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Taxus wallichiana (Zucc.), an Endangered Anti-Cancerous Plant: A Review

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Abstract:

Taxus one of the slow growing species, is found to be the major source of Taxol *(anti-cancer agent). Since very less is known about the biology of Taxus genus alongside the controversies going on with the species, profitable mechanism for large scale* Taxol *production is still in debate. Commercially,* Taxol *synthesis is unlikely because of the limited sources and probability of over consumption of natural product, which would be an issue concerning the endangered species of Taxus like Taxus wallichiana. The only possible alternative way for high production of* Taxol *is by generating* Taxol *in plant-based mediums without exploiting the plant in its natural vegetation. Before that, one should have detailed understanding about Taxus. Here, in this study Taxus wallichiana, an endangered Taxus species, native to Nepal has been briefly reviewed.*

Keywords

Taxus wallichiana, Taxol, *Paclitaxel*, *Anti-cancerous.*

1. Introduction

As most conifers, *Taxus wallichiana* is an evergreen tree species belonging to Taxaceae family. Also commonly known as Himalayan Yew or Common Yew, the tree of *T. wallichiana* is dioecious in nature, having the male and female parts on separate trees. It grows as small-to medium sized canopy tree or a shrub; found in montane, temperate and warm-temperate forest [1]. Mature tree is usually 6 to 10 m (rarely up to 30m) in height, 1.5 to 1.8m in girth.

1.1. Distribution

Found in temperate Himalayas at an elevation range of 1800 to 3300m, the plant is said to be native to Nepal, Bhutan, India, China and Myanmar also extending to other neighboring countries from Thailand, Vietnam, Philippines and Indonesia in east to Pakistan and Afghanistan in west according to the revised population distributions. According to Ethnobotanical Society of Nepal (1997), there are 22 representative districts enlisted for *T. wallichiana* which are: Baglung, Bajhang, Darchula, Dhankuta, Dolakha, Dolpa, Doti, Gorkha, Humla, Jajarkot, Jumla, Kaski, Kathmandu, Lamjung, Manang, Mustang, Myagdi, Parbat, Rasuwa, Sankhuwasabha, Solukhumbu and Taplejung.

In Nepal, it is distributed in the Western, Central and Eastern sub-alpine and temperate regions in the range of 1800-3000m in association with *Quercus semecarpifolia, Abies spectabilis, Picea smithiana, Cedrus deodara, Tsuga dumosa, Pinus wallichiana* and *Rhododendron campanulatum* [2]. In Vietnam, conifers like *Dacrycarpus imbricatus*, *Keteleeria evelyniana*, *Nageia wallichiana*, and *Podocarpus neriifolius* has been found associated with *T. wallichiana* where they grow in sub-montane evergreen mixed forests. In the Philippines it occurs on high ridges and mountain summits in mossy forest, or sometimes in rocky grass and scrubland [1].

1.2. Botanical Description

The bark is thin reddish brown, and scaly. Leaves are dark green above, paler beneath, linear, flattened and up to 3 cm long, with a pointed tip, and appear to spread in two rows on either side of the shoot. Male cones are ovoid whereas female does not bear a cone but a fleshy fruit which is a solitary seed, half enclosed in a red aril. Unlike many other conifers, *Taxus* does not actually bear its seeds in a cone. Instead, each seed grows alone at the tip of a dwarf shoot, enclosed in a fleshy, usually red, aril which is open at the tip. In the Himalayan region, needles are shed in May and June; flowers appear from April to October and the fruits ripen from October to December. The tree is long lived, but the growth is extremely slow; growth rate of 12-14 annual rings per 2.5 cm of radius and girth increment 0.4 to 1.3cm per year. Dormancy of 1.5-2 years has been recorded [3] with poor regeneration [4]. Seeds are easily dispersed by birds and animals, pollinated by wind however, not much is known about the habitat preferences the species exhibits.

Taxus is also called the 'tree of death' because the tree is poisonous however, its leaves, twigs and barks are found to possess medicinal value. The species was made more unique when Wani and his

coworkers first discovered the drug Taxol which is anti-cancerous in nature increasing its excessive exploitation for Taxol extraction in 1971 [5]. That's why within last 25 years there has been major decline in global population of the species and is still continuing leading the plant to be enlisted as endangered species by IUCN red list.

