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Standard Review

Taxoids: Biosynthesis and i*n vitro* production

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Taxoids viz. paclitaxel and docetaxel are of commercial importance since these are shown to have anti-cancerous activity. These taxoids have been isolated from the bark of *Taxus* species. There is an important gymnosperm, *Taxus wallichiana* (common name, 'yew') used for the isolation of taxoids. Due to cutting of the trees for its bark, population of the plant species are threatened to be endangered. Therefore, these are required to be protected globally. Plant cell culture techniques have been exploited for the isolation of mutant cell lines, production of secondary metabolites and genetic transformation of the plants. *In vitro*, culture of *Taxus* not only helps in conservation but is also helpful in the production of paclitaxel and other taxoids. Various strategies tested globally for the commercial production of taxoids are discussed. Different *Taxus* species, their origin, diterpenoids obtained from different parts of the tree and their applications are discussed. Although, detailed taxoid biosynthetic pathway is not well known, an overview of the pathway has been described. Micropropagation of *Taxus* and regeneration of transgenic plants has been described. Although, several protocols have been reported for the production of some important taxoids, a rapid, reproducible and economically viable protocol required for the efficient production of taxoids has yet to be established. Supplementation of the biotic and abiotic elicitor(s) to the cell suspension cultures of *Taxus* has been shown to increase the growth of the cell biomass as well as paclitaxel production due to pathway stimulation. Up-scaling of *Taxus* cell lines capable of over-producing taxoids could only make the industrial production of paclitaxel feasible. Here, we have reviewed *Taxus wallichiana* cell cultures in terms of their capabilities of biomass and secondary metabolites production.

Key words: Docetaxel, paclitaxel, taxanes, taxus.

INTRODUCTION

Plant tissue culture techniques have made the isolation and culture of cells, tissues and organs of many plant species possible, and have been exploited for the production of various secondary metabolites including several economically important metabolites (Wink et al., 2005). Most of the current strategies for the improved secondary metabolites production involve use of the bioreactors and genetically engineered whole plants and / or cultivation of cells derived from such plants or tissues (Rischer and Oksman-Caldentey, 2005; Ho et al., 2005). These techniques allow manipulation of the pathways by engineering rate limiting genes and cell cultures to obtain enhanced production of desired metabolites. The plant tissue culture techniques also involve induction of the hairy roots and development of transgenic plants through crown gall formation. *Taxus* species belong to the family Taxeaceae (common name 'Yew') and are the source of taxoids, a group of potent chemotherapeutic anti-cancerous agents. The most important compounds are paclitaxel (Taxol);

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Abbreviations: BA - benzylaminopurine; 10-DAB - 10-deacetyl baccatin III; DR - Dragendorf's reagent; GA_3 - gibberellic acid; GI - growth index; IAA - indole – 3 – acetic acid; JA - Jasmonic acid; MDR - multi-drug resistance; MS - Murashige and Skoog; NAA - ∝-naphthalene acetic acid; NCI - National Cancer Institute; PVP - polyvinyl pyrrolidone; WPM - woody plant medium.

Table 1. Distribution of the different Taxus species

Taxus species	Distribution
T. baccata	Europe and Asia (European yew)
T. brevifolia	Northwest Pacific (Pacific yew)
T. canadensis	Canada
T. celebica	Asia
T. cuspidata	Japan (Japanese yew)
T. floridana	Northwest Florida
T. globosa	Mexico / El Salvador
T. wallichiana	Indian Himalayas (Himalayan yew)

and its biogenetic precursor, 10-deacetyl baccatin III (10-DAB) that is chemically transformed to docetaxel (Lavelle et al., 1995). Taxoids are complex polyoxygenated diterpenoids having phenylisoserine moieties. These compounds are active in various *in vitro* and *in vivo* pre - clinical models viz. cell lines, human tumor stem cells, murine grafted tumors, human xenografts etc. and have been extensively used as drugs against ovarian, breast, lung, head, neck, prostate, cervical cancers and AIDS related Kaposi's sarcoma (Ketchum et al., 2007; Itokawa, 2003).

The toxicity of the yew tree is known since long time and has been explained due to the presence of taxine, a complex mixture isolated from the leaves of the tree. Taxine has been shown to be a mixture of seven alkaloids with taxine A and B as major components (Prasain et al., 2001). Contrary to taxoids, taxines do not exhibit anti-tumor activity and are relatively abundant in plants. These taxines can be used as starting material for the semi-synthetic production of paclitaxel derivatives (Croteau et al., 2006).

The first taxoid was discovered in 1960s by the National Cancer Institute (NCI) during a large scale plant screening program. It was shown that the bark of Pacific Yew, *Taxus brevifolia* was active against many cancers (Baloglu and Kingston, 1999; Itokawa, 2003). The active ingredient of the bark was later identified as paclitaxel. It exhibits anti-tumor activity against murine leukemia cells (Appendino, 1993), ovarian (McGuire et al., 1998) and breast cancers (Gotaskie and Andreassi, 1994). These findings stimulated an interest in the chemistry, biology and pharmacology of different species of *Taxus* (Wani et al., 1971). In 1983, NCI carried out phase I clinical trials. Afterwards, John Hopkins Oncology Center reported striking clinical results for the treatment of breast cancer. In the same year, Bristol Myers Squibb (Princeton, NJ) became the partner of NCI to commercialize the drug and was granted the patent for Taxol®. Yew tree pharmaceuticals (Haarlem, The Netherlands) developed Yew taxan®, a paclitaxel formulation. Rhone Poulenc (Paris, France) was granted approval for Taxotere® (a semi - synthetic taxane with the generic name docetaxel) in more than 33 countries.

After the FDA approval of paclitaxel and docetaxel as anticancer drugs, efforts are made for the search of new anticancer drugs and a number of new analogues are prepared for getting taxoids with better potency and availability to the targeted cells. Besides, efforts are made to search taxoids efficient in targeting multi-drug resistant cancers or capable of acting as modulators in multi-drug resistance (MDR) (Odgen, 1988; Ketchum et al., 2007). Studies have also been carried out for searching improved methods for getting natural or synthetic preparations of these important anticancer drugs (Dubois et al., 2003). Since, there are reports indicating undesired side effects on using paclitaxel, it has been considered important to develop new taxoids with fewer side effects, superior pharmacological properties, and improved activity against various classes of tumors. This approach resulted in the discovery and development of "Second generation taxoids" with various modifications in the baccatin skeleton and the phenylisoserine side chain (Lin and Ojima, 2000).

The contents of paclitaxel have been found to be very low in the bark (0.017% dry weight in *T. brevifolia*). Therefore, globally, efforts are made to overcome the supply problem since yield by isolation from the bark of the slow-growing yew trees is limited (Witherup et al., 1990). Alternative sources of paclitaxel viz. use of needles, stem bark, heart wood, roots of several other species of *Taxus* have been tried (Donovan, 1995). Besides, plant cell cultures as well as chemical and biotechnological semi-synthesis have been intensively investigated for the production of paclitaxel (Taxol) and docetaxel (Taxotere) in last few years (Chattopadhyay et al., 1995; Nicolaou et al., 1994). Besides, production of paclitaxel using fungal cultures has also been reported (Stierle et al., 1993). In this review, an effort has been made to correlate various aspects of *in vitro* production of taxoids.

