In vitro Cultures for the Production of Some Anticancer Agents (Review Article)

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Abstract: Organ, tissue and cell culture and other biotechnological techniques are useful ways to obtain biologically active constituents those play an important roles in our life (They have pharmaceutical, medicinal and economical importance in our life). These ways (in vitro) of obtaining secondary metabolites are better than the classical methods since: They are natural in origin, so they are of little toxic side effects compared with synthetic drugs. They are safe sources and do not cause any environmental pollution as like as those occurred by applications of pesticides and insecticides to farm lands. They are performed under controlled conditions (since the yield can be increased, with increasing replicates number and by using elicitors, fomenters and bioreactors in a large application scale), they are performed under aseptic conditions (This means that, they are system free of contaminating microbes “ fungi and bacteria”, consequently they are clean sources of drugs). Using these techniques we can direct the culture for producing the organ that contains the highest amounts of the needed product (for example, root culture can be produced using Agrobacterium rizogenes to obtain substances that produced in root cells only). Production cycle is smaller than that of normal culture in land (it takes little time), since callus cultures with short life cycle may be a good source for production of needed phytochemicals. Using these methods we can conserve our natural resources (wild plants) instead of over-collection by herbalists. Finally, the cost can be decreased if done on a large scale (instead of Fedens language “large areas” we use jars in small place = higher productivity of secondary metabolites). This review will discuss with some detailed examples the in vitro production of some anticancer agents.

Key words: Anticancer - In vitro cultures – Pharmaceutical products- Callus cultures- Cell suspension cultures.

1. Introduction:

The culture of microorganisms (Penicillium, Streptomyces etc,) has long been used industrially to obtain substances such as penicillin, streptomycin etc., which are important pharmaceutically. Higher plants are also an important source of all types of substances, especially medicines (flavonoids, alkaloids, saponins, antheraquinones, cardiac glycosides, ethereal oils, steroids etc.,)

Traditionally, the medicinal plants have been grown and then active components extracted and this is likely to remain the normal procedure. However the production of medicinal plants can present problems, which have led to the search of other ways to produce naturally occurring substances:

1- Production in the field is strongly dependent on season, weather, climate, diseases and pests etc.,

2- Naturally occurring sources, especially in the tropics and subtropical zones are becoming limited and some medicinal plants are extremely scarce.

3- There may be technical and economic problems in production.

4- Production is labor intensive and therefore costs are high.

5- There may be political instability in the country where the plants are available resulting in an interrupted supply (Pierik, 1987).

The biotechnological production of valuable secondary metabolites in plant cell, tissue or organ cultures is an attractive alternative source to the extraction of whole plant material (Alam, 2012 a,b, 2013).

Field of the plant biotechnology is focusing on the production of plant secondary metabolites. For most compounds of interest, e.g., morphine, quinine, vinblastine, atropine, scopolamine and digoxin, one has so far not been able to come to a commercially feasible process (Kumar et al., 2012).

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 case of shikonin and berberine with success, in many others with limited success.

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. With the culture of differentiated cells one has in most cases been able to get the production of the desired compounds in levels comparable to that of the plant; however the culture of such differentiated tissues on a large scale in bioreactors is a major constraint. For studies of
the biosynthesis, such systems are very useful. The second approach mentioned, the use of elicitors has been successful in several cases. However, it remains limited to a certain types of compounds for each plant, compounds which almost likely act as phytoalexins in these plants. Therefore attention is more and more focused on metabolic engineering which includes increasing activity of enzymes which are limiting in a pathway; include expression of regulatory genes; block competitive pathways; block catabolism.

Plant cell cultures provide an excellent system for studying the biosynthesis of secondary metabolites. For the large scale production of these compounds in most cases the production is too low for a commercialization. To improve yields metabolic engineering offers promising prospective, but requires understanding the regulation of the secondary metabolite pathways involved on the levels of products, enzymes and genes, including aspects as transport and compartmentation. Unraveling the regulation requires a joint effort of several disciplines, e.g., phytochemistry, plant physiology, cell biology and molecular biology (Purohit, 2007).

The present review aimed at discussing some successful examples of in vitro production of active pharmaceutical products against cancer.

2. Materials and Methods:
In this review in vitro cultures were used as great sources of active pharmaceutical products against cancer as following:

A. Production of carotenoids from in vitro cultures of tomato cell suspension cultures (Lycopersicon esculentum) as anticancer agents (Campbell et al., 2006, Lu et al., 2008 and Engelmann et al., 2010)

B. In vitro cultures of Rheum palmatum L. for the production of anthracene derivatives as anticancer agents (Kasperová et al., 2003).

C. In vitro cultures of Polygonum hydropiper for the production of some anticancer agents (Ono et al., 1998).

D. In vitro cultures of Rumex alpinus L. for the production of some anthraquinones as anticancer agents (Van and Labadie, 1981).

E. Quercetin production from in vitro cultures of Pluchea lanceolata Oliverr and Hiern as an anticancer agent (Arya et al., 2008).

F. Terpenoids and flavonoids production from in vitro cultures of Azadirachta indica as anticancer agents (Babu et al., 2008).

G. Tropane alkaloids production from in vitro cultures of Brugmansia suaveolens as anticancer agents (Zayed and Winka, 2004).

H. Anthraquinones production from in vitro cultures of Cinchona robusta as anticancer agents (Schripsema et al., 1999).

I. In vitro cultures of Fagopyrum esculentum for the production of some flavonoids as anticancer agents (Trotin et al., 1993).

J. Podophyllotoxin production from in vitro cultures of Podophyllum (Sauerwein et al., 1992, Benisril et al., 2004 and Bhattacharyya et al., 2012).


M. Indole Alkaloids: Vinblastine derivatives from in vitro cultures of Catharanthus (Kurz, G.W. and Constabel, F. National Research Council of Canada publication no. 38467).