2. History of *Taxus*

Gymnosperms are ancient plant groups having their origin in the Permian era, 200-300 million years ago. Though outnumbered by angiosperms in terms of species richness, gymnosperms, especially the conifers dominate the world's forest types, occurring across huge landscapes of the temperate zones of both hemispheres. A unique family among the gymnosperms, whose position in the classification systems has been a source of controversy, is Taxaceae. The generic name *Taxus* comes from the poisonous taxanes found in the tree. Some botanists did not consider yew to be a true conifer, since it does not bear its seeds in a cone. However, proper consideration of its evolutionary relationships now places the yew family (Taxaceae) firmly within the conifers. Earlier belonging to the order Coniferales (cone bearing plants), the family was shifted out to a separate order called Taxales [6] with the justification that Taxaceae did not bear cones. In India, the family is represented by two genera, namely *Taxus* and Amento *Taxus* [6].

3. Species and Varieties

Taxus wallichiana (Himalayan Yew) is the first ever Himalayan species of *Taxus* to be discovered by Joseph Gerhard Zuccarini in 1843, hence giving him the authority of the species and associating the code, Zucc., after his name while naming *Taxus wallichiana*. Initially, *Taxus baccata* (European Yew) was the first *Taxus* species to be identified by Carl Linnaeus in 1753. Soon, other varieties of *Taxus* was discovered and since the botanists didn't follow proper rule of International Code for Botanical Nomenclature (ICBN), there has been a long history of controversy in naming *Taxus* species. Till date 24 species and 55 varieties (still counting) have been identified based on morphological features [7], however the count has reduced to 12 species based on geographical boundaries. ICBN has recognized and validated these 12 species showing its support to geographical method of separation most probably because of the strong morphological and molecular evidences supporting the separation. Remaining identified species are yet to be validated. Details about the species outline can be found in http://www.worldbotanical.com/Nomenclature.htm# nomenclature (Nomenclature, Keys and Descriptions for species of *Taxus* with discussion and citation of specimens studied).

In Nepal, *T. wallichiana* was first collected by Nathaniel Wallich in 1822 as determined from a review of herbarium specimens and references. He reported its occurrence in Nepal in his *Tentaman Florae Nepalensis*, "Sheopore (meant Shivapuri)," near Kathmandu). The name *Taxus nucifera* was assigned to the specimen that was referred to be collected from Nepal in 1822 and referred the number 6054a [8]. Linnaeus described it as a species that occurs in Japan in his Species Plantarum, which was later reported by Siebold and Zuccarini to be of Torreya genus not *Taxus*. Later Zuccarini published in his illustration in 1843 that Wallich's *Taxus* was indeed a new species, which then he described and named as *Taxus wallichiana*.

T. wallichiana was listed as endangered in the China Plant Red Book [9], although this listing relates only to variety *wallichiana* and not to the species as whole. However, in 2004 all varieties of *T. wallichiana* and other closely related Asian yews were added to Appendix 2 of the Convention on International Trade in Endangered Species (CITES) to protect them from illegal trade. In 2011, IUCN Red List published *Taxus wallichiana* as endangered species A2acd ver 3.1.

4. Chemical Ingredients

Taxol, (Paclitaxel, as commonly called) is the diterpenoid alkaloid prominently found in foliage and barks of several *Taxus* species. Complete chemical synthesis of Taxol is now possible, however the method is uneconomic [10]. At present, must Taxol is prepared by semi-synthesis from baccatin III or 10-deacetylbaccatin (10-DAP) which are the precursors to Taxol. Another taxane synthesized from advanced 10-DAB is Docetaxel, commonly called Taxotere [11]. The commercial supply of Taxol depends on the natural sources and the level varies within the plant parts. Nadeem *et. al.* (2002) reported Taxol content in the bark of Himalayan Yew of Jageshwor region, India in relation to tree age and sex [12].

Krauze-Baranowska M. in 2004 isolated various flavonoids from the needles of *Taxus baccata* which were; 3-O-rutinosides quercetin, myricetin and kaempferol, 7-O-glucosides kaempferol and quercetin, kaempferol, quercetin, myricetin. Also, by HPLC separation, the composition of flavonols and biflavones in some species of the genus *Taxus* was also performed [13].