Origin and distribution of *Taxus* species

The different *Taxus* species are considered as a single species descending from *Paleotaxus rediviva*, a fossil angiosperm that was abounded about 200 million years ago in the Triassic era, and later got confined to the temperate zones of the northern hemisphere during various glaciations (Hartzell, 1991). Although, the members of taxeaceae family are apparently similar to the conifers, but due to the absence of cones and resin ducts, are placed in a separate order named as Taxales. Yews are dioecious evergreen trees or shrubs and produce seeds having an edible fleshy aril. Many scientists believe that eight to ten species of *Taxus* are there (Table 1) (Krusmann, 1972; Appendino et al., 1993) whereas, others consider these species as simply different geo-graphical varieties. Besides the regular species, two hybrids are also known namely *Taxus x media* Rehder which is a cross between *Taxus baccata* and *Taxus cuspidata* and

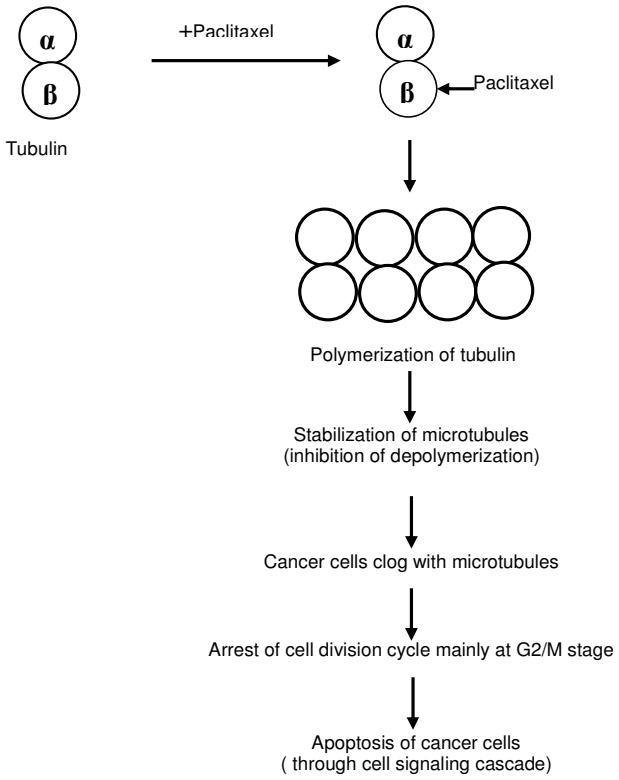

Figure 1. A flow diagram showing mode of action of paclitaxel in the apoptosis of cancer cells.

Taxus x hunnewelliana Rehder, a cross between *T. cuspidata* and *Taxus canadensis*.

Taxus diterpenoids and their uses

Ancient Greeks named the yew as '*Taxus'* due to two important aspects of the tree: Taxon means bow and Toxicon is poison (Hartzell, 1991). The Irish Druids had high regards for the tree due to its poisonous nature and was believed to be efficacious against fairies and witches (Wani et al., 1971). The structure of paclitaxel was first reported in 1971 (Wani et al., 1971). It is a complex and highly oxygenated diterpenoid amide. The compound and its related precursors/ products are commonly called as taxanes or taxoids. Generally, a taxoid compound contains an oxetane ring and is oxygenated at positions C-1, C-2, C-5, C-7, C-9, C-10 and C-13. However, rarely taxoids are oxygenated at C-14 position. Presence of a phenyl isoserine ester group at C-13 position of the diterpenoid taxanes is a critical feature for the anti – cancerous property of taxoids (Ojima et al., 1992). Taxol C is found to be more potent anti-cancerous agent than paclitaxel (Kobayashi et al., 1994). In addition, docetaxel, an analogue of taxol, has almost the same potency against cancers as paclitaxel (Guenard et al., 1993). Shen et al. (1999) isolated many diterpenoids namely chinentaxunine, 10-deacetyl taxol A, 10-deacetyl-7-*epi*-taxol, 10-deacetyl-10-oxo-7-*epi*-taxol, taxinine M, taxchinin A, 10-deacetyl taxinine B and taxuspine X from the seeds of *Taxus chinensis*. Shi et al (1999) isolated several new taxane diterpenoids from the seeds of *Taxus chinensis* var. *mairei* and *Taxus yunnanensis*. Brubaker et al. (2006) showed that zoledronic acid, an osteolysis inhibitory agent, in combination with docetaxel effectively inhibits growth of the prostate and bone tumors and therefore is a potential treatment option. By the use of fluorescent taxoids, paclitaxel binding site has been shown on the outer side of the microtubules (Diaz et al, 2005). Chen and Hong (2006) studied chemical modifycation of Taxus diterpenoids isolated from *T. brevifolia* and bisindole alkaloids isolated from *Catha-ranthus roseus* for efficient use as antitumor agents. Shen et al. (2005) isolated two new bicyclic taxoids namely tasumatrols M (1) and N (2) from the leaves and twigs of *Taxus sumatrana* and determined their structures by using 1D and 2D NMR and chemical derivatization. Zhang et al. (2006) isolated a new abeotaxane diterpenoid from the seeds of *Taxus mairei* and identified its structure as 2α, 5α, 13α-trihydroxy-7β, 10β-diacetoxy-2(3----20) abeotaxa-4(20), 11-dien-9-one by using 1D and 2D spectral analysis.

Mode of action

Taxoids inhibit the growth of cancer cells by affecting microtubules. During normal cell growth, microtubules are formed during cell division. However, once the cell stops dividing, the microtubules are broken down or destroyed. The microtubules produced in the presence of taxoids are resistant to disassembly by physiological stimuli, and cells exposed to these agents exhibit an accumulation of disorganized microtubule arrays. It affects the normal mitotic process and eventually results in cell death (Schrijvers and Vermorken, 2000). Paclitaxel binds to the ß-subunit of the tubulin heterodimer. After binding, the complex stimulates the polymerization of tubulin and stabilizes the resultant microtubules. It leads in the inhibition of their de-polymerization. Thereafter, cancer cells get clogged with the microtubules and are unable to divide resulting in the arrest of cell division cycle mainly at the G2 / M stage. This causes apoptosis of the cancer cells through cell signaling cascade. Hence, taxoids may be considered as a new class of anti - mitotic agents different from other plant derived anti-microtubule agents such as colchicine, podophyllotoxin and *Vinca* alkaloids that inhibit microtubule assembly. The mode of action of paclitaxel for the apoptosis of the cancer cells has been shown in Figure 1. Paclitaxel/taxoids are lipophilic in nature and most likely accumulate into the extracytoplasmic compartments (the cell wall) and not sequestered within the cellular compart-ments viz. vacuoles / plastids. *Taxus* cells avoid its toxic effect on self by excluding it

Table 2. Characteristics of the enzymes involved in taxoid biosynthetic pathway.

Enzyme	Opt. pH	Km	ORF of cDNA	Residues	Mol. wt. (kDa)	Reference
Geranyl geranyl pyrophosphate synthase	--	--	1179	393	42.6	Stierle et al., 1993
Taxadiene synthase	8.5	3μM for prenyl substrate	2586	862	98.3	Nicolaou et al., 1994
Taxadien-5α-yl acetate 10β – hydroxylase (Taxane 10β – hydroxylase)	--	--	1494	498	56.69	Krusmann, 1972
Taxadien-5α-ol-*O*-acetyltransferase	9.0	4.2μM for taxadienol and 5.5μM for acetyl CoA	1317	439	49.079	Hartzell, 1991
10-Deacetylbaccatin III-10β-*O*-acetyltransferase	7.5	10 μM for 10-deacetylbaccatin III & 8 μM for acetyl CoA	1320	440	49.052	Grothaus et al., 1993
Taxane 2α-*O*-benzoyltransferase	8.0	0.64μM for 2-debenzoyl-7,13-diacetylbaccatin III & 0.30μM for benzoyl coenzyme A	1320	440	50.089	Saikia et al, 2000

from the protoplast and excreting it into the cell wall. This system is considered as a natural defense system in cells against toxic effects of their own compounds.

