secondary metabolites when cultured as undifferentiated cells (e. g. *Atropa belladonna, Duboisia leichhardtii, Cinchona ledgeriana, Digitalis lanata*[2]). The biosynthesis of the secondary metabolites seems to be related to the differentiation of the cells or plant organs such as shoots or roots[3,4]. The cultivation of intact plants or their organs, however, is in most cases either hormone-dependent or of poor growth, so that these systems are not very useful for industrial purposes[5]. A recent development to overcome those difficulties is the genetic transformation of plants with *Agrobacterium rhizogenes* or *A. tumefaciens*. The transformed plant cells develop fast growing "hairy roots" or "crown galls" which are able to produce the secondary metabolites, which are nomally formed in the roots or all parts of a plant at a stable and a high yield. In this article we shall review the progress that has been made to produce useful secondary metabolites by *in vitro* cultures of plants. We will discuss some examples of recent work which has been done in this field.

Cell Cultures

1. *Podophyllum*

Two anti-cancer drugs, etoposide and teniposide, are synthesized from the plant derived lignan

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podophyllotoxin [**Fig. 1**]. This compound is extracted from two *Podophyllum* species, *P. hexandrum* and *P. peltatum*. The former contains up to 4 % dry wt. of podophyllotoxin in its roots. As the natural sources of podophylloxin are very restricted, the attempt has been made to produce it by tissue cultures. The callus culture of *P. hexandrum* was established from roots of the *in vitro* cultures on a modified Gamborg B5 medium[42]. Suspension cultures were derived from the calli in the same liquid medium. The callus and cell suspension cultures showed a wide variety in producing podophyllotoxin (up to 0.1 % dry wt.). High yielding cell lines were selected by their dark brown color. The highest content which was obtained in callus and cell suspension cultures was 0.3 % dry wt., produced by calli grown in the dark on modified Gamborg B5 medium[42,44]. Precursor feeding (coniferyl alcohol and coniferin) increased the productivity of the low producing cell suspension cultures, but not over a maximal yield of 0.3 % dry wt[43]. The production of podophyllotoxin derivatives has been reported from root and cell suspension cultures of *Linum flavum*. The highest amount of methypodophyllotoxin which was produced in these culture systems was 1.3 % dry wt. in organized roots and 0.2 % dry wt. in cell suspension[45,46].

2. Rheum

The roots and rhizomes of rhubarb, *Rheum palmatum, R. tanguticum* or *R. officinale* are used in medicine for chronic constipation. The main purgative principle of rhubarb are the dimeric anthraquinone derivatives sennoside A and B [**Fig. 2**]. Callus cultures of a hybrid of *R. palmatum* x *R. coreanum* were established from seedlings[39]. The seeds germinated after treatment at 4°C for 3 weeks. Calli were initiated on MS solid medium containing 2, 4-D and kinetin at 21°C. Other phytohormone combinations were less effective to induce callus formation. Cell suspension cultures showed best growth in medium containing IAA and the synthenic cytokinin 4-PU-30 [*N*-(2-chloro-4-pyridyl)-*N'*-phenylurea] . The highest yield of sennosides (0.012 % dry wt.) were obtained on MS medium containing IAA, 4-PU-30 and maltose (2 %) as carbon source. When kinetin was used instead of 4-PU-30, the calli failed to produce sennoside B, but the largest content of sennosid A was observed.

3. Digitalis

Digoxin [**Fig. 3**] is one of the important drugs for treatment of cardiac diseases. Sources for the compound and its chemically synthesized derivatives are *Digitalis* species, mainly *Digitalis lanata*. Many efforts have been made to obtain *in vitro* cultures of *Digitalis*, which are able to synthesize significant amounts of cardiac glycosides. However, undifferentiated cultures did not have the capability of *de-novo* synthesis of digoxin so far, but the biotransformation of digitoxin and β-methyldigitoxin to more useful 12-hydroxy-derivatives digoxin and β-methydigoxin has been