5. Biological and Pharmacological Consequences

Taxanes are effective against a large number of human tumors particularly in breast and ovarian carcinomas [14]. As the number of cancers cases and Taxol treatment is increasing, major use of Taxol is observed in treatment of metastatic carcinoma of ovary, breast and and non-small cell lung cancer as well as in the second-line treatment of AIDS-related Kaposi's sarcoma [15]. Taxol is currently being studied for the treatment of diseases not related with cancer that require microtubule stabilization and avoidance of cell proliferation and angiogenesis, for example psoriasis [16], polycystic kidney diseases [17], and multiple sclerosis [18]. Taxol is also being studied for the treatment of taupathies (affections in tau proteins), such as Alzheimer's or Parkinson's linked to chromosome 17, among others [19].

Besides breast and ovarian cancer, clinically Taxol's use has been increased for the treatment of lung cancer [20], head and neck cancer [21], renal, prostrate, colon, cervix, gastric and pancreatic cancers [22-24]. Paclitaxel thus represents a new class of antineoplastic agents [25].

Various parts of this species are used in home-remedies and domestic purposes. Kayastha (2002) reported a tincture made from young shoots can be used for treatment of headache, giddiness, falling pulse, cold and diarrhoea. Extract of *Taxus* can be used in cosmetics, such as hair lotion, rinses, shaving and beauty creams and dentifrices [26]. Leaves have anti-spasmodic properties used in treatment of hysteria, epilepsy, nervousness and frequently used in Unani system of medicine [2].

Poudel *et. al.* (2013) explored the ethno-pharmacological relevance of Yews (*Taxus*) among communities of Mongol and Caucasian origins of Nepal and reported along with the similar reports collected from other parts of Hindu Kush-Himalayan region. Also, they enlisted vernacular names used by indigenous people for the species found in that region [27].

6. Taxol as Anti-cancer Agent

Wani *et al.* (1971) first discovered the drug Taxol which is anti-cancerous in nature. As it was proven that taxane alkaloids from yew barks can traet cancer, use of Taxol as cancer drug got approval after 1990s. Taxanes represent a new class of antitumor drugs endowed with a peculiar mechanism of action which is inhibition of microtubule disassembly [28]. As the news spread harvesting of *Taxus bark increased rapidly*, especially *Taxus brevifolia* (growing in North America) and other yew species. Later it was found that yew leaves also bear starting material for the synthesis of Paclitaxel, the active anti-cancer compound in the drugs Taxol and Taxotere. Besides bark and leaves, roots are also reported as Taxol source.

Taxol (Paclitaxel) is an important antitumor agent. It was first isolated from the barks of the Pacific yew (*Taxus brevifolia* Nutt.) in 1971 [5] and later found in other *Taxus* species, including *Taxus wallichiana* var. *mairei* [29]. In 1981, USDA led a research for the isolation of paclitaxel at 0.001% from a mixture of leaves, stems and roots of *T. wallichiana*. Among the 300 known members of taxane diterpenoids, 34 so far have been isolated and identified from *T. wallichiana* [30]. At present, it is mainly extracted from the bark and needles of various *Taxus* species, but since this species are rare and slow growing, the needles which, unlike bark, are renewable are receiving consideration as an alternative source for Taxol.

Taxanes are effective against a large number of human tumors particularly in breast and ovarian carcinomas [14]. Taxol inhibits cell proliferation by promoting the stabilization of microtubules at the G2-M phase of the cell cycle, by which depolymerisation of microtubules to soluble tubulin is blocked [31]. Reports show that 10-deacetylbaccatin III (10-DAB) which is the starting material to produce active paclitaxel analogues was originally obtained from the needles of *T. baccata* [32]. Because it can kill tumor cells by enhancing the assembly of microtubules and inhibiting their depolymerization, Taxol has been well established and approved by FDA (Food and Drug Administration) as a very important effective chemotherapeutic agent against a wide range of tumours since 1992.

Ho *et. al.* (1997) tested Taxol concentration in needles of *Taxus mairei*, a native Taiwan species and selected superior trees with respect to high Taxol and 10-deacteyl baccatin III concentration. They found that rooted cutting steckling's needle showed higher

Taxol content than needles from mature trees, thus suggesting these vegetative propagated plantlets can serve as the alternative source for Taxol and hence also confirmed that Taxol yield is a heritable trait.