Taxoid biosynthetic pathway

The detailed taxoid biosynthetic pathway is not well known. Floss and Mocek (1995) carried out feeding studies with advanced labeled taxoids, and gave an overview of the pathway. Taxoid biosynthesis occurs in non-photosynthetic stem tissue *in vivo*, and in non-photosynthetic, undifferentiated cells in culture systems *in vitro*. It involves several enzymatic steps leading to the formation of a tetracycline skeleton by cyclization of the universal diterpenoid precursor, geranylgeranyl pyrophosphate and afterwards oxygen and acyl groups are added to this taxane core by oxygenation at multiple positions mediated by cytochrome P450 mono-oxygenases. Croteau et al. (2006) showed that taxol biosyn-thetic pathway involves 19 discrete enzymatic steps from primary metabolism. It is shown that there is precursor flux to the production of taxoids other than taxol (Kikuchi and Yatakai, 2003; Takeya, 2003, Ketchum et al., 2007). Any effort to improve the yield of paclitaxel and its precursors must consider numerous and diversionary taxoid bio-synthetic pathway outlined by Ketchum et al. (2007). A brief summary of the characteristics of the few enzymes studied is given in Table 2. Over the past years, significant advances have been made in the identification of the genes that code the enzymes involved in this metabolic pathway. In a study, cDNAs of five enzymes involved in taxoid biosynthesis have been isolated, cloned, expressed and characterized (Walker and Croteau, 2001). These are the enzymes of the first committed step: taxadiene synthase (which constructs the taxane skeleton), a cytochrome P450 taxane hydroxylase and three taxoid *O* – acyltransferases. Long and Croteau (2005) sho-wed involvement of a microsomal 2'- hydroxyl-lase in the cytochrome P450 mediated C-13 side chain hydroxylation of β-phenylalanoyl baccatin III to form phenylisoserinoyl baccatin III. There are several enzymes involved in taxoid biosynthesis whose genes are yet to be identified. A line diagram (Figure 2) describes various known steps involved in the biosynthesis. The entire pathway can be summarized in the following steps.

Taxadiene formation

The first committed step of taxol biosynthesis is catalyzed by taxadiene synthase, an enzyme first isolated from *T. brevifolia* stem. The enzyme catalyzes the cyclization of the universal diterpenoid precursor, geranylgeranyl pyrophosphate to taxa-4 (5), 11(12)-diene resulting in the formation of a taxane skeleton. This enzyme mediates a unique intramolecular hydrogen migration in the B / C ring closure step. It is a monomer (mol wt ~79 kDa) and requires Mg^{2+} for the enzyme activity (Hezari et al., 1995). Its cDNA has been cloned

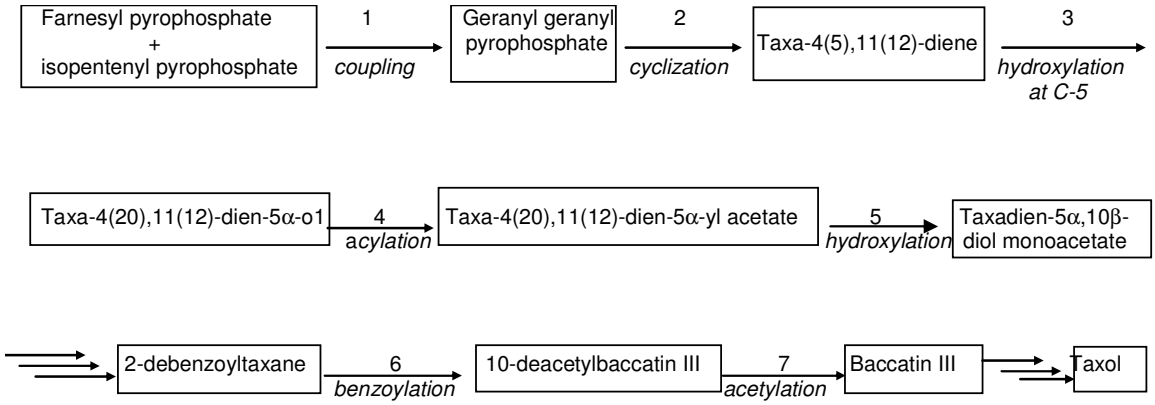

Figure 2: Taxoid biosynthesis pathway. Enzymes involved in various steps are: 1. Geranylgeranyl pyrophosphate synthase; 2. Taxadiene synthase; 3. Cytochrome P450 taxadiene 5α-hydroxylase; 4. Taxa-4(20),11(12)-diene-5α-ol-*O*-acetytranferase; 5. Cytochrome P450 taxane 10β-hydroxylase, 6. Taxane 2α-*O*-benzoyltransferase and 7. 10-deacetyl baccatin III-10-*O*-acetyltransferase. Multiple arrows indicate multiple convergent steps.

and functionally expressed in *E. coli* (Wildung and Croteau, 1996). Geranylgeranyl pyrophosphate is synthesized by a prenyl transferase named geranylgeranyl pyrophosphate synthase. Its gene has been isolated and sequenced. This enzyme is of special interest as it leads to the formation of a branched point progenitor of a variety of diterpenoids and tetraterpenoids (Wildung and Croteau, 1996; Hefner et al., 1998). Methyl jasmonate modulates the production of this enzyme at transcriptional level.

Taxadienol formation

Kingston et al. (1993) suggested hydroxylation of taxa (4)5, 11(12) taxadiene at C-5 position catalyzed by the enzyme, taxadiene 5α-hydroxylase. This hydroxylation results in the formation of taxa-4 (20), 11(12)-dien-5α-ol, the second step in taxoid biosynthesis. The cytochrome P450 monooxygenases bring about hydroxylation of various intermediates of the pathway (Jennewein et al., 2003). Subsequent oxy-genations depend on the relative availability of oxygen functional groups on the taxane ring. The suggested order of oxygenation after C5 is C10, C2, C9, C13 (Hefner et al., 1996).

Acylation of taxadienol

Since naturally occurring taxoids bear an acylation at C-5 position, it is considered that acetylation of taxa-4 (20),11(12)-dien-5α–ol in the presence of Acetyl CoA could be the next step in paclitaxel biosynthesis (Kingston et al., 1993). An another important enzyme, taxane 10β -hydroxylase has also been isolated that catalyzes the conversion of taxadien-5α-yl acetate into 10β -hydroxy taxadien-5α-yl acetate. The third specific intermediate in the pathway is taxa-4(5), 11(12)-diene 5α-yl acetate. Reverse genetic approach led to the isolation of a full length cDNA encoding taxa-4 (20), 11(12)-dien-5α-ol-*O*-acetyltransferase, an enzyme that catalyzes the first acylation step using taxadienol and acetyl coA as substrates and is highly specific towards the C5 hydroxyl position. The taxa-4 (20), 11(12)-dien-5α-acetate provides a functional group that is further used for the extension of oxetane ring by epoxidation of 4(20)- double bond. Subsequent reactions include additional oxygenations, hydroxy group acylations, oxidation to ketone and ultimately generation of the oxetane ring. The formation of an oxetane ring is a central step in the biosynthesis of all the bio-active taxoids. Another identified transacetylation reaction in the taxol biosynthetic pathway involves hydroxylation at C10 position of the 10-DAB. The reaction is catalyzed by the enzyme, 10-deacetylbaccatin III–10-O-acetyltransferase. It leads to the formation of the last diterpene intermediate, baccatin III in the pathway using 10- DAB and acetyl coA as substrates. A full length cDNA clone of this enzyme has been isolated from *T. cuspidate* (Walker and Croteau, 2000). The last enzymatic step (acylation) in the taxol biosynthetic pathway is catalyzed by several full length acyl transferases (CoA thioester dependent), amongst which 3'–N-debenzoyl – 2'- deoxytaxol N–benzoyltransferase is identified, cloned and expressed in *E. coli*. The enzyme catalyzes the conversion of 2-debenzoyl-7,13-diacetylbaccatin III and benzoyl coenzyme A into 7,13- diacetylbaccatin III. This enzyme can be exploited to improve the production yield of taxol in genetically engineered systems (Walker et al., 2002). Structural studies of these acyltransferases have revealed presence of a conserved sequence HXXXDG. The histidine residue is considered to be essential for the catalytic activity of these enzymes.

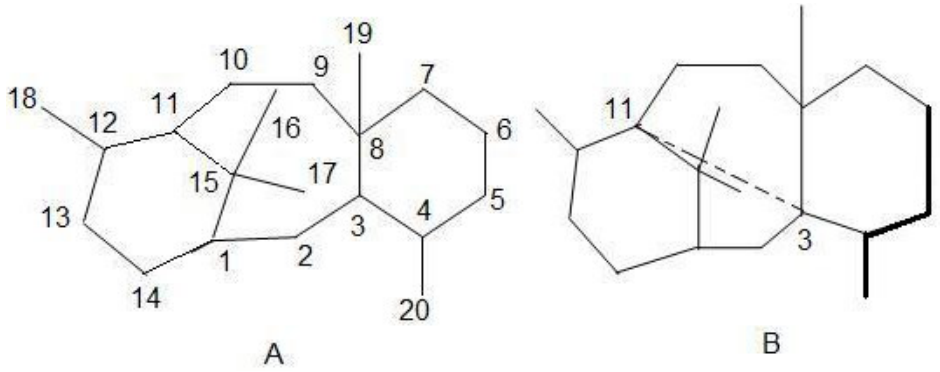

Figure 3. 6/8/6 and 5/7/6 ring systems of taxoids.