N. Camptothecin derivatives from in vitro cultures of Camptotheca (Watase et al., 2004 and Benisril et al., 2004).


P. Production of shikonin derivatives from in vitro cultures of Arnebia (Royle) Johnst (Ruanzicao) as an antitumor agent (Xiong et al., 2009).

Q. Anticancer activity of in vitro cultures of rice (Deshpande A et al., 2011).

3. Results and Discussion:
Some detailed examples of in vitro cultures producing anticancer agents:

A- Production of carotenoids from in vitro cultures of tomato cell suspension cultures (Lycopersicon esculentum) as anticancer agents:

An abundance of epidemiological evidence suggests that, a diet rich in fruits and vegetables is associated with a reduced risk of several forms of cancer. Phytochemicals present in fruits and vegetables have been proposed to provide protection from carcinogenesis through various biological actions. With respect to tomatoes, increased consumption of tomatoes and tomato products has been significantly associated with a reduced risk of prostate cancer in several epidemiological studies.

Carotenoids are natural yellow, orange, and red pigments present in tomatoes and possess a wide range of proposed biological functions, including antioxidant and anticarcinogen properties and immunoprotection. Lycopene, the most abundant tomato carotenoid, has been the primary focus of
both in vitro and in vivo studies examining the relationship between increased intake of tomatoes and reduced risk of prostate cancer. However, recent studies suggested that, other tomato phytochemicals may also modulate prostate cancer.

The herbicide norflurazon was added to establish cell suspension cultures of tomato (Lycopersicon esculentum cv. VFNT cherry), to induce the biosynthesis and accumulation of phytoene precursors, phytoene and phytofluene, in their natural isomeric forms (15-cis-phytoene and two cis-phytofluene isomers). Norflurazon concentrations, solvent carrier type and concentration, and duration of the culture exposure to norflurazon were screened to optimize phytoene and phytofluene synthesis. Maximum yields of both phytoene and phytofluene were achieved after 7 days of treatment with 0.03 mg norflurazon/40 mL fresh medium, provided in 0.07 % solvent carrier. Introduction of 14C-sucrose to the tomato cell culture medium enabled the production of 14C-labeled phytoene for subsequent prostate tumor cell uptake studies. In DU 145 prostate tumor cells, it was determined that, 15-cis-phytoene and an oxidized product of phytoene were taken up and partially metabolized by the cells.

The ability to biosynthesize, radiolabel and isolate these carotenoids from tomato cell cultures is a novel, valuable methodology for further in vitro and in vivo investigations into the roles of phytoene and phytofluene in cancer chemoprevention.

The carotenoid biosynthetic enzyme, phytoene desaturase, causes two consecutive desaturation reactions to convert phytoene to α-carotene, with phytofluene as an intermediate product. Norflurazon is a known inhibitor of phytoene desaturase.

Therefore, it was hypothesized that, through the provision of norflurazon to this tomato cell suspension culture system, this enzyme would be primarily blocked at the first desaturation step, thus ultimately eliciting phytoene production within the tomato cells. Subsequently, phytofluene was expected to accumulate to a lesser extent than phytoene in the tomato cells following norflurazon addition.

In preliminary trials, experiments were conducted to determine whether various concentrations of norflurazon and duration of culture exposure to norflurazon affected tomato cell yield of phytoene. Tomato cells were treated with norflurazon (0.3, 0.03 and 0.003 mg norflurazon/40 mL fresh media, with final media concentration of 0.29 % ethanol) for 3, 5, 7, or 9 days. Results indicated that, a norflurazon treatment of 0.03 mg norflurazon/40 mL fresh media for 7 days provided the highest phytoene yield (170.3 µg phytoene/L tomato cell suspension), as compared to the other treatments. The addition of norflurazon to the tomato cell cultures was effective in the inhibition of phytoene desaturase, as no quantities of α-carotene or lycopene were detected in the treated cells.

Data from these preliminary trials were utilized to conduct further experiments in this tomato cell suspension culture system. A novel approach has successfully developed to produce naturally accumulating tomato isomers of phytoene and phytofluene in tomato cell suspension cultures through the incorporation of a phytoene desaturase inhibitor to the culture medium.

Moreover, 14C-labeled and 15-cis-phytoene have been biosynthesized and isolated through this culture system. Preliminary data from a human prostate tumor cell type suggested that, indeed 15-cis-phytoene is taken up by cells, as well as, a potential oxidized product of 15-cis-phytoene, and are apparently metabolized to unidentified polar phytoene metabolites. Through biosynthesizing naturally accumulating isomers of radiolabeled phytoene and phytofluene through in vitro plant techniques, further in vitro and subsequent in vivo bioavailability and metabolism experiments can be conducted. Only then can studies begin to adequately evaluate the potential roles of phytoene, phytofluene, and/or their metabolites in health, including prostate cancer prevention.

Isotopically labeled tomato carotenoids, phytoene, phytofluene and lycopene, are needed for mammalian bioavailability and metabolism research but are currently commercially unavailable. Multiple in vitro tomato cell lines were established and screened for carotenoid production, test the best producers with or without the bleaching herbicides, norflurazon and 2-(4-chlorophenylthio)triethylamine (CPTA) and to use the greatest carotenoid accumulator for in vitro 13C-labeling. Different Solanum lycopersicum allelic variants for high lycopene and varying herbicide treatments were compared for carotenoid accumulation in callus and cell suspension cultures of the hp-1 line were chosen for isotopic labeling. When grown with [U]-13C-glucose and treated with CPTA, hp-1 suspensions yielded highly enriched 13C-lycopene with 45% of lycopene in the M+40 form and 88 % in the M+35 to M+40 isomomer range.