7. Toxicity, Carcinogenicity

Eating just a few leaves can make a small child severely ill. All parts of the tree are poisonous, with the exception of the bright red arils. The arils are harmless, fleshy, cup-like structures, partially enveloping the seeds, which are eaten by birds (which disperse the seeds). However, the black seeds inside them should not be eaten as they contain poisonous alkaloids. Hence, direct consumption of plant part for Taxol is not possible and alternative source for Taxol intake is necessary to cure the diseases.

8. Need of Biotechnological Approach

The original source, *T. brevifolia*, is the only FDA approved source of paclitaxel [33]. Because of the limited availability (0.01% of dry weight of the bark) [34], slow growth (takes several decades to increase a few inches in diameter) and ban on the export of the plant product as removal of the bark massively results in death of tree, the availability and demand for paclitaxel has increased extensively. The search for alternative source increased because the drug itself is costly because of the complex chemical process involved in the production and purification and low yielding. It takes 10,000 kgs of *Taxus* bark or approx. 3000 yew trees to produce one kg of the drug [35] and a cancer patient approximately needs 2.5-3gm of paclitaxel [36] i.e. the treatment of each cancer patient consumes about 8 sixty year old yew trees. Total synthesis of paclitaxel has been achieved [37], but the process is complicated and not economically feasible. Thus, pharmaceutical companies still rely heavily on plant sources. High demand, combined with such low yields from such slow-growing trees has prompted researchers to explore alternative sources of paclitaxel [25].

Taking into consideration of the above facts, supported by the seasonal variation in taxane concentration in *Taxus* [38] and high demand of the drug, there is an urgent need to find other alternative sources of Taxol production i.e., plant cell cultures. This methodology offers several advantages, not being subjected to weather, season or contamination, and the material can be grown independently of its original, potentially remote, location [39]. In addition, to increase the productivity different strategies can be implemented such as, optimization of culture conditions, selection of high-producing cell lines, and addition of precursors, additives and elicitors.

9. Propagation/Tissue Culture of *Taxus wallichiana*

9.1. In-vitro clonal propagation/shoot induction by Stem-tip culture/nodal explants

Although the clonal propagation of forest tree species has progressed significantly over the decade, coniferous plants are still considered difficult to propagate [40]. Regardless of the tissue source, all shoot-tip cultures produced only one elongated shoot per explant whereas, stem explants were capable of producing multiple shoots [41]. However, addition of activated charcoal, antioxidants like ascorbic acid and light conditions (16h photoperiod) are found to promote shoot induction in significant manner [34, 42].

9.2. Establishment of Callus Culture

Callus is best starting material for variety of cultures. We can generate shoots from callus as well as establish suspension cultures. Brunakova *et. al.* (2004) induced callus cultures using stems from different genotypes of the same *Taxus* species and examined the fresh weight when using two different basal media i.e., B5 and MS. They found that Gamborg's B5 medium favored callus growth irrespective of the genotype compared to modified MS medium. Cell suspensions were then initiated by inoculating friable calli into liquid medium [43]. These fast growing systems hence can be used for large-scale culture of plant cells to obtain the valuable products [44].

In *T. wallichiana*, earlier studies indicated that callus was induced from stem and needle explants cultured on B5 basal medium supplemented with 2,4-D and kinetin [45].

Datta *et. al.* (2005) initiated callus from axenic, zygotic embryos of *T. wallichiana* collected from West Bengal in ½ WPMSH media. Irrespective of the maturity stage of the embryos used, BA in combination with either 2,4-D or NAA induced callus at a significant level than kinetin under identical conditions[46]. Cytokinins have been reported to be essential for inducing adventitious buds in conifers [47], among which BA was most effective cytokinin for induction of adventitious shoot buds in callus cultures of *T. wallichiana*.

Datta and Jha (2008) tried analysis of paclitaxel and related taxanes in embryogenic and non-embryogenic calli extracted in chloroform. The taxane analysis revealed that embryogenic calli were found to accumulate more paclitaxel than non-embryogenic calli [48], indicating that cellular organization greatly influenced taxane accumulation.

9.3. Organogenesis and Somatic Embryogenesis

Investigations by Le Page-Degivry in early 1970s [49] showed that the dormancy of yew embryos are because of one immaturity of embryos and two the occurrence of endogenous inhibitors like abscisic acid, ABA. The first one can be hence overcome by using *in vitro* techniques whereas for the latter soaking, chemical treatment, temperature treatment have been reported to work. And hence, scientists started working on various culture medium taking various *Taxus* species for their study, resulting in variable choice of basal medium for culture along with supplementation of various hormones in various concentration.