TAXOID COMPOUNDS IDENTIFIED IN *TAXUS*

During the search for improved analogues of paclitaxel, a large number of chemical constituents in *Taxus* species are identified (Parmar et al., 1999). There are several reports on the isolation of taxoids, flavonoids, abeotaxanes, lignans and glycosides from *Taxus.* In addition to taxoids, *Taxus wallichiana* has been used for the isolation of a wide range of non - taxoid compounds viz. higher isoprenoids, lignans, flavonoids, sugar derivatives, apocarotenoids and phenolic compounds. Approximately 120 taxoids have been isolated to date from the Japanese yew (Kobayashi and Shigemori, 2004). The taxoid compounds identified in various *Taxus* species have been summarized in Table 3. The normal taxoids contain 6/8/6-membered ring system (Figure 3A) but rearranged taxoids (naturally occurring) possess a 5/7/6/ or 6/10/6 membered ring system (Figure 3B). Taxoids with 5/7/6 type of ring system are known as (15→1) abeotaxoids or nortaxoids whereas those with 6/10/6 type of ring systems as 2 (3 → 20) abeotaxoids.

Normal taxanes

The naturally occurring diterpenoids containing taxane ring are the typical constituents. In addition to paclitaxel, taxine also belongs to this class. The compounds of this group are responsible for the toxicity of leaves and berries.

3, 11-Cyclotaxanes

The 3,11-cyclotaxanes can be produced upon irradiation of corresponding 13-oxo-taxa-11-enes (Appendino et al., 1992b, 1993).

11(15 →1) Abeotaxanes

Taxoids isolated from different *Taxus* species have been found to possess a rearranged nor-taxoid skeleton, 11 (15→1) abeotaxanes. Taxchinin A was the first naturally occurring rearranged taxoid identified as 11 (15→ 1) abeotaxane. Whereas, Taxchinin B isolated from *Taxus chinensis* was the first identified 11 (15→1) obeotaxoid with an oxetane ring. The first natural taxoid, identified to have 11(15→1) abeotaxane ring was brevifoliol (Balza et al., 1991). It was initially assigned a normal taxane skeleton but later corrected to 11(15→1) abeoskeleton (Datta et al., 1994; Georg et al., 1995). Wollifoliol with 11 (15→1) and 11(10→9) are the only bisabeotaxanes isolated from *T. wallichiana.*

Pretaxoids

Epitaxol, acetyltaxol and taxol C can be categorized under this class. These contain ring structure as 6/8/6/4 (A/B/C/D) (Wani et al., 1971).

New taxoids

A series of new taxoids, named taxuspines possessing various skeletons containing 5/7/6, 6/10/6, 6/5/5/6, 6/8/6, or 6/12-membered ring systems have been isolated from *T. cuspidata* (Kobayashi and Shigemori, 2002).

ISOLATION OF TAXOIDS

In spite of the low yield, until recently, *T. brevifolia* was the sole source of paclitaxel (100 mg/kg of dry bark). Due to short supply compared to increased demand for the drug, investigations started searching for alternate sources viz. other species or tree parts, cell cultures, fungi, synthetic and semisynthetic approaches. Extensive chemical investigations on the Himalayan yew have resulted in the isolation and characterization of several unique taxoids in addition to paclitaxel (Shinozaki et al., 2002; Shi et al., 2001; Cheng et al., 2000; Kiyota et al., 2002; Shen et al., 2002). The contents of paclitaxel and 10-DAB in the shoots of *T .wallichiana* have been repor-

Table 3. Taxoids from different plant parts of *Taxus wallichiana.*

Taxoids	Plant part(s)	Reference (s)
10,13-deacetyl obeobaccatin IV	Stem bark	Wickremesinhe & Arteca, 1993
2-deacetoxydecinnamoyl taxinine J	Stem bark	Navia-Osorio et al., 2002
Taxol and 10-deacetyl baccatin III	Needles	Floss & Mocek, 1995
2-deacetyltaxinineJ, Brevifaliol, 13α-acetoxy brevifoliol, 2α-acetoxy brevifoliol, baccatin IV	Stem bark	Fett-Neto et al., 1992
1 ß-hydroxybaccatin I	Stem bark, needle	Fett-Neto et al., 1992, Kim et al., 1995
Dihydrotaxol, 9-o-benzoyl-9, 10-dideacetyl-11(15→1) abeobaccatinVI and	Stem bark	Menhard et al., 1998
9-o-benzoyl-9-o-acetyl-10-de-o-acetyl 11(15→1) Abeobaccatin VI		
19-hydroxybaccatin III	Needles	Seki et al., 1997
10-Deacetyltaxol	Stem bark, needle	Seki et al., 1997
1-hydroxy-2-deacetoxy taxinine J and 7, 2'-bisdeacetoxy austrospicatine	-do-	Yukimune et al., 2000
Pactitaxel	-do-	Kim et al., 1995; Yukimune et al.,, 2000
7-Xylosyl-10-deacetyl taxol C	Needles	Walker et al., 2002; Yukimune et al., 2000
10-deacetyl cephalomannine	-do-	Seki et al., 1997
Taxuspinanane A	-do-	Kobayushi et al., 1994
Brevifoliol	Needles	Furmanowa et al., 1997
7-deacetoxy-10-debenzoyl brevifoliol	-do-	Fett-Neto et al., 1992; Guenard et al., 1993
Taxichinin A (2-acetoxy brevifoliol)	Needles	Yukimune et al., 2000
Taxacuotin & Taxayuntin	Heartwood	Walker et al., 2002
10-debenzoyltax-chinin C and 7, 9, 13-deacetyl abeobaccatin	Stem bark	Furmanowa et al., 1997
1-hydrocybaccatin I and 2-deacetoxy decinnamoyltaxinine J	Needle	Guenard et al., 1993
Wallifoliol	Heart wood	Walker et al., 2002
Yunnaxane and taxusin	Aerial parts	Ketchum et al., 1999
Dantaxusin (II) and dantaxusin (D2)	Needles	Phisalaphong & Linden, 1999
5 alpha O – (3' – dimethyl amino – 3' – phenyl proionyl) taxinine M (1)	Needles	Seki et al., 1997
19-hydroxybaccatin III [3], 10-deacetylcephalomannine [4)] and 10-deacetyltaxol [5].	Leaves	Baebler et al., 2002
5 alpha,13 alpha-diacetoxy-taxa-4[20],11-diene-9 alpha,10 beta-diol [1], 7 beta, 13 alpha-diacetoxy-5 alpha-cinnamyloxy-2[3-->20]-abeo-taxa-4[20],11-diene-2 alpha, 10 beta-diol [2], and 2 alpha,10 beta,13 alpha-triacetoxy-taxa-4[20],11-diene-5 alpha,7 beta,9 alpha-triol [3]	Needles	Linden, & Phisalaphong, 2000
l-beta-hydroxy-7beta-acetoxytaxinine [1] and lbeta,7beta-dihydroxytaxinine [2]	Roots	Wu & Lin, 2003
Taxumairols G (1), H (2), I (3), J [4], and L [5]	Needles	Morrita et al., 2005
Taxezopidines M and N	Seeds	Morrita et al., 2005
2,20-*O*-diacetyltaxumairol N (1) and 14ß-hydroxy-10-deacetyl-2-*O*-debenzoylbacatin III (2)	Needles and stems	Xia et al., 2005

ted to vary between 18.3-40.6 mg/kg and 247.6-594.9 mg/kg, respectively on dry weight basis (Poupat et al., 2000). Further, the bark and needle leaves have been found to be enriched in taxoids along with other non-