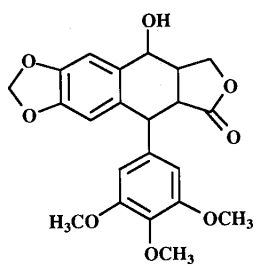


Fig. 1 1) Podophyllotoxin 2) Anti-tumor activity, substrate for chemical synthesis of anti-cancer drugs 3) *Podophyllum* spp., in rhizome and resin (up to 50 %) derivatives in *Linum flavum*

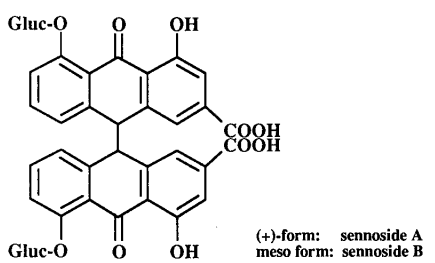
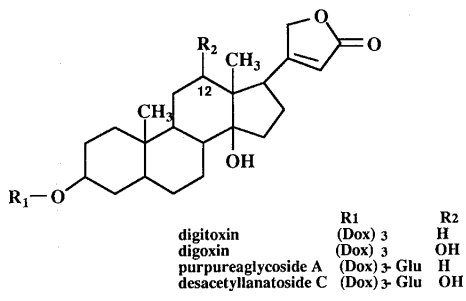


Fig. 2 1) Sennoside A and B 2) Purgative, antiobstipans 3) *Cassia senna, C. angustifolia* leaves and fruits, *Rheum palmatum, R. officinale, R. coreanum* rhizome

Table 1. Recent examples of production of useful secondary metabolites by *in vitro* cultures.

PRODUCT	PLANT	CULTURE	YIELD+	REFERENCE
Alkaloids				
Indole alkaloids				
vinblastine,	*Catharanthus roseus*	shoot	0.015	[5]
catharanthine,	*Catharanthus roseus*	hairy root	4.0	[6]
pleiocarpamine	*Amsonia elliptica**	hairy root	0.25	[7]
Quinoline alkaloids				
quinine, quinidine	*Cinchona ledgeriana**	crown gall	1.5	[8]
	C. pubescens	hairy root	0.5	[9]
Berberine	*Thalictrum* spp.	cell suspension	0.02	[10] [11] [12]
	Coptis japonica	immobilized cell	0.02	[13]
Tropane alkaloids				
hyoscyamine	Solanaceous spp.	hairy root	15.0	[14] [15]
Emetine, cephaeline	*Cephaelis ipecacuanha**	callus and root	13.0	[16] [17] [18] [19] [20]
Terpenoids				
Monoterpenes				
linalool, linalyl-acetate	*Mentha citrata**	transformed shoot	n. d. a	[21]
limonene	*Pelargonium fragans*	callus with shoot	n. d. a	[22]
chrysanthemic acid	*Chrysanthemum cinerariaefolium*	callus	0.001	[23]
amarogentin	*Swertia japonica*	hairy root	0.005	[24]
Sesquiterpenes				
dihydroleucodin	*Artemisia douglasiana*	callus	0.7	[25]
s.-lactones	*Ambrosia tenuifolia*	callus	1.8	[26]
santonin	*Fossombronia pusilla**	plantlet	0.001	[27]
hernandulcin	*Lippia dulcis**	shoot	25.0	[28]
		hairy root	1.25	[29]
Diterpenes				
perrottetianal A, B	*Fossombronia pusilla**	plantlet	1.8	[27]
sclareol	*Salvia sclarea,*	cell suspension	0.003	[30]
Steroids				
cardiac glycosides	*Digitalis lanata** (biotransformation)	somatic embryo	0.8	[31]
		cell suspension	2.5	[32]
		crown gall	0.02	[33]
		hairy root	0.02	[34]
	*Digitalis purpurea**	hairy root	0.001	[35]
Phenolics				
Tannins	*Sanguisorba officinalis*	root	49.3	[36]
	Geranium thunbergii	cell suspension	2.5	[37]
		hairy root	8.3	[38]
Quinones	*Rheum palmatum**	callus	0.12	[39]
Anthocyanins	*Ajuga reptans*	cell	15.0	[40]
	Euphorbia millii	cell suspension	15.0	[41]
Podophyllotoxines	*Podophyllum** *hexandrum*	cell suspension callus	3.1	[42] [43] [44]
	Linum flavum	root and cell suspension	13.0	[45] [46]
Naphthoqinones	*Lithospermum erythrorizon*	cell suspension	156	[47]
		hairy root	25.5	[48]