Response surface methodology (RSM) was employed, which combines fractional factorial design and a second-degree polynomial model. Tomato cells were homogenized with ethanol, saponified by KOH, and extracted with hexane, and the lycopene content was analyzed by HPLC-PDA. Five factors were varied at five levels: ethanol volume (1.33–4 mL/g); homogenization period (0--
The maximal yield of total catechins in suspension and about cultured cells was about 1.5 times as much as callus
range of 2.6 wt and that of (−)epicatechin was in the range of 1.1–7.7 mg/g dry wt after 6 days from the start of subculture. This is the highest value of (+)-catechin content among reports on catechin production in vitro so far published. The amount of (−)-epicatechin was in the range of 1.1–7.7 mg/g dry wt and that of (−)-epicatechin-3-O-gallate was in the range of 2.6–6.4 mg/g dry wt for a culture period of 15 days, respectively. Comparing with plant parts in regard to (+)-catechin, the amount of suspension-cultured cells was about 1.5 times as much as callus cells, about 9 times that of leaves (3.2 mg/g dry wt) and about 7 times that of stems (4.0 mg/g dry wt). The maximal yield of total catechins in suspension-cultured cells was 4.3% dry wt. It is well known that (+)-catechin, (−)-epicatechin and (−)-epicatechin-3-O-gallate can play an important role as anticancer agents.

B- In vitro cultures of Rheum palmatum L. for the production of anthracene derivatives as anticancer agents:

Kasparová et al., 2003 examined the effect of four concentrations of calcium ions on the production of anthracene derivatives by means of the callus and suspension cultures of Rheum palmatum L. (Polygonaceae), which was elicited by an abiotic elicitor "20 µM solution of lead dichloride". The culture was cultivated on a Murashige-Skoog medium with an addition of 10 mg/l of alphahydroxybenzylacetate. It follows from the results that, the maximal content of anthracene derivatives, determined by the photometric determination according to PhBs4, was demonstrated in the suspension culture after 24 hours administration of the elicitor and 10 mM calcium chloride solution. In comparison with the control and elicited groups, the production was stimulated by 44 and 17%, respectively. After an addition of calcium ions to the elicited callus culture, no positive influence on the production was stimulated by 44 and 17%, respectively. After an addition of calcium ions to the elicited callus culture, no positive influence on the production was observed. It is well known that anthracene derivatives can play an important role as anticancer agents.

C- In vitro cultures of Polygonum hydropiper for the production of some anticancer agents:

Callus and suspension-cultured cells were induced from hypocotyls of Polygonum hydropiper seedlings. The callus and suspension-cultured cells produced mainly (+)-catechin accompanied by (−)-epicatechin and (−)-epicatechin-3-O-gallate. The (+)-catechin production of suspension-cultured cells increased with cell growth and reached the maximal value (29.0 mg/g dry wt) after 6 days from the start of subculture. This is the highest value of (+)-catechin content among reports on catechin production in vitro so far published. The amount of (−)-epicatechin was in the range of 1.1–7.7 mg/g dry wt and that of (−)-epicatechin-3-O-gallate was in the range of 2.6–6.4 mg/g dry wt for a culture period of 15 days, respectively. Comparing with plant parts in regard to (+)-catechin, the amount of suspension-cultured cells was about 1.5 times as much as callus cells, about 9 times that of leaves (3.2 mg/g dry wt) and about 7 times that of stems (4.0 mg/g dry wt). The maximal yield of total catechins in suspension-cultured cells was 4.3% dry wt. It is well known that (+)-catechin, (−)-epicatechin and (−)-epicatechin-3-O-gallate can play an important role as anticancer agents.

D- In vitro cultures of Rumex alpinus L. for the production of some anthraquinones as anticancer agents:

Tissue cultures set up from roots of Rumex alpinus L. were grown on Murashige and Skoog medium with kinetin and either 2,4-D or naphthaleneacetic acid (NAA) as growth factors. The 2, 4-D cultures produced a broader spectrum of hydroxynaphthacene, hydroxyanthracene and hydroxybenzene derivatives than the NAA cultures. In the 2,4-D cultures the anthraquinones chrysophanol, physcion and emodin, the diheterodianthrone and the monoglucoside of chrysophanol were identified. The naphthalene-1,8-diols nepodin, nepodinmonoglucoside and methoxynepodin were identified. The occurrence of 2-acetylorcinol and its monoglucoside was established. In the NAA cultures accumulation of only chrysophanol, physcion and 2-acetylorcinol were proved. The yields of these secondary metabolites produced in the NAA cultures were much lower than those produced by the 2,4-D cultures. It is well known that anthraquinone derivatives (emodin, chrysophanol and physcion) can play an important role as anticancer agents.

E- Quercetin production from in vitro cultures of Pluchea lanceolata Oliverr and Hiern as an anticancer agent:

Arya et al., 2008 found that, maximum quercetin content (0.23 mg/g dry weight of tissues) was obtained in 6 weeks old callus tissues derived from leaf explants of Pluchea lanceolata Oliverr and Hiern. It is well known that quercetin can play an important role as anticancer agents.

F- Terpenoids and flavonoids production from in vitro cultures of Azadirachta indica as anticancer agents:

Babu et al., 2008 found that, the most significant enhancement of terpenoid and flavonoids productivity can be obtained by feeding sucrose to the cell suspension cultures media of Azadirachta indica (A. Juss), sucrose added to the media (liquid MS medium containing 5% sucrose, 1% mannitol and 0.1 mg/l IBA) prolonged the stationary phase and sustain viability of cells in cultures without subculturing process for 60 days, thus accumulates higher amounts of secondary metabolites including three terpenoid compounds (azadirachtin, mimbin and salamin) and two flavonoids (quercetin and kaempferol). It is well known that flavonoids
(especially quercetin) and terpenoids can play an important role as anticancer agents.