taxoids viz. polyphenols, glycosides, lignans and flavornoids. The needles are renewable sources of both taxoid and non-taxoid compounds. A few taxoids and some sugar derivatives have also been isolated some twigs, seeds, roots and heart wood of *T. wallichiana* (Chattopadhyay et al., 1994, 1997, 1999a, 1999b). The needles also contain a group of 11 toxic alkaloid compounds, collectively called as taxines (0.4 – 0.7% of the fresh plant material). The taxines were isolated even before paclitaxel was discovered. The needles of *T. baccata* were the first identified source for 10-DAB (Appendino et al., 1992a). Furthermore, the needles of *T. wallichiana* have also been identified as the only source of a unique taxane, wollifoliol, having 5/6/6/6/4 ring system (Vander Velde et al., 1994). Various organs including stem bark and leaves of *T. wallichiana* and a few other species, *Taxus yunnanensis* and *T. cuspidata* have been found to accumulate C-14 oxygenated taxoids (Zhang et al., 1995). All C-14 oxygenated taxoids exhibit poor cytotoxicity due to lack of the side chain and C-4 (20), 5-oxetane ring. In 1984, docetaxel was semi - synthetically produced from 10-DAB, by combining it with a fully synthetic side chain. This was an efficient process of using a renewable source i.e. needles in place of bark which opened possibilities for the pharmaceutical deve-lopment of taxoids.

Micropropagation of *taxus* species

Several tissue culture techniques used for the micropropagation of *Taxus* are summarized in Table 4. The *Taxus* species are found recalcitrant towards direct regeneration from axillary buds, nodal, internodal and leaf explants. *Taxus* seeds have a long dormancy period of 1 - 2 years, hence dormancy breaking agents such as gibberellic acid (GA_3) are used for *in vitro* germination. Regeneration through *in vitro* germination of zygotic embryos of several *Taxus* species has been successful (Chee, 1994). Washing of mature zygotic embryos under tap-water for 7 days, followed by culturing for 7 days on modified Murashige and Skoog (MS) or Heller's media is also used for breaking the dormancy. However, embryo derived seedlings showed nutrient deficiency and that could be overcome by the supplementation of additional magnesium sulfate in the media (Flores et al., 1993). Rooting in seedlings is induced by the supplementation of boric acid in the liquid medium. Another approach involves culture of zygotic embryos of *T. baccata* on woody plant medium (WPM) supplemented with 5% activated charcoal, resulting in 65% shoot induction and complete regeneration after 2 months of incubation (Majda et al., 2000). Besides dormancy, light conditions play a critical role in embryo germination. An initial dark incubation of 2 - 4 weeks, followed by subsequent transfer of embryos to white cool light conditions proved efficient (Majda et al., 2000). Chee (1995) reported conversion of adventitious bud primordia into multiple shoots arising from the zygotic embryos on ½ B5 medium supplemented with 10 µM BA. However, by this method only 58% of the shoot primordia explants could be developed into complete plantlets. Micropropagation of *Taxus* trees is also achieved by culturing microcuttings of *T. x media* in a medium supplemented with 1 mg/lkinetin and 2% sucrose. Induction of somatic embryogenesis has been reported from friable calli and immature zygotic embryos in the pre-sence of 2, 4-D, 1 mg/l glutamine, cytokinin, BA and auxin, NAA. Subsequent transfer of embryo-genic calli onto the WP medium supplemented with 4 µM BA, 1 µM kinetin and 1 µM NAA for 6 to 8 weeks supported maturation of the somatic embryos (Chee, 1996).

Taxus: *In vitro* expression of taxoids

Christen *et al*. (1989) for the first time reported the production of paclitaxel using *Taxus* cell cultures. Afterwards, many scientists attempted to optimize conditions for prolific growth of cells and enhanced taxane production in several tissue culture systems viz. cell, hairy root, shoot and embryogenic cultures of different *Taxus* species (Mirajalili and Linden, 1996; Ketchum et al., 1995). Amongst various approaches available for *in vitro* production of taxoids, cell suspension cultures have been found to be the most reliable yielding 0.8% taxoids on dry biomass basis (Jaziri et al., 1996; Yukimune et al., 1996; Cusido et al., 1999). Moreover, cell suspension cultures secrete 90% of the total taxoids synthesized into the culture medium (Srinivasan et al., 1995). In addition to paclitaxel and 10-DAB, a range of other regular C-14 taxoids has also been identified in cells and culture media from cell cultures of various *Taxus* species (Banerjee et al., 1996; Vanek et al., 1999; Agarwal et al., 2000).

Establishment of cell cultures of *taxus* species

Rohr was the first scientist who developed callus cultures from different explants of *T. baccata* including microspores (Rohr, 1973). Subse-quently, David and Plastira (1976) standardized mineral and phytohormonal composition of the culture medium required for efficient prolixferation of the calli generated from mature stem explants in *T. baccata* L. A number of culture parameters affect the callus and cell suspension culture of various *Taxus* species (Table 5). Basal media such as Gamborg's (B5) (Gamborg et al., 1968; Wickremesinhe and Arteca, 1994), MS (Murshige and Skoog, 1962) and Woody Plant Medium (WPM) (Loyd and McCown, 1981) have been used for the initiation, proliferation and maintenance of the callus and cell suspension cultures in *Taxus* species. Fre-quently, supple-mentation of organic substances such as casein hydrolyzate, polyvinyl pyrrolidone (PVP), ascorbic acid and other essential amino acids such as glutamine, aspartic acid, proline, phenyl alanine along with vitamins in the medium enhanced callus/cell growth.

Table 4. Organ cultures in various Taxus species.

Culture type	Taxus sp.	Explants	Media	Hormone(s)	Remark (s)	Reference (s)
Embryo culture	*T. brevifolia*	Embryos	B5	NS[a]	63% full seedlings	Hezari et al., 1995
	T. baccata	Embryos	MS/ Heller	GA_3	100% full seedlings (1 week)	Hefner et al., 1998
	T. brevifolia	Embryos	B5	NS	Mature embryos, 36% full seedlings	Wildung & Croteau, 1996
	T. brevifolia	Embryos	½ B5	BA	Multiple shoots , 50% rooting	Chee, 1995; Chang et al.,2001
	T. baccata	Zygotic embryos	WPM	BA/ Zeatin	65% seedlings development	Kingston et al., 1993
Shoot multiplication	*T x media*	Shoot tips	Hogland	Kinetin	NS	Walker & Croteau, 2000
	T. floridana	Needles	MS/ B5	NAA	Glutamine used to induce somatic embryos in callus	Wu & Lin, 2003
Microcutting	*T. cuspidata*	Embryos	WPM	NAA+ 2,4 –D+ Kinetin	Immature embryos, mature in hormone free medium	Appendino et al., 1992b
Somatic embryogenesis	*Taxus species*	Zygotic embryos	MCM	2,4 –D/ BA	2 stages cultures	Parmar et al., 1999

[a]NS- not specified

Most of the research groups have observed that auxins (IBA, NAA, 2,4-D and Picloram) at the varying level of 1.0-10.0 mg/l used in combination with cytokinins (BAP/Kinetin) at the level of 0.1 - 0.5 mg/l were effective for the initiation of callusing. Inorganic compounds like $VOSO_4$ have also been reported to be instrumental in enhancing the cell growth and multiplication in different *Taxus* species. The influence of plant growth factors on the rate of cell proliferation is complex and depends on both the basal medium composition and plant genotype. A major problem associated with Taxus cultures is the tendency to secrete phenolics, hampering the growth of calli and after a certain period leading to blackening / browning that results in death of the cells (Gibson et al., 1993). Use of anti-oxidants (phenolic binding compounds) like PVP, citric acid, ascorbic acid, activated charcoal, and fre-quent sub-culturing of cell cultures have been found to prevent ill effects of phenolics leach-outs. Simultaneous incubation of cultures under dark conditions leads to lesser leaching of phenolics. The inoculum size, period of subculture, light intensity and photoperiod are also the deciding factors for the multiplication, phenotypic appearance and proliferation of Taxus cells (Wickremsinhe and Arteca, 1993; Navia-Osorio et al., 2002).