Seed maturity is very important for *in vitro* cultures. The results of Chee, Le Page Devigry and Zarek confirms that, among the five maturity stages (stage I-V), all of them have the ability to germinate in *in vitro* conditions but the youngest embryos (stage I) germinate only in a small percentage.

Regeneration of *T. wallichiana* plants via shoot organogenesis from callus cultures derived from zygotic embryos has been elaborated by Datta *et. al.* which showed zygotic embryos cultured on ½ WPMSH basal medium supplemented with BA and charcoal led to maximum shoot elongation[46].

Datta and Jha (2008) carried out regeneration of *T. wallichiana* (Zucc.) plants from callus cultures derived from zygotic embryos on ½ WPMSH media (Lloyd and McCown's basal media with SH vitamins). They were able to regenerate only 10% of somatic embryos into plantlets however the regeneration was complete and obtained after 7-8 months of initiation of culture.

Embryos of *Taxus* cultivars undergo precocious germination at a high frequency when isolated from the appropriate developmental stage.

9.4. Establishment of Suspension Culture

Continuous harvesting of plant part for Taxol cannot be the adequate source to meet the clinical demands. So, the interest has shifted to production of Taxol in the suspension culture of plant cells. Improved yield of Taxol in cell suspension culture of *T. wallichiana* Zucc., has also been reported following cell line selection and addition of IAA-conjugates (IAA-glycine, IAA-phenylalanine, IAA-alanine and IAA-aspartic acid) [45]. Using three different cell lines with different Taxol-producing capacities, it has been demonstrated that 2,4-D and IAA-phenylalanine when present alone favored growth and Taxol production but when combined enhanced biomass without enhancing Taxol accumulation. This suggests that two-stage culture is beneficial for optimization of Taxol accumulation.

9.5. Regeneration Protocols

Additional improvements in Taxol production can be achieved through genetic engineering; however, an effective plant regeneration system is needed to facilitate genetic modification. However, regeneration of *Taxus* plants have often been reported to be difficult with regard to embryo formation and synchronous development (*T. brevifolia* [50] and *T. chinensis* [51]. Still, plant regeneration via direct organogenesis from zygotic embryos has been reported in *T. brevifolia* [42].

The most difficult stage of plant regeneration in woody species is the induction of roots on new shoots. For root induction, IBA has been reported to be more effective than NAA from *in vitro* shoots derived from both tree and steckling cultures [41, 52]. Shoots grown on rooting medium with IBA showed less callus formation at their cut ends than grown on medium with NAA. Datta *et. al.* regenerated roots from microshoots of *T. wallichiana* in MS basal medium by adjusting the concentration of nitrate.

10. Uses [2]

Various parts of this species are used for food, medicine, fuel and other domestic purposes. The red and fleshy cup-shaped aril that surrounds the seed is eaten by villagers. The foliage is used as litter and fed to cattle. Wood is used as fuel, somewhere as incense, as bow during middle ages for hunting, for cabinet-work and other fancy articles, such as handles of knives and back of combs, and for wood carving, etc. The wood of this species has poor timber value, but widely used for making doors, windows etc. Green twigs are used to decorate houses in Nepal during religious festivals. Red juice of the bark is used as an inferior dye and utilized by Brahmins for staining the forehead.

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Besides anti-cancer property, leaves have anti-spasmodic properties used in the treatment of hysteria, epilepsy, nervousness and frequently used in Unani system of medicine. Dried leaves are considered to be useful in asthma, bronchitis, hiccough, dizziness, diarrhoea and headache. Ayurvedic products like Talisadi Bati, Talisadi Churna have also been marketed. Extract of *Taxus* can be used in cosmetics, such as hair lotion, rinses, beauty and shaving cream and dentifrices.

11. Commercialization and Demands

Taxol is also known as generic drug Paclitaxel and as its registered trade name Taxol® BMS [Bristol-Myers Squibb]. Based on the current bark extraction procedure, approx. 7200 kg of yew bark is necessary to obtain one kilogram of paclitaxel [53]. Yearly, about 800 metric tons of crude Lodhsalla is collected by Dabur-Nepal from seven districts of Nepal namely: Sindhuli, Dolakha, Sindhupalchowk, Manang, Gorkha, Lamjung, Ramechhap (source: Dabur-Nepal).