G- Tropane alkaloids production from in vitro cultures of Brugmansia suaveolens as anticancer agents:

Hairy root cultures of *Brugmansia suaveolens* were set up by infection of root tips with *Agrobacterium rhizogenes*. The successful transformation was confirmed by analyzing rolC and virC genes using polymerase chain reaction (PCR). Hairy root cultures were employed to study the formation of tropane alkaloids, such as hyoscyamine. The transformed cultures were incubated with potential elicitors, such as methyljasmonate, quercetin and salicylic acid in order to stimulate the biosynthesis of tropane alkaloids. Profile and amounts of tropane alkaloids were analyzed using capillary GLC-MS. At least 18 different tropane alkaloids could be identified. Treatment of the cultures with 200 μm methyljasmonate increased the alkaloid accumulation 25-fold up to a level of 1 mg/g fresh weight as compared to untreated controls. Quercetin enhanced the alkaloid production 10 fold (0.4 mg/g fresh weight) within 24 hours. In contrast 100 μM MeJA increased the alkaloid productivity up to a level of 1 mg/g fresh weight. It is well known that tropane alkaloids can play an important role as anticancer agents.

H- Anthraquinones production from in vitro cultures of Cinchona robusta as anticancer agents:

A suspension culture of *Cinchona robusta* which under normal culture conditions does not produce anthraquinones but produces a range of these anthraquinones after elicitation [Eight new anthraquinones were identified as robustaquinones, in addition to two known anthraquinones 0-2-7 trihydroxy-1-methoxyanthraquinone and copareolatin 5-methyl ether]. Their oxygenation pattern characterized by a 5-6 disubstitution in the A ring is rare and raises questions about their biogenetic origin. They might be derived from phenylpropanoids and not from o-succinyl-benzoic acid. It is well known that anthraquinone derivatives can play an important role as anticancer agents.

I- In vitro cultures of *Fagopyrum esculentum* for the production of some flavonoids as anticancer agents:

Hairy roots culture of *Fagopyrum esculentum* was established by infection with *Agrobacterium rhizogenes* strain 15834. Faster growth (four-fold higher than normal roots culture) in B5 liquid medium was observed and the synthesis of five flavonols obtained : (+)-catechin, (-)-epicatechin, (-)-epicatechin-3-O-gallate, procyanidin B2 and procyanidin BZ3'-O-gallate also present in normal roots culture with a common prominency of epicatechin-3-0-gallate synthesis and inversion in the contents rates of (+)-catechin and (-) tepicatechin. The highest content of procyanidin B2-3'-O-gallate was obtained in hairy roots which can be considered as a better production source for flavonols, especially of the two galloylated derivatives. It is well known that flavonoids (catechin and epicatechin derivatives) can play an important role as anticancer agents.

J- Podophyllotoxin production from in vitro cultures of Podophyllum:

Podophyllotoxin (PTOX), the precursor for semi-synthesis of cancer therapeutics like etoposide, teniposide and etopos, is primarily obtained from an endangered medicinal herb, *Podophyllum hexandrum* Royle. PTOX, a lignan is biosynthetically derived from the phenylpropanoid pathway.

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 podophyllotoxin 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. 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. 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. The production of podophyllotoxin derivatives has been reported from root and cell suspension cultures of *Linum flavum*. The highest amount of methylopodophyllotoxin which was produced in these culture systems was 1.3 % dry wt. in organized roots and 0.2 % dry wt. in cell suspension.

Since 5-methoxypodophyllotoxin, an analogue of podophyllotoxin, has strong cytostatic activity, many researchers have tried to improve its yield through tissue cultures.

The HPLC analysis showed approximately 7-8 fold change in accumulation of PTOX, in the 12d old cell suspension culture (i.e., after 9 days of elicitation) elicited with 100 μM MeJA as compared to the control. The phenylpropanoid and monolignol pathway enzymes were identified, amongst these, chalcone synthase, polyphenol oxidase, caffeoyl
increased the production rate of paclitaxel, which addition of carbohydrate during the growth cycle medium containing 6% fructose. cultures following 10 days in culture with optimized concentrations used. The free cells in suspension depended on the gel and cell amounts released depended on the amount of air supplied. Furthermore, some of the paclitaxel produced in the immobilized cells was accumulated in the culture medium (14.78 mg/L). In addition to paclitaxel, several other taxoids have been identified in both cell and culture medium of Taxus cultures. Parc et al., (2002) reported that, the production of taxoids by callus cultures from selected Taxus genotypes.

In order to increase the taxoid production in these cultures, the addition of different amino acids to the culture medium were studied and phenylalanine was found to assist in maximum taxol production in T. cuspidata cultures.

The influence of biotic and abiotic elicitors was also studied to improve the production and accumulation of taxol through tissue cultures.

To enhance the productivity of Taxus cell cultures, biogenetic precursors, Nutrient feeding, in situ extraction, elicitors, a combination of inducing factors and jasmonic acid, have all been used. On the other hand, production of paclitaxel by immobilized cell cultures of Taxus sp. with a bioreactor has been reported before. The amount of paclitaxel released into the medium ranged from about 0.4 to 4 mg/L. The production could be enhanced by adding an elicitor or biogenetic precursor. However, the productivity was not as high as that in free cell cultures.

Paclitaxel produced after the addition of elicitors, chito-oligosaccharides and chito-heptaose, the addition of a biogenetic precursor, phenylalanine and the addition of chito-heptaose or jasmonic acid and another biogenetic precursor, phenylisoserine, with a combination of two elicitors and with a combination of one elicitor and the biogenetic precursor, in cell suspension cultures. The amount of paclitaxel produced was increased by supplying air to the cell suspension cultures, though productivity depended on the amount of air supplied. Furthermore, some of the paclitaxel produced in the cultures was released into the medium when cells from T. cuspidata var. nana were immobilized with calcium alginate.