* : Examples further discussed in thds review. + : Highest content in the cultures, calculated as mg/g dry wt., n.d.a.: no data available.



	R1	R2
digitoxin	(Dox) 3	H
digoxin	(Dox) 3	OH
purpureaglycoside A	(Dox) 3- Glu	H
desacetyllanatoside C	(Dox) 3- Glu	OH

Dox: digitoxose, Glu: glucose

Fig. 3 1) Cardiac glycosides, digitoxin, digoxin 2) Cardiac diseases 3) *Digitalis lanata* (digoxin and derivatives), *D. purpurea* (digitoxin and derivatives) leaves

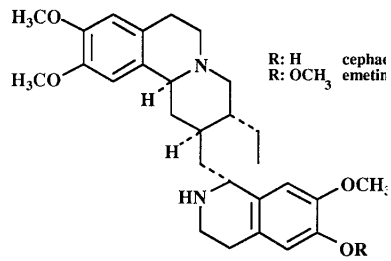


Fig. 4 1) Emetic alkaloids, emetine, cephaeline 2) Emetic, expectorant, amoebacide 3) *Cephaelis ipecacuanha, C. accuminata* root

scaled un to 200 *l* stage fermenter[49]. Cell suspension cultures of *D. lanata*, grown in modified MS medium, were subcultured to the medium containing 8 % glucose. In this "production medium" up to 82 % of the added digitoxin (0.75 g/*l*) was biotransformed to the main product of desacetyllanatoside C, after 11 days of incubation. If digitoxin was added in a lower concentration (0.3 g/*l*), over 75 % was hydroxylated to digoxin within 3 days[32]. Organized cultures of *Digitalis* have been developed, and recently a fermenter system for somatic embryos producing a high yield of cardenolides has been reported[31]. Therefore, cardiac glycoside biosynthesis seems to be closely related to the differentiation of the cultures.

Organ cultures

1. Ipecacuanha

Cephaelis ipecacuanha, Rubiaceae, is an Amazonian plant, the roots of which are used as an expectorant, amoebacide and emetic. The pharmacological activity of its roots is related to their alkaloid content, mainly to cephaeline and emetine [**Fig. 4**]. Callus[16,17], root[17,18] and adventitious shoot cultures[19,20] of *C. ipecacuanha* have been shown to be able to produce emetic alkaloids. The shoot cultures of *C. ipecacuanha* were established from the shoot tips of plants grown in the greenhouse. Multiple shoots were then obtained on B5 medium supplemented with NAA and kinetin. From these shoots the adventitious root and callus cultures were established on MS solid medium. But the callus cultures produced the emetic alkaloids in trace amounts and only for the first two passages of the cultures[17]. On the other hand, calli induced from hypocotyl on MS medium containing 2, 4-D and NAA accumulated 0.346 % dry wt. emetine and 0.93 % dry wt. cephaeline after 3～4 weeks of culture, though the growth index was relatively low[16].