However, Himalayan yew, known for the treatment of ovarian and breast cancer has been overexploited and smuggled heavily. A medium size tree normally gives a harvest of two quintals of fleshy leaves worth providing Rs. 4,000 net without tree felling. The leaves are pruned from September to April. Earlier, villagers used to cut down the trees for leaves but these days they cut only the small branches. It is estimated, from a single district Sankhuwasabha, traders export around hundred tons of semi dried leaves every year.

12. Other Studies on *Taxus*

12.1 Tissue Culture

In vitro culture and precocious germination of *Taxus* embryos (*T. brevifolia and T. media*) were done by Flores and Sgrignoli (1991) to overcome the lengthy dormancy requirement of yew seeds. They determined that germination was high in White's and MS media. Effects of temperature on storage of seeds was completed and found that green seeds and seeds with developing arils could be stored at 5°C without large loss in germination [54]; seeds with fully developed arils could be stored frozen at -20°C for 1 week while still allowing about 50% of embryo germination.

Flores and Sgrignoli (1991) reported that a short freezing (-20˚C) of fully matured seeds could break dormancy, but led to lower germination ratio than in younger seeds. Both Flores *et. al.* (1993) and Deyu and Zhongchen (1999) reported storing seeds at 4˚C for about 30-40 days improved germination ability of isolated embryos up to 70% [55-56].

Calluses have been induced from stem and needle explants of *T. brevifolia*, T. *baccata*, T. *cuspidata* and *T. media* on Gamborg's B5 medium supplemented with phytohormones like auxins; 2,4-D, NAA and IBA in various concentrations in combination with 0.2mg/l Kinetin by the duo of Arteca and Wickremesinhe (1993) [57].

Chee in 1994 came up with *in vitro* culture methods for zygotic embryos of different *Taxus* species which included *T. brevifolia*, T. *cuspidata*, T. *baccata* and *T. stricta* in Gamborg's B5 culture media, in order to overcome lengthy dormancy of *Taxus* seeds. He also studied whether seed maturity influenced the frequency of radicle emergence and seedling development [58]. Again in 1995 he reported, plant regeneration via direct organogenesis from zygotic embryos in *T. brevifolia.* Adventitious bud development from calli derived from hypocotyls of germinated zygotic embryos has been reported in *T. chinensis* var. *mairei* [5].

Chang *et. al.*, in 2001 carried out micropropagation of mature *T. mairei* growing in central Taiwan at an elevation of 2000m, using bud explants derived from approximately 1000 year old field grown trees and from 1 year old stecklings raised from rooted cuttings of those trees on MS medium supplemented with various cytokinins and charcoal to inhibit browning. Orthotropic growth was restored in 25% and 10% of the steckling and mature tree derived cultures respectively suggesting micropropagation to be a useful technique for mass propagation of superior yew trees and production of high quality plantlets for Taxol production. While studying the seasonal effects, they found that the survival rate of tree buds collected in the summer and autumn were comparable to those of steckling buds and were significantly greater than that of tree buds collected in the winter but only few explants that survived produce lateral shoots. Stecklings grown in greenhouse at lower elevation produced long shoots, while those grown in field at high elevation gave little shoot elongation.

Ewald (2007) based on his experiences in micropropagation of conifers, worked out in detail on bud formation-propagation-elongation, shooting,

rooting and hardening methods to multiply selected yew cones of *T. baccata* L in Woody Plant Medium (WPM) supplemented with zeatin and thidiazuron.

Conditions for obtaining an efficient mass propagation procedure to overcome dormancy in *T. baccata* seeds were investigated in MS basal media supplemented with activated charcoal [60]. Before culturing, the seeds were soaked in distilled water at low temperature (4° C) at least for 48 hrs instead leaching them under running water which led to better germination and 100% sterility.

Shrubs have been reported to assist the establishment of Yew woodlands [61]. But once the woodlands oust the shrubs, regeneration of *Taxus* is hampered. *Taxus* also inhibits self-regeneration and seedling survival, prompting concerns for the long-term preservation of the species. Pre germination, factors that influence establishment are availability of mother trees (seed source), availability of perch sites, availability of dispersers and availability of suitable habitat for germination, presence of seed predators [62]. Post germination factors like optimum light, nutrient requirement, and protection from grazing are also keys to the survival of the species.