For the production of taxol (paclitaxel) by immobilized cells cultured in a bioreactor, cells prepared from Taxus cuspidata var. nana were entrapped with calcium alginate and the amount of paclitaxel released into F4G4 liquid medium was investigated. Immobilized cell cultures were developed using various calcium alginate gel concentrations (2, 2.5 and 4% w/v) and cell concentrations (3, 5 and 10% w/v). Some of the paclitaxel produced in the immobilized cells was found to be released into F4G4 liquid medium. The amount released depended on the gel and cell concentrations used. The free cells in suspension cultures accumulated paclitaxel in intracellular compartments, while the cells immobilized with the

**K- Paclitaxel from in vitro cultures of Taxus:**

(Taxol; paclitaxel) and taxanes are complex oxygenated diterpenes, initially isolated from the stem bark of Pacific yew (Taxus brevifolia Nutt.) by Wani et al., [1971]. More recently, these compounds have also been demonstrated to occur in needles of various Taxus spp. and in the mycelium of Taxomyces andreaneae an endophytic fungus of Pacific yew. Little is known about the biosynthesis of paclitaxel and the possibility of engineering its production remains frustratingly remote.

For commercial use, paclitaxel was first extracted from the bark of the yew T. brevifolia, which has brought this species to the verge of extinction. Later, paclitaxel was produced semi-synthetically from precursors (baccatin III and 10-deacetyl baccatin III) isolated from needles of other yew species.

*Taxus* tree, is one of the most promising anticancer agents known due to its unique mode of action on the micro tubular cell system. At present, production of taxol by various *Taxus* species cells in cultures has been one of the most extensively explored areas of plant cell cultures in recent years owing to the enormous commercial value of taxol, the scarcity of the *Taxus* tree, and the costly synthetic process.

In 1989, Christen et al., reported for the first time the production of taxol (paclitaxel) by *Taxus* cell cultures.

Fett-Neto et al., [1995] have studied the effect of nutrients and other factors on paclitaxel production by *T. cuspidata* cell cultures (0.02% yield on dry weight basis). Srinivasan et al., [1995] have studied the kinetics of biomass accumulation and paclitaxel production by *T. baccata* cell suspension cultures.

Paclitaxel was found to accumulate at high yields (1.5 mg/L) exclusively in the second phase of growth. Kim et al., [1995] established a similar level of paclitaxel from *T. brevifolia* cell suspension cultures following 10 days in culture with optimized medium containing 6% fructose.

Ketchum and Gibson, [1996] reported that, the addition of carbohydrate during the growth cycle increased the production rate of paclitaxel, which
calcium alginate gel released some paclitaxel into the medium. The gel forced some of the paclitaxel produced in the cells to be released into the medium. When the cultures using immobilized cells entrapped at a 3% cell concentration and 2.5% gel concentration with an air-lift bioreactor were conducted for 30 days, the amount of paclitaxel released into the medium increased with time on the addition of an elicitor, chito-heptaose.

The release of paclitaxel was enhanced to 2.4 mg/L of culture medium after the 30 days of incubation. This is the first report that the release of paclitaxel into the medium by immobilized cell cultures of Taxus sp. was enhanced by the addition of an elicitor. Eight five percent of the paclitaxel produced in the cells was released by the immobilized cell cultures in 30 days. The results obtained in this study, with immobilized T. cuspidata var. nana cells in a 2.5 % calcium alginate gel, may pave the way for the production of paclitaxel by immobilized cell cultures in a bioreactor.

The production of taxol from nodule cultures containing cohesive multicultural units displaying a high degree of differentiation has been achieved from cultured needles of seven Taxus cultivars.

Factors influencing stability and recovery of paclitaxel from suspension cultures and the media have been studied in detail by Nguyen et al., (2001). The effects of rare earth elements and gas concentrations on taxol production have been reported.

Results in Table 1 are significant inasmuch as they show paclitaxel synthesis and accumulation in cells cultured in vitro. One may, thus, assume that, paclitaxel occurs in parenchyma cells, likely in older cells and according to one observation, in cells that tend to be rich in phenolics, for they easily turn brown over time. Stimulation of paclitaxel production may well be achieved by permutation of concentrations of various media components, by application of two-phase systems, or by application of biotic and abiotic stress agents (elicitors). Phyton Catalytic (New York) is said to have increased paclitaxel yields to 1-3mg/L of supernatant using elicitors. The idea of a cocultivation, as a mixture or separated by a membrane, of Taxus cells and fungus (Taxomyces andreanae) is intriguing.

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<td>0.04</td>
<td>Fett-Neto et al., 1993</td>
</tr>
<tr>
<td>T. andreanae</td>
<td>Mycelium</td>
<td>50 ng/L</td>
<td></td>
<td>Strobel et al., 1993</td>
</tr>
</tbody>
</table>

"Medium after Gamborg et al., (1968) with 1 mg/L 2,4-D and 0.2% casamino acids. "Agrobacterium tumefaciens" gall-derived cells. Woody plant medium after Lloyd and McCown, (1980) without hormones. "Habituated callus. Medium after Gamborg et al., (1968) with 2 x B₅ vitamins, without hormones. "Medium as for e with 1 mg 2,4-D and 1 mg/L kinetin plus 20 g/L sucrose + 2.5 g/L glucose + 2.5 g/L fructose. "Medium after Gamborg et al., (1968) with 1 mg/L 2,4-D and 1 mg/L kinetin, plus 1.5% polyvinylpyrrolidone. "Medium after Gamborg et al., (1968) as for e plus 0.1 mg/L phenylalanine.

For the bioengineering of paclitaxel production, prospects of success are slim, at the moment. For one, its biosynthetic pathway is undefined. The likely precursor is geranylgeranylpyrophosphate. Isolation and partial purification of an enzyme that catalyzes cyclization of this precursor has been reported. Second, transgenic conifers have been demonstrated. Still, a workable concept on how to genetically manipulate paclitaxel synthesis and accumulation has not been devised. ESGA genetics (California) may succeed in using a by-way by employing hairy root cultures for improved production. As it stands, 1 kg paclitaxel would require the harvest of 500-1000 bioreactors of 500 L each filled with a heavy suspension of Taxus cells.
Recently, commercial production of paclitaxel by Taxus cell suspension cultures was established. Yew cell suspension culture is used as an alternative source of paclitaxel (Taxol), an effective anti-cancer drug (for advanced breast, ovarian and other cancers).