The adventitious root cultures were established and maintained in MS liquid medium, containing NAA[18]. Cephaeline was the main product in the adventitious roots. Between the 4th and the 8th week, large amounts of cephaeline were found in the medium and in the roots. Emetine was produced in the later culture stage, the production starting after 7 weeks of culture. Emeine was detected only trace amounts in the medium compared to cephaeline. The addition of IAA derivatives increased the alkaloid productivity of the roots, although they were added to the medium at a very low concentration. The adventitious roots cultured in one 100 m*l* flask for only 7 weeks yielded 0.6 mg emetine and 2.4 mg cephaeline. This yield is comparable to that in roots of one-year-old regenerated plants. Adventitious shoot cultures without roots could synthesize alkaloids as well as root cultures, and both of them accumulated much more cephaeline than emetine though intact plant contained almost the same levels of emetine and cephaeline in its roots. It might

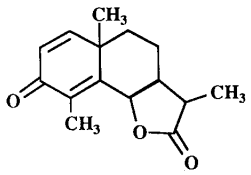


Fig. 5a 1) α-Santonin 2) Anthelmintic 3) *Artemisia cina, A. annua* essential oil, leaves

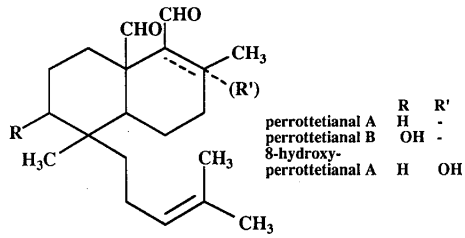


Fig. 5b 1) Perrottetianal 2) Bacteriostatic 3) *Porella perrottetiana, Fossombronia pusilla* (Hepaticae)

indicate the different capability of alkaloid biosynthesis between *in vitro* culture and the intact plant.

2. Fossombronia

Liverworts (bryophyta) are known to produce a large variety of terpenes. Some of them exert interesting biological activities such as insecticidal, molluscicidal or tumor inhibitory activity. It is quite difficult to investigate secondary metabolites in *Fossombronia pusilla*, a small liverwort, since there is difficulty collecting a sufficient amount in the wild. Therfore, the *in vitro* culture of *F. pusilla* was established in order to analyze its secondary metabolites (sesqui- and diterpenes [**Fig. 5**])[27]. The plantlets grew well on Gamborg B5 solid medium, but when they were transferred to Gamborg B5 liquid medium the cultures died. On the other hand, the addition of vitamin B_{12} to the liquid culture of *F. pusilla* stimulated its growth and the production of the terpenes. After 32 days of culture the 5-fold amount of terpenes was produced compared to the solid medium. The main products were the diterpenedialdehydes perrottetianal A, B and 8-hydroxyperrottetianal A. Santonin, which was reported for the first time as constituents of a bryophyte, was produced in a lower amount. The *in vitro* culture of *F. pusilla* made it possible to obtain the material for the first phytochemical analysis of this liverwort.

Transformed cultures

1. Cinchona

Cinchona species produce a large variety of quinoline alkaloids, of which quinine and quinidine [**Fig. 6**] are commercially important. Quinine is used as an antimalaria drug and as bittering agent in many soft-drinks. Quinidine is an anti-arrhythmic drug which is used for several heart diseases. A culture of *Cinchona ledgeriana* transformed with *A. tumefaciens* has been obtained which is able to produce the cinchona alkaloids[8]. The initial alkaloid production in these crown gall cultures (6.7 mg/*l*) was ca. 5-fold greater than the reported value of nontransformed cell suspension cultures. In the non-transformed cultures, up to 140 μg alkaloids/g fresh wt. were produced when the medium was optimized for production (medium containing IAA and zeatin riboside). Cell suspension cultures released up to 75 % of the alkaloids into the culture medium when they were cultured with low concentration of 2, 4-D and BA[50]. In contrast to the findings with cell suspension and callus cultures, the addition of phytohormones (such as IAA or IBA in combination with zeatin) did not affect the alkaloid production in the transformed cells. Incubation of the transformed cultures under white or blue light inhibited the alkaloid production. On the other hand, dark-grown cultures produced up to 150 μg alkaloids/g fresh wt., while 34 % of the total alkaloids were released to the medium. The main product was cinchonine, the precursor of quinidine[8]. The hairy root cultures, transformed with *A. rhizogenes* LBA 9402, produced a maximum of 50 μg alkaloids/g fresh wt. after 45 days. The main alkaloid produced by the hairy roots was quinine[9]. Only about 1 % of the