Synthesis and accumulation of paclitaxel and 10-DAB in the cell cultures of *Taxus* species

For optimum cell growth and taxoid production, selection of explants, nutritional/ phytohormonal supplementation, pH, use of antioxidants and amino acids have been observed as the deciding factors (Jaziri et al., 1996). Several protocols have been reported for the production of some important taxoids and up-scaling of these cell cultures (Navia-Osorio et al., 2002). Almost every *Taxus* species has been studied in terms of the optimization of the growth media to yield maximum biomass (Mirajalili and Linden, 1996). Ketchum et al.(1995) formulated a new TMS growth medium for the callus cultures of *T. brevifolia*. Similarly, Fett- Neto et al. (1992) studied the effect of various nutritional components on paclitaxel production in *T. cuspidata* cell cultures. Srinivasan et al. (1995) studied the kinetics of biomass accumulation and paclitaxel production in *T. baccata* cell suspension cultures. Fur-thermore, a strong correlation is found between biomass production and taxol accumulation in callus cultures. Paclitaxel has been found to accumulate in high amounts (1.5 mg/l of culture) in the second phase of the growth curve. A similar level of paclitaxel has also been observed in *T. brevifolia* suspension cultures (Kim et al., 1995). Seventeen known taxoids and 10 abietanes have been isolated from the dark brown callus culture of *T. cuspidata* cultivated on a modified Gamborg's B5 medium with 0.5 or 1.0 mg/l NAA (Bai et al., 2005). Production of the

Table 5. Effect of variation in the nutrient and phytohormones used in the media for culturing the explants from *Taxus* species

Taxus sp.	Explants used	Basal medium	Phytohor-mones (mg/l)	% Carbohydrate	Anti-oxidant (mg/l)	Additive(s)	Type of culture(s)	Reference (s)
T. baccata	Gametophyte	B&N	NS[a]	Sucrose	NS	NS	Callus	Chattopadhyay et al., 1999a
T. baccata	Old stem segments	Heller, MS	2,4-D + kinetin	NS	NS	Thiamine + Nurse culture	Callus	Lucas, 1956
T x media	Stem	B5	NS	NS	NS	Casein hydrolysate	Cell suspension	Mirajalili & Linden, 1996
T. cuspidata	Young needle	B5	2,4-D + GA_3	Sucrose	PVP	Casein hydrolysate	Callus	Wu & Ge, 2004
T. floridana	Young needle	MS	NAA+BA	Sucrose	PVP	Glutamine	Callus, embryo	Wu & Lin, 2003
T. brevifolia	Stem, bark and needle	B5	2,4-D	NS	NS	Casamino acids	Callus	Gibson et al., 1993
T. baccata	Stem	WPM	NAA+BA	Sucrose	NS	NS	Callus	David & Plastira, 1976
		B5	Picloram+ kinetin+ ABA/ GA3	NS	NS	Glutamine	Cell suspension cultures	Kobayashi & Shigemori, 2002
		B5	Picloram + kinetin + ABA	Sucrose + fructose	Ascorbic acid, citric acid	Aspartic acid + arginine+ proline+ KM,N&N vitamins	Cell suspension	Wang et al., 2001
		B5	NS	NS	NS	NS	Cell suspension	Flores et al., 1993
		Eriksson medium	2,4-D + kinetin + BA	Sucrose	NS	Glutamine	Cell suspension	Chattopadhyay et al., 1994
		B5	2,4-D+ Kinetin	Sucrose or fructose	NS	Casein hydrolysate, Kao Michayluk vitamins	Protoplasts , callus, cell suspension	Wu et al., 2001
		B5	2,4-D+ Kinetin	Sucrose	NS	2 x B5 vitamins & $VOSO_4$ as elicitor	Callus, cell suspension	Wickremesinhe & Arteca, 1994
T. baccata	Young stems	B5	NAA+BA	Sucrose	Ascorbic acid	Glutamine	Cell suspension	Christen et al., 1991
T. wallichiana	Leaf needle, stem	B5	2,4-D + kinetin	Sucrose	NS	Casein hydrolysate	Callus, cell suspension	Wang et al., 2000
T. cuspidata	mature and immature seeds	B5	2,4-D+GA	Sucrose	NS	KCl,KI,NaH_2PO_4,$MgSO_4.7H_2O$, $(NH4)_2\ SO_4$	Callus, cell suspension	Xu et al., 1998
T. media	Zygotic embryo	WPM	NAA	Sucrose	NS	Methyl jasmonate	Callus, cell suspension	Agarwal et al., 2000; Chee, 1995

[a]NS- not specified

taxoids does not occur in the stationary growth phase of the cell cultures. Seki et. al. (1997) and Morita et al. (2005) focused their studies on plant cell cultures of *T. cuspidata* for the continuous production of taxoids. Addition of carbohydrates (sucrose and fructose) in the midway of the growth cycle increased the rate of the cell growth and paclitaxel accumulation. Supplementation of biotic and abiotic elicitor(s) in the cell suspension cultures of *Taxus* has been shown to affect the growth of cell biomass as well as paclitaxel production by pathway stimulation. Several reports have shown that methyl jasmonate (abiotic chemical elicitor) if added in the second growth phase of suspension cultures, strongly promoted taxane biosynthesis (Menhard et al., 1998; Yukimune et al., 1996, 2000; Ketchum et al., 1999; Phisalaphong and Linden, 1999; Mirajilili and Linden, 1996). Jasmonic acid (JA) in 100 µM concentration if added at the 7th day after subculture also increased taxane content in the culture medium (Baebler et al., 2002). Oligosac-charide addition stimulates the effect of methyl jasmonate in *Taxus canadensis* culture (Furmanowa et al., 1997; Linden and Phisalaphong, 2000). Paclitaxel and baccatin III production in suspension cultures of *T. media* can be improved by a two-stage culture method by adding methyl jasmonate (220 µg/l FW) together with mevalonate (0.38 mM) and N-benzoylglycine (0.2 mM). Under these conditions, 21.12 mg/l of paclitaxel and 56.03 mg/l of baccatin III were obtained after 8 days of the culture in the pro-duction medium (Cusido et al., 2002). Ten known taxoids, paclitaxel, 7-*epi*-taxol, taxol C, baccatin VI, taxayuntin C, taxuyunnanine C, yunnanxane and an abietane, taxamairin A, have been produced in the callus culture of *T. cuspidata* cultivated on a modified Gamborg's B5 medium in the presence of 0.5 mg/l NAA. After stimulation with 100 µM methyl jasmonate, five more taxoids have been found in addition to the above-men-tioned compounds (Bai et al, 2004). *Aspergillus niger*, an endophytic fungus, isolated from the inner bark of *T. chinensis*, if added as an elicitor (40 mg/l) in the late exponential-growth phase resulted in more than two-folds increase in the yield of the taxol and about a six-folds increase in the yield of the total taxoids (Wang et al., 2001). Addition of a trivalent ion of a rare earth element, lanthanum (1.15 to 23.0 µM) also promoted taxol production in suspension cultures of *Taxus* species (Wu et al., 2001). Maximum synthesis and accumulation of taxoids occurred in the period just after subculture. Thereafter a steady decrease in the paclitaxel content occurred (Wickremesinhe and Arteca, 1994). Consistent pro-duction of taxoids in a continuous culture system can be achieved only by their removal just after optimum production. Commercial resins are used for the removal of paclitaxel from *T. brevifolia* suspension cultures (Christen et al., 1991). It is observed that higher initial sucrose concentration in culture medium repressed cell growth leading to a longer lag phase. This could be overcome by a low initial sucrose concentration (20 g/l) and subsequent sucrose feeding (fed-batch culture) resulting in a high taxane yield of 274.4 mg/l (Wang et al., 2000). It has also been shown that initial addition of 1.0 – 2.0 mmol/l phenylalanine into the medium, followed by addition of 73.0 mmol/l sucrose and 173.3 mmol/l mannitol at the 28th day of culture, strongly promoted cell growth and taxoid production. The variable amount of taxoids in callus lines of different *Taxus* species and in the callus lines of the same *Taxus* species could be explained on the basis of the fact that secondary metabolite accumulation occurred due to dynamic equili-brium between the product formation, transport, storage, turnover and degradation of compounds. The levels of paclitaxel obtained in cell/organ culture systems of various *Taxus* species are described in Table 6. Taxol production can be enhanced by the use of self-immo-bilized cell aggregates, free and calcium alginate gel particle-immobilized cells (Xu et al., 1998). Besides, taxol biosynthesis has also been enhanced up to 40 - 70% by the addition of adsorbents like ion exchanger, XAD-4 in cell cultures. A new method for the production of taxoids involved culturing bacteria isolated from yew (*Sphingo-monas* sp., *Bacillus* sp., *Pantoea* sp. or *Curtobacterium* sp. isolated from *T. canadensis*) or its mutated forms. Taxane production has also been reported using bio-transformation of pro–taxanes namely paclitaxel, 10-deacetylcephalomannine, 7-epitaxol, 10-deacetyl-7-epitaxol, 7-epicephalomannine, 7-epibaccatin-III, 7-xylosyltaxol, 7-xylosyl-cephalomannine, taxagifine, delta-benzoyloxy taxagifine, 9-acetyloxy taxusin, 9-hydroxy taxusin, taxane-Ia, taxane-Ib, taxane-Ic or taxane-Id into taxanes. Another method used for the high yield of taxane production from *Taxus* sp. was fed–batch mode of suspension culture with inclusion of an inhibitor of phenylpropanoid metabolism. This method is high yielding, producing 15 mg taxane/l/day, 10 mg taxol/l/day and / or 15 mg baccatin-III/l/day. The culture medium is exchanged at least once during taxane production, and taxane may be removed from the culture during production (Roberts et al., 2003).