To optimize the initiation protocol, highly dormant yew seeds (Taxus X media redh) were germinated in vitro and the seedlings used to establish callus culture. The best sources of explant for callus initiation and growth were seedling stems and roots, and the most successful medium was modified B5 medium containing 2,4-dichlorophenoxyacetic acid and kinetin. Calluses were friable and suitable for establishing cell suspension cultures, which were maintained for over 3 years.

Flow cytometric analysis of nuclear DNA content revealed that, 2 years old cell suspension cultures consisted predominantly of putative euploid and aneuploid cells coexisting as sub-populations. Additional measurements performed 3 and 7 mo. later revealed further genomic instability, with a tendency towards a higher proportion of cells with elevated nuclear DNA content. In a selected cell line, which showed significant taxane production, the addition of 100 mM jasmonic acid strongly enhanced total taxane production and slightly inhibited growth while no effect on nuclear DNA content was noted.

In conclusion, chromosomal rearrangements causing major genome size variation occur regularly in long-term yew cell suspensions. Such major genomic changes could affect the metabolic activity of cells, which is of major importance when they are used for paclitaxel production. The structure and stability of nuclear DNA content should be carefully monitored in large-scale suspension cultures.

L- Terpenoids from in vitro cultures of 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. Therefore, the in vitro culture of F. pusilla was established in order to analyze its secondary metabolites (sesqui- and diterpenes). 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 B2 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 diterpenodialdehydes perrottetian 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.

M- Indole Alkaloids: Vinblastine derivatives from in vitro cultures of Catharanthus:

Vinblastine, a dimeric compound composed of catharanthine (1) and vindoline (2), two monoterpen indole alkaloids, is found in Catharanthus roseus (L.) G. Don (Apocynaceae), Madagascar periwinkle. The biosynthetic pathway has been elucidated to the extent that initial steps from tryptophan and mevalonic acid to strictosidine and catharanthine and from tabersonine to vindoline have been well analyzed and described. Natural dimerization to vinblastine includes 3’4’-anhydrovinblastine. Chemical dimerization has been accomplished by way of the Polonovsky reaction.

In plants vinblastine occurs at very low concentrations (5 g/2 tons FW). Owing to its cytotoxic effect, it may well function as a phytoalexin, a chemical defense agent. Vinblastine may occur in latexifers and idioblasts of Catharanthus, it accumulates in parenchyma throughout the plant. Of the two monomers, catharanthine has the highest concentrations in youngest, fully expanded leaves; the vindoline concentration was highest in leaves 5 and 7 from the apex.

Ever since 1958 when vinblastine and its analogue vincristine were recognized as anticancer agents and established as important drugs in the chemotherapeutic treatment of Hodgkin’s disease and acute leukemia, its supply has been of great concern. Plantations of C. roseus in various parts of the world could match the demand, were it not for the extreme cost associated with extraction and purification. Alternative sources of higher value (i.e., rich in vinblastine and unaccompanied by a host of related alkaloids) are the goals targeted by R&D. Not surprisingly, the development of cell cultures of C. roseus began as soon as in vitro culture had been established as a method in plant physiology, with callus, crown-gall, and habituated tissue cultures. More recently, these efforts have been complemented by DNA technologies.

Despite many years of effort and remarkable success in increasing the production of catharanthine in cell suspensions by treatment with biotic and abiotic elicitors (Table 2).
Table. 2: Production of catharanthine, vindoline, and vinblastine in plant tissues derived from *Catharanthus roseus* and cultured *in vitro.*

<table>
<thead>
<tr>
<th>Material</th>
<th>Medium Catharanthine</th>
<th>% DW</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf 5</td>
<td>—</td>
<td>0.2</td>
<td>Balsevich and Bishop, 1989</td>
</tr>
<tr>
<td>Leaf 3</td>
<td>—</td>
<td>0.3</td>
<td>Balsevich and Bishop, 1989</td>
</tr>
<tr>
<td>Cell suspension</td>
<td>B₅&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.001</td>
<td>Eilert <em>et al.</em>. 1986</td>
</tr>
<tr>
<td>Cell suspension</td>
<td>MS&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.15</td>
<td>Smith <em>et al.</em>. 1988</td>
</tr>
<tr>
<td>Hairy roots</td>
<td>SH&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.3</td>
<td>Jung <em>et al.</em>. 1994</td>
</tr>
<tr>
<td>Cell suspension</td>
<td>B₅&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.8</td>
<td>Fujita <em>et al.</em>. 1990</td>
</tr>
<tr>
<td>Leaf 5</td>
<td>—</td>
<td>0.3</td>
<td>Balsevich and Bishop, 1989</td>
</tr>
<tr>
<td>Leaf 3</td>
<td>—</td>
<td>0.4</td>
<td>Balsevich and Bishop, 1989</td>
</tr>
<tr>
<td>Hairy roots</td>
<td>B₅&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Traces</td>
<td>Parr <em>et al.</em>. 1988</td>
</tr>
<tr>
<td>Shoot cultures</td>
<td>MS&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.0015</td>
<td>Miura <em>et al.</em>. 1988</td>
</tr>
<tr>
<td>Regenerated shoots</td>
<td>—</td>
<td>0.01</td>
<td>Hirata <em>et al.</em>. 1993</td>
</tr>
</tbody>
</table>

**Vinblastine/anhydrovinblastine**

<table>
<thead>
<tr>
<th>Material</th>
<th>Medium Catharanthine</th>
<th>% DW</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf 5</td>
<td>—</td>
<td>0.3</td>
<td>Balsevich and Bishop, 1989</td>
</tr>
<tr>
<td>Leaf 9</td>
<td>—</td>
<td>0.4</td>
<td>Balsevich and Bishop, 1989</td>
</tr>
<tr>
<td>Hairy roots</td>
<td>B₅&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Traces</td>
<td>Parr <em>et al.</em>. 1988</td>
</tr>
<tr>
<td>Shoot cultures</td>
<td>MS&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.0015</td>
<td>Miura <em>et al.</em>. 1988</td>
</tr>
<tr>
<td>Regenerated shoots</td>
<td>—</td>
<td>0.01</td>
<td>Mirata <em>et al.</em>. 1993</td>
</tr>
</tbody>
</table>