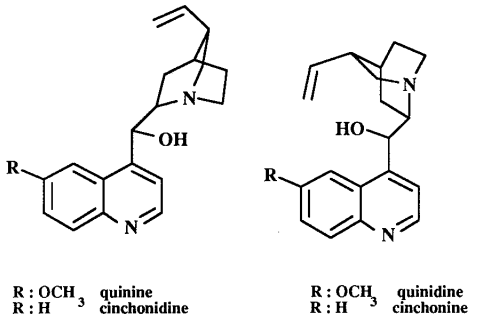


Fig. 6 1) China alkaloids 2) Anti-malaria, antiarrhythmic 3) *Cinchona ledgeriana, C. pubescens, C. succirubra* bark

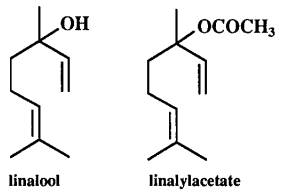


Fig. 7 1) Linalylacetate 2) Bacteriostatic, fragancy 3) Lamiaceae plants, *Mentha* spp., *Lavandula angustifolia* essential oil

alkaloids produced by the hairy roots were released into the medium.

2. Mentha

Stems of *Mentha citrata* were transformed with *A. tumefaciens* T37, which usually causes the development of shooty teratoma[21]. Three weeks after infection, galls appeared at the infected sites and shoots were initiated after 4 weeks. The shooty teratoma were subcultured onto MS solid and in Gamborg B5 liquid media. The growth rate of the galls was faster in liquid medium, but this might also be caused by the different medium compositions as well as the absence of agar. The shooty teratoma produced the same terpenes as the mother plant which was confirmed by GC/MS analysis. Linalool and linalylacetate [**Fig. 7**] were the main constituents (94 % of the total essential oil). The total yield of essential oil produced by the shooty teratoma was ca 1/4 the amount obtained from the mother plant. Electron microscopy of the galls revealed the presence of secretory glands on the surface of the leaves of the transformed shoots. Their appearance was similar to those observed on the surface of the mother plant.

3. Green hairy root cultures

The production of secondary metabolites by hairy root cultures has been reported over a wide range of plants. On the other hand the influence of culture conditions namely light on the productivity of the transformed root cultures has not been examined deeply. Three examples of the influence of light on the productivity of hairy root cultures are given with 1) alkaloid production in *Amsonia elliptica*[7], 2) terpene production in *Lippia dulcis*[29] and 3) cardiac glycoside production in *Digitalis lanata*[34] and *D. purpurea*[35].

The hairy roots of *A. elliptica* cultured in Gamborg B5 liquid medium produced the same indole alkaloids [**Fig. 8**] as the normal root cultures and the mother plant, but at a lower level. In addition, the growth of the hairy roots cultured in the dark was rather poor, while the hairy roots cultured in 16 h light turned dark green and showed rapid growth. In both cultures pleiocarpamine was the main alkaloid. In the hairy roots cultured in 16 h light the indole aldehyde vallesiachotamine was produced during the later growth stage at 10- fold amount compared to the hairy roots cultured in the dark. Hairy roots transferred to medium containing NAA showed rapid growth either in the dark or in 16 h light and about a 10- fold amount of alkaloids was obtained.

The sweet sesquiterpene hernandulcin [**Fig. 9**] is the minor constituent (0.004 % dry wt.) of the Verbenaceae *Lippia dulcis*. Although (+)-hernandulcin could be synthesized from *R*-(+)-limonene, the overall yield was only 1.09 %, which makes the use of hernandulcin as a sweetener difficult. The hairy roots of *L. dulcis* transformed with *A. rhizogenes* A4 grew well in MS liquid medium. The hairy roots cultured in the dark in hormone-free MS liquid medium did not produce