Production of various other taxoids in cell cultures of *Taxus* species

Cell cultures have been found to produce a variety of irregular and unique taxoids in addition to regular taxoids. A few species produced detectable amounts of other related taxoids such as baccatin, epitaxol, 10-deacetyl taxol, 7-epi-10-deacetyl taxol etc. (Table 7). Cell cultures of *T. baccata* produced small quantities (4.4 µg/l FW) of the new bioactive taxoids including 7-o-xylosides of taxol C; 10-deacetyl taxol C; N-methyl taxol and a C-13 oxygenated taxoid taxucultine (Ma et al., 1994a). In a subsequent report, Ma et al. (1994 b) reported four new C-14 oxygenated taxoids, along with yunnaxane. Similarly, Cheng et al. (1996) reported production of seven C-14 oxygenated taxoids from the cell suspension cultures

Table 6. Content of paclitaxel in cell lines and organ cultures of different *Taxus* species.

Taxus species	Content of Paclitaxel	Reference
(a) Cell Line		
T. baccata	1.50 mg/l	Chattopadhyay et al., 1999
T. cuspidata	0.30 mg/l	Roberts et al., 2003
T. canadensis	5.95 mg/l	Wang et al., 2001
T. media	110.00 mg/l	Agarwal et al., 2000
T. baccata	48.30 mg/l	Agarwal et al., 2000
T. brevifolia	0.50 mg/l	Agarwal et al., 2000
T. baccata	13.00 mg/l	Christen et al., 1991
T. wallichiana	0.50 mg/l (d.w.)	Wang et al., 2000
T. yunanensis	3.00 mg/l (d.w.)	Loyd & McCown, 1981
Organ culture (culture type)		
T. brevifolia (shoot)	1.0µg /gFW[a]	Wildung & Croteau, 1996
T. brevifolia (Root)	6.2 µg /g FW	Wildung & Croteau, 1996
T. x media(shoot)	2.1 µg /gFW	Wildung & Croteau, 1996
T.x media (Root)	8.1 µg /gFW	Wildung & Croteau, 1996
T. media Hicksii *(Root)*	619µg /g DW[b]	Wildung & Croteau, 1996
T. cuspidata (Root)	423µg /g DW	Wildung & Croteau, 1996
T. cuspidata var special (Root)	332 µg /gDW	Wildung & Croteau, 1996
T. baccata (seedling)	40,000 µg /gDW	Kingston et al., 1993

[a] = FW, fresh weight; [b] = DW, dry weight

of T. yunnanensis. Out of these, three were entirely new compounds characterized as 10-de-acetyl yunnaxane; 2α-hydroxy-5α, 10β, 14β-triacetoxy-4 (20), 11-taxadiene and 2α, 10β, 14β-triacetoxy-5α-acetoxy-4(20), 11-taxadiene. 4(5), 11(12)-taxadiene and their deo-xygenated derivatives are potential biosynthetic precu-rsors of paclitaxel and other taxoids. Microbial mediated hydroxyllation of 4(5),11(12)-taxadiene by *Absidia coerula* resulted in a 20-hydroxylated metabolite with a molecular formula of $C_{28}H_{40}O_9$. Menhard et al. (1998) reported production of a mixture of 16 different C-13 and C-14 oxygenated taxoids in cell cultures of *T. chinensis*. Twelve of these taxoids were found to be esterified at C-13 position, whereas four of them were oxygenated at C-14 position. A cell suspension line of *T. wallichiana* has been found to produce a group of three C-14 oxygenated taxoids and an epoxide of baccatin.

OTHER TISSUE CULTURE TECHNIQUES USED FOR TAXOIDS PRODUCTION

Several other tissue culture techniques such as organ culture, genetic transformation and protoplast culture have been found to play an important role in taxoids production.

Organ cultures

These cultures possessed a high potential for paclitaxel production. A yield of 40 mg of paclitaxel per g dry weight *Taxus* has been reported by Majda et al. (2000).

Genetic transformation and regeneration of transgenic plants

Successful transformation of *Taxus* cells with *Agro-bacterium rhizogenes* and *Agrobacterium tumefaciens* has been achieved (Han et al., 1994). *Taxus* embryos serve as a good starting material for establishing hairy root cultures (Plaut- CarCasson, 1994). Use of *Nicotiana* feeder cells and acetosyringone further enhanced the transformation rate of the embryos. The highest hairy root biomass has been obtained in B5 liquid medium supplemented with 0.5 g/l L-glutamine. Transgenic plantlets are regenerated from these hairy roots in 2 steps, by culturing in B5 liquid medium supplemented with 1 mg/l NAA, followed by transfer into a medium containing 5 mg/l BAP. Both hairy roots and the plants possessed detectable amount of paclitaxel. In another report, hairy root cultures of *Taxus x media var. Hick*sii were established from shoot tips, young leaves and hypocotyls of 8 weeks old seedlings cultured on plant growth factor free modified DCR medium with 20 g/l sucrose and solidified with 6 g/l phyto-agar. On addition of 100 µM methyl jasmonate, paclitaxel contents in these hairy root cultures increased from 69 to 210 µg/g dry wt. whereas, the 10 DAB contents were not affected (Furmanowa and

Table7. Type and level of various taxoids other than paclitaxel and DAB in cell cultures of *Taxus* species.