<sup>a</sup>Medium after Gamborg *et al.*. (1968) with *Pythium apherrnatum* homogenate. <sup>b</sup>Medium after Murashige and Skoog, (1962) with 0.1 mg/L NAA, 0.1 mg/L L-tryptophan, plus SO mg/L vanadysulfate (selected cell line JOH). <sup>c</sup>Medium after Schenk and Hildebrandt, (1972), two-phase culture with inorganic salts x 1/3 in second phase. <sup>d</sup>Medium after Gamborg *et al.*. (1968) using a high-yielding cell line at high inoculation density. <sup>e</sup>Medium after Gamborg *et al.*, (1968) without hormones. <sup>f</sup>Medium after Murashige and Skoog, (1962) with 1 mg/L benzyladenine.

Vinblastine has eluded all laboratories. The problem is a lack of vindoline synthesis and accumulation in cell suspension cultures. Still, vindoline recurs in shoots regenerated from callus and accumulates in multiple shoot cultures. This observation underpinned the notion that vindoline synthesis in material cultured *in vitro* requires prior differentiation of cells. Incidentally, differentiation of material as expressed in shoot cultures also stimulated the formation of vinblastine. Also, vindoline and catharanthine production in shoot cultures was influenced by phytohormone levels in the medium and treatment with near-ultraviolet light. Evidence for vindoline synthesis as a function of differentiation may be deduced from seedling analyses that show that enzymes involved in the transformation of tabersonine to vindoline are localized in the chloroplast, the endoplasmic reticulum, a putative alkaloid synthesizing vesicle, and cytoplasm, and are phytochrome-dependent, respectively. It remains to be seen whether photoautotrophic cells of *C. roseus* would produce sizable amounts of vinblastine. So far, photoautotrophic cell cultures have been incapable of synthesizing vindoline.

Recently, vindoline-producing cell cultures of *C. roseus* have been presented. These cultures had been treated with *A. tumefaciens* and characterized as habituated (i.e., growing in medium without hormones) under constant fluorescent light of 2500 lux. Hairy root cultures generated by employing *A. rhizogenes* have shown remarkable levels of catharanthine and traces of vinblastine.

Enhanced production of catharanthine and vindoline in *planta* and *in vitro* is expected to be effected by genetic manipulation of their biosynthesis or by genetically directed changes of the cytoenvironment of alkaloid synthesis and accumulation. Early work has shown that, elicitors would induce tryptophan decarboxylase and strictosidine activities in cell cultures of *C. roseus*. Since then, initial steps have been completed with the isolation and characterization of tryptophan decarboxylase cDNA and the isolation and heterologous expression of cDNA and genes of strictosidine synthase from *R. serpentina* and from *C. roseus*.

Finally, given sufficient yields of catharanthine from cell cultures and vindoline from shoot cultures or plants, vinblastine can be obtained through dimerization catalyzed by cell-free extracts of cells cultured in vitro, by enzymes of such cultures and by horseradish peroxidase with 3’ 4’-anhydrovinblastine as an intermediate. Following a procedure by Vucovic, a chemical reaction involving Fe<sup>3+</sup> as a catalyst may result in coupling of the two precursors to anhydro vinblastine at a yield of 90 % and in a
second step in the formation of vinblastine at a yield of 50%.

The latter would appear to be the most promising technology for vinblastine production, to date. Cryopreservation of *C. roseus* cells has been successfully demonstrated.

Another important study reported that the periwinkle, *Catharanthus roseus* L. (G.) Don, produces several commercially valuable secondary metabolites including the anticancer agent, vinblastine, vincristine and the hypertension drugs, ajmalicine and serpentine.

Periwinkle seeds, after sterilization were cultured on MS medium. Petiole segments of seedlings (4 days old) were subcultured to medium containing various concentrations of NAA accompanied with Kinetin and subcultured to regenerate the callus and root. Callus and roots were obtained from petioles in some of treatments. The extracts of callus and roots from different treatments were analyzed by spectrophotometer, TLC and HPLC with respect to the indole alkaloids producing capacity. Alkaloids were produced from callus and roots from petiole of *C. roseus* in the presence of 0.1, 5, 10 and 20 mg/L Kinetin and NAA. MS with 0.1 mg/L NAA + 0.1 mg/L Kinetin had the highest vindoline, catharanthine, vincristine and root organogenesis capacity. But the level of these alkaloids and ajmalicine were very low compared to that in petiole of intact plant, and the level of serpentine was similar.

New roots, callus roots, and callus from MS medium containing 0.1 mg/L NAA + 0.1 mg/L Kinetin were subcultured in hormone-free and 0.1 mg/L NAA + 0.1 mg/L Kinetin media and for organogenesis and growth.

The most alkaloids amount was produced in new roots and callus roots. The indole alkald levels of new roots in new media were higher than in petioles of intact plants. 10-fold catharanthine, 125-fold serpentine, 0.5-fold vindoline and 0.34-fold ajmalicine were produced by new roots. The most interesting result was presentation of two important ant-cancer dimeric alkaloids, vinblastine and vincristine with amounts of 20-fold vinblastine and 6-fold vincristine to compare that in the petioles of intact plants.

N- Camptothecin derivatives from in vitro cultures of *Camptotheca*:

Camptothecin, a potent antitumor alkaloid was isolated from *Camptotheca acuminata*. Sakato and Misawa, (1974) induced *C. acuminata* callus on MS medium containing 0.2 mg/L 2,4-D and 1 mg/L kinetin and developed liquid cultures in the presence of gibberellin, L-tryptophan, and conditioned medium, which yielded camptothecin at about 0.0025 % on a dry weight basis. When the cultures were grown on MS medium containing 4 mg/L NAA, accumulation of camptothecin reached 0.998 mg/L. 10-Hydroxycamptothecin, a promising derivative of camptothecin is in clinical trials in the US.