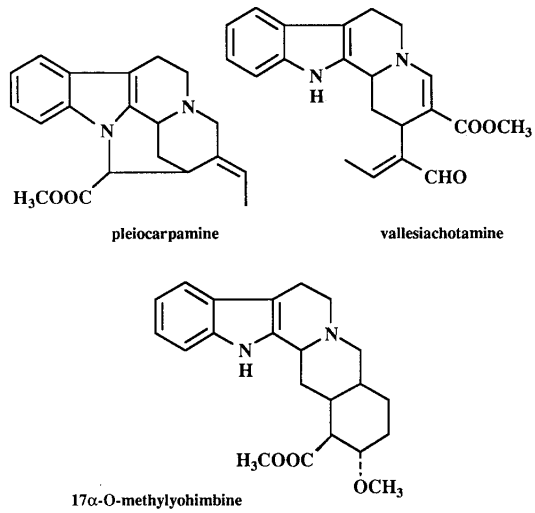


Fig. 8 1) Indole alkaloid, vallesiachotamine 2) Anti-leucemia 3) *Amsonia brevifolia, A. elliptica, Tabernaemontana* spp. root

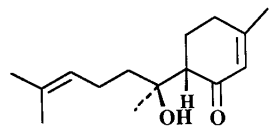


Fig. 9 1) Hernandulcin 2) Natural sweetner 3) *Lippia dulcis* leaves and flowers

any detectable level of the sweet sesquiterpene hernandulcin or other mono-and sesquiterpenes. On the other hand, when these cultures were placed under 16 h light, the hairy roots turned green and produced hernandulcin together with several terpenes. However the relative composition of the n-hexane extract obtained from the green hairy roots was different from that of the aerial parts of mother plant. The proportion of monoterpenes produced by the transformed roots was much larger than that in the mother plant. During the first 3 weeks of culture in the light, the hernandulcin content in the hairy roots was low, but at the beginning of the logarithmic growth the production of hernandulcin increased. In contrast, hernandulcin was not detected in normal root cultures, although the roots turned partially green when they were cultured in 16 h light. The addition of a low concentration of chitosan (0.2-10.0 mg/*l*) enhanced the production of hernandulcin 5-fold (up to 1.2 mg/g dry wt.).

Axenic plants of *Digitalis purpurea* were transformed with *A. rhizogenes* 15834. Hairy roots were cultured on Gamborg B5 medium and analyzed by enzyme-linked immunosorbent assay (ELISA) for their cardenolide content[34]. The production of cardenolides was directly correlated to the content of chlorophyll in the cells and a maximum of 250 ng/g fresh wt. was obtained. *D. lanata* hairy roots, transformed with *A. rhizogenes* A4, were cultured in half-strength MS liquid medium. In the light a maximal amount of 16.5 μg digoxin/g dry wt. was detected in the green hairy roots. The same clone cultured in the dark produced only 0.04 μg/g of digoxin[34]. Contradictory results were obtained by the transformation with *A. tumefaciens*. The crown galls, derived from the leaves, did not contain chlorophyll, but produced up to 20 μg/g dry wt. of total cardenolides (calculated from radio immuno assay for digitoxin)[33].

Conclusions

The use of cell and organized cultures for production of secondary metabolites has a number of attractions. The capability of a wide number of plants to synthesize their secondary products *in vitro* has been demonstrated. In general it has been shown that the productivity of the cultures is related to their organization, e. g, organized roots of *C. ipecacuanha* produce more alkaloids than calli. However, to lead to an economical process further optimization of the culture systems is required. This implies the deeper investigation of the biological process which leads to the formation of secondary metabolites, the development of high producing cell lines and the scale up of the production process. One way to investigate the product formation *in vitro* are the culture of transformed organs, such as hairy roots. Here it has been shown, that the green hairy root cultures have the capability to produce the secondary metabolites of the intact plant, including the aerial parts. To explain this phenomenon further investigations are required. Another attempted application of plant tissues *in vitro* is the development of culture systems which secrete the desired products to the medium. In this case also the biotransformation of exogenous compounds must be regarded as worth of further development.

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