Taxus species	Type of culture	Name of the taxoid expressed	Max.level of taxoid detected	Reference(s)
T. baccata	Callus culture	Taxol C	4.4 mg /kg FW	Banerjee et al., 1996
T. baccata	-do-	10-deacetyl taxol C	1.0 mg /kg FW	Banerjee et al., 1996
T. baccata	-do-	N-methyl taxol	2.2 mg /kg FW	Banerjee et al., 1996
T. baccata	-do-	Taxcultine	1.6mg/kg F.W.	Banerjee et al. 1996
T. chinensis and *T. wallichiana*	cell suspension	2α, 5α, 10ß, 14ß-tetraacetoxy-4[20], 11-taxadiene C and Yunnanaxane	145 mg/ l	Chattopadhyay et al., 1997
-do-	-do-	2α, 5α, 10ß-triacetoxy-14ß-propionyloxy-4[20], 11-taxadiene	11.2 mg /l	Banerjee et al., 1996
T. chinensis and *T. wallichiana*	-do-	2α, 5α, 10ß-triacetoxy-14ß-(2-methyl)-butyryloxy-4[20], 11-taxadiene	2.5 mg/ l	Chattopadhyay et al., 1997
T. chinensis	-do-	9-dehydrobaccatin III	0.2 mg /l	Flores et al., 1993
T. media, T. baccata, T. brevifolia,	-do-	Baccatin III	53.6 mg /l	Flores et al., 1993; Agarwal et al., 2000
T. chinensis	-do-	13-dehydroxy-10-deacetyl baccatin III	0.9 mg /l	Flores et al., 1993,
-do-	-do-	9-dihydro-13-acetoxy baccatin III	0.9 mg /l	Flores et al.,1993
-do-	-do-	9-dihydro-13-dehydroxy baccatin III	3.2 mg /l	Flores et al.,1993,
-do-	-do-	13-dihydroxy baccatin III	1.9 mg /l	Flores et al.,1993
-do-	-do-	Baccatin VI	0.5 mg /l	Flores et al.,1993,
T. canadensis	-do-	13-deacetoxy baccatin I	0.75 mg /l	Flores et al.,1993
T. baccata	-do-	2α, 5α, 10ß-triacetoxy-14ß-propionlofy-4[20], 11-taxadiene	1.4 mg /l	Flores et al.,1993
-do-	-do-	10-deacetyl-7-xylosyl taxol B	0.027 mg /l	Ketchum et al., 1999
-do-	-do-	10-deacetyl-7-xylosyl taxol C	0.087 mg /l	Ketchum et al., 1999
-do-	-do-	7-epitaxol	0.102 mg /l	Ketchum et al., 1999
-do-	-do-	10-deacetyl-7-epitaxol	0.064 mg /l	Ketchum et al., 1999
-do-	-do-	10-deacetyltaxol	0.122 mg /l	Vander Velde et al., 1994

Table 8. Comparison of the paclitaxel recovery from *in vivo* and *in vitro* tissue biomass of *Taxus* species.

Plant material	Average paclitaxel content (% D.W.)	Reference
Bark of mature tree (100 yrs old)	0.017	Yukimune et al., 2000
Taxus needles	0.005	Ma et al., 1994a
Taxus cell cultures	0.800	Agarwal et al., 2000
Taxomyces andreanae	---	Rohr, 1973

Syklowska, 2000).

Production of paclitaxel through protoplast culture

Luo et al. (1999) isolated viable protoplasts from 20 day old friable calli of *T. yunnanensis*. These protoplasts were cultured and maintained on B5 salts having KM-vitamins and supplemented with 0.45 M fructose, 3.0 mg/l 2, 4-D and 0.1 mg/l kinetin (Luo et al., 1999). Protoplast derived colonies varied in terms of the growth and paclitaxel production. Though these colonies were not promising for paclitaxel production, but provided a source for obtaining

cell lines with high paclitaxel productivity after mutagenesis. Aoyagi reported 6 times more paclitaxel accumulation in protoplast culture of *T. cuspidata* after immobilization in agarose gel (in shaking cultures) compared with the cell suspension cultures (Aoyagi et al., 2002). This may also be a promising approach for taxane production.

COMMERCIAL FEASIBILITY OF *IN VITRO* TAXOID PRODUCTION

Several problems have been observed in the isolation of taxoids from *Taxus*, for instance the growth of yew trees is very slow. Complete chemical synthesis of taxoids on an industrial scale seems to be impractical. A comparison of different sources for paclitaxel isolation (stem bark of mature trees, needles from 4 year old trees, cell suspension culture and cultures of *Taxomyces andreaneae*) indicated that cell cultures of *Taxus* are the only potential source for the commercial production of paclitaxel as well as other taxoids (Table 8). The growth rate of callus cultures is slow, hence, repeated use of *Taxus* cells is necessary for economical production of paclitaxel. The highest concentration of paclitaxel reported is 0.8% on dry cell weight basis (Yukimune et al., 1996). This value is approximately 470 times higher than the paclitaxel contents observed in the bark. In a study, out of the 27 different genotypes screened, *T x media* " Sargentii" was found to be enriched in paclitaxel, yielding 0.069 and 0.032% taxoids from the leaves and callus, respectively (Parc et al., 2002). Large scale production of taxoids (3 mg/l taxol and 74 mg/l of total taxanes) was achieved from the suspension cultures of *T. cuspidata* using a balloon type bubble bioreactor. In cell suspension cul-tures of *T. wallichiana,* established in shake flasks and in a 20 l airlift bioreactor running for 28 days in a batch mode, the maximum yield of paclitaxel was 20.84 mg/l on the 24th day and of baccatin III was 25.67 mg/l on the 28th day (Yukimune et al., 2000).

Taxol® is manufactured by Bristol-Myers Squibb in a 9-steps semi-synthetic process from 10-DAB originally isolated and purified from yew species such as *T. baccata* in a multi-steps process. Aphios Corporation manufactures Taxol in a 4-steps process that is cost-effective and environmental-friendly. This process produces 10-DAB, a precursor of taxoids and cephalomannine as by-product, that may be semi-synthetically converted to Taxol in a 3-steps process. Total synthesis of taxane is costly, low yielding and therefore is not a good alternate for its commercial supply. Therefore, at present, semi-synthesis is the major industrial mode for the production of paclitaxol and other related taxoids (Commercon et al., 1992, 1995). Although semi-synthesis is efficient, but purification of the precursors required from the plant tissues is difficult. On the other hand, isolation of taxol from *Taxus* cell cultures requires fewer steps than purification from intact tissue.

A few other organisms have been found capable of producing taxol. These include a diverse group of endophytic fungi of *Taxus* species viz. *Taxomyces andreanae*, *Pestalotiopsis*, *Fusarium* and *Alternaria*, but these are yet to be exploited for the commercial production of these drugs (Stierle et al., 1993; Strobel et al., 1996; Kim et al., 1999). Induced *Taxus* cell cultures provide a good model for the study of complex biosynthetic pathway for the synthesis of Taxol. The most promising approach for taxol production is *Taxus* cell cultures elicited with methyl jasmonate for increased taxol production (Yukimune et al., 2000; Baebler et al., 2002). Novel taxane derivatives can also be obtained by genetic manipulation (Han et al., 1994). Hence, in the present scenario, biological method of taxol production is commercially feasible. It requires understanding of the complete taxol biosynthetic pathway and the enzymes involved in it. This will be helpful in improving taxol production by manipulation of several steps. Over-expression of the genes in transgenic plants may also be helpful in increasing the level of the taxoids to commercially significant levels. Once the entire pathway is studied and the genes identified, it might be possible to suppress the undesirable side routes by various sense and anti-sense techniques and also to direct the pathway towards other intermediates possibly some new taxoid derivatives. Furthermore, cell cultures can produce a range of other taxanes that may prove a powerful way in knowing the biochemistry, enzymology and molecular biology of taxoids production. Hence, it may be concluded that up-scaling of *Taxus* cell lines that are capable of overproducing taxoids could only make the industrial production of paclitaxel feasible.

Conclusion

Taxoids viz. paclitaxel and docetaxel are of commercial importance since these are shown to have anti-cancerous activity. These taxoids have been isolated from the bark of *Taxus* species. Plant cell culture techniques have been exploited for the isolation of mutant cell lines, production of secondary metabolites and genetic transformation of the plants. *In vitro*, culture of *Taxus* not only helps in conservation but is also helpful in the production of paclitaxel and other taxoids. Various stra-tegies tested globally for the commercial production of taxoids are discussed. Different *Taxus* species, their origin, diterpenoids obtained from different parts of the tree and their applications are discussed. Although, detailed taxoid biosynthetic pathway is not well known, an overview of the pathway has been described. Micropro-pagation of *Taxus* and regeneration of transgenic plants has been described. On the basis of comparative studies on paclitaxel production in different systems, it is concluded that at present *Taxus* cell suspension cultures are relatively more suitable and could be considered as an alternate for the direct production of paclitaxel as well as other related taxanes. Of course, feasibility of a cost effective produc-

tion of paclitaxel through tissue/cell culture requires more advancement in optimization of the amount and rate of the biomass and taxoid production. Commercial feasibility of *in vitro* taxoid production has also been discussed.

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