Elicitor-induced accumulation of secondary metabolites in plant cell culture has received increasing attention. This is due to, in part, the fact that elicitation can improve the efficiency of secondary metabolite accumulation in culture systems where product formation appears near or after the late growth phase. Elicitation of secondary metabolite production is a function of the source material used for elicitor preparation. Some elicitors can cause induction and accumulation of secondary metabolites, whereas others may result in no response. In some cases, different elicitors may also result in unique responses within the same cell culture. The elicitor concentration is a factor that strongly affects the intensity of the response. The accumulation rate is highly affected by elicitor concentration at low elicitor concentration, but virtually unaffected at high elicitor concentration. Inhibition by overdosed elicitor can reduce the accumulation of secondary metabolites as well as cell growth. Camptothecin production was increased with elicitors, methyl jasmonate, jasmonic acid, yeast extract elicitor, and ferulic acid in suspension cultures of *Camptotheca acuminata*. Jasmonic acid was found to be the most efficient elicitor. Camptothecin production increased 11 times by using the optimum dosing concentration of jasmonic acid which was 50 μM. The kinetics of camptothecin accumulation in response to the treatment with jasmonic acid showed that, the camptothecin accumulation reached the maximum value at 4 days after jasmonic acid dosing and then a rapid decrease in camptothecin accumulation was observed (Song and Byun, 1998).

O- Antitumor activity of in vitro cultures of green tea seed (*Camellia sinensis* L.):

The antitumor activity of suspension cultures of tea callus cells grown in the presence of different concentrations of the growth regulator 2,4-dichlorophenoxy acetic acid (2,4-D) with or without light irradiation. The methanol and ethanol extracts of precipitated cells (MEP, EEP) exhibited stronger inhibitory effects on the growth of tumor cell lines than the water extract of precipitated cells (WEP) or the supernatant.

Compared to culture under dark conditions, exposure to light irradiation led to significantly higher antitumor activity. The MEP from light irradiated cells at 250 μg/mL with 2.0 mg/L 2,4-D displayed more than 64 % growth inhibition of HEP-2 cells, whereas normal cells showed less than 25 % growth inhibition. The some fractions of MEP obtained from Diaion HP-20 column chromatography displayed the
majority of inhibitory activity against the HEP-2 cell line. These results showed that, 2,4-D, and light stimulated the synthesis of antitumor compounds.

P- Production of shikonin derivatives from in vitro cultures of Arnebia (Royle) Johnst (Ruanzicao) as an antitumor agent:

Shikonin derivatives have cytotoxic and antitumor effects. The antitumor effects of acetylsinonol isolated from a Chinese medicinal herb Arnebia euchroma (Royle) Johnst were investigated. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the in vitro antitumor effects of acetylsinonol on human lung adenocarcinoma cell line A549, human hepatocellular carcinoma cell line Bel-7402, human breast adenocarcinoma cell line MCF-7 and mouse Lewis lung carcinoma (LLC) cell line. Acetylsinonol suppressed tumor growth in C57BL/6 mice with LLC model were used to study the in vivo antitumor effects of acetylsinonol. The expression of bax, bcl-2 and caspase-3 proteins in LLC tissue was determined with immunohistochemical staining. In A549, Bel-7402, MCF-7 and LLC cell lines, acetylsinonol inhibited cell growth in a dose-dependent manner. IC50 (means ± SD) were 5.6 ± 0.86 μg/mL, 6.82 ± 1.5 μg/mL, 3.04 ± 0.44 μg/mL and 2.72 ± 0.38 μg/mL, respectively. Acetylsinonol inhibited tumor growth in C57BL/6 mice with LLC. The inhibition rate of acetylsinonol (2 mg/kg) was 42.85%.

Immunohistochemical staining revealed that, in the acetylsinonol groups the expression of bax and caspase-3 increased, whereas the expression of bcl-2 decreased, suggesting that, acetylsinonol induced tumor cell apoptosis through activating the proapoptotic bcl-2 family and caspase-3. Acetylsinonol isolated from Arnebia euchroma (Royle) Johnst cell suspension cultures exhibits specific in vivo and in vitro antitumor effects.

Q- Anticancer activity of in vitro cultures of rice:

A multitude of natural products from plant extracts have been tested for their ability to inhibit the progression of several diseases including cancer. A novel approach of evaluating plant (rice) callus suspension cultures for anticancer activity is reported.

The ability of different dilutions of rice callus suspension cultures to inhibit growth of two human cancer cell lines was tested employing varying cell numbers and different incubation times. A crystal violet assay was performed to assess cell viability of the cancer cell lines. Furthermore, microscopic analysis was carried out to determine the effect of the rice callus culture on the morphology of the cancer cells. Rice callus suspension cultures significantly inhibited the growth of human cancer and renal cell lines at densities of 5000 and 10000 cells/mL when incubated for 72 and 96 hours.

Rice callus suspension culture was more efficient than paclitaxel (Taxol®) and etoposide in selectively killing human colon and renal cancer cell lines compared with a control cell line (human lung fibroblasts). The use of plant cell suspension cultures is a novel approach for inhibiting the growth of cancer cells, which will lead to the development of new agents for selectively killing cancer cells.

It could be concluded that, in vitro cultures of some plants (Lycopersicon esculentum, Rheum palmatum L., Polygonum hydropiper, Rumex alpinus L., Pluchea lanceolata Oliver and Hirn, Azadirachta indica, Brugmansia suaveolens, Cinchona robusta, Fagopyrum esculentum, Podophyllum, Taxus, Fossombronia, Catharanthus, Camptotheca, Camellia sinensis L., Arnebia (Royle) Johnst (Ruanzicao) and rice) can be considered rich sources of anticancer agents (alkaloids, flavonoids, anthraquinones, etc.).

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