12.2. Suspension Culture

As already mentioned, production of the drug from native plant of *Taxus* is not quite possible because of the low availability of the plant and slow growth. Hence, by using biotechnological approaches semi or total synthesis of *Taxus* has been done by using production by fungi and bacteria and production by cell culture technique in suspension cultures. Accumulating drug in the large scale suspension culture has been taken as the next best, stable and long-term alternative approach for taxoid production [63]. However, since suspension culture has its own limitations like the enzymatic pathway involved, degrades the product and also leads to the conversion of the product, which not only affects the productivity moreover may be toxic to the plant cells itself. Many scientists prefer two phase culture along with using elicitor to increase the productivity along with maintaining the integrity of the cells. Molecules of biological and non-biological origin that stimulate secondary metabolism are called elicitors [64].

Taxol producing gene cloning has also been thought of but it is still underway. Large scale bioprocess engineering of plant cells have been proven to be cost effective approach and the responsiveness of various elicitors studied has further highlighted the applicability of this methodology. Endophytic fungi of *Taxus* species has also largely been studied to produce Taxol, however, sustainable amount generation from these microorganisms has yet not been established and also purification from intact tissues is also presumably lengthy and costly as compared to the plant cell cultures [65].

Various components have been studied so far for the induction of Taxol in the cell cultures. Phenylalanine and vanadyl sulfate were shown to prefer *Taxus baccata* L. cells [66]. L-Phenylalanine, being the precursor of paclitaxel's side chain, has found to be considerably supportive in increasing Taxol content in the suspension culture as reviewed by Zhong (2002) [67]. In 2005, Syklowska-Baranek and Furmanowa conducted a comparative study of biomass growth and examined the influence of L-phenylalanine on production of paclitaxel and 10-DAB III in suspension culture of *Taxus* media var. Hicksii in flasks and bioreactor and got positive results with visible increase in taxane accumulation [68]. Hence, L-phenylalanine when added along with optimized dose of precursors, additional carbon source, increased the paclitaxel content in the cells [69-71].

Ethylene inhibitors like silver nitrate and cobalt chloride, along with their combination also promote Taxol production [72]. Inducing factors, addition time, culture days and stage of cultivation have also shown their influences on the cell culture and Taxol production [73].

Usually researchers are focused on the effects of an individual elicitor. Methyl jasmonate is one of the highly studied elicitor to induce paclitaxel biosynthesis [74-77]. However, understanding the complexity of signal transduction in plant secondary metabolism, Zhang *et. al.* (2000) worked on enhanced paclitaxel production by the combination of chitosan, methyl jasmonate, fungal elicitor and Ag+ compared to that of each elicitor treatment. They determined that the combination of chitosan, methyl jasmonate and Ag+ at their certain concentrations resulted in increased paclitaxel production, being almost 40 times higher than that of control culture, 10 times higher than that of culture exposed to Ag+, 6 times higher than that of culture elicited by chitosan and twice than that of culture elicited by methyl jasmonate.

Wang *et. al.* (2001) published paper entitled "Enhanced Taxol production and release in *Taxus chinensis* cell suspension cultures with selected organic solvents and sucrose feeding " in which he exclaimed the value added by sucrose and organic

solvents in the suspension culture of *T. chinensis* [78].

Khosroushahi *et. al.* (2005) made attempts to improve Taxol production by combining different inducing factors in suspension culture of *Taxus baccata*. After callus induction and cell line selection, they developed 2-stage suspension culture of *Taxus baccata* in Gamborg's B5 medium supplemented with various inducers like vanadyl sulfate, silver nitrate, cobalt chloride, ammonium citrate, phenylalanine, methyl jasmonate, salicylic acid and fungal elicitor. These inducers were supplemented in different phase of cell cultures solely or in combination [79]. The study revealed supplementation of B5 medium with combination of biomass growth factors at stage I and mixture of elicitors at stage II significantly increased Taxol production.

Kajani *et. al.* (2012) examined the ability of dimethylsulfoxide (DMSO) to induce taxane synthesis and release in cell suspension culture of *Taxus baccata* [80], showed that addition of 5% DMSO at the late exponential phase of cell culture (day 14) and culturing for 21 days gave best yield of taxanes both in cell biomass and extracellular taxane portion.

For the quantification of Taxol in the culture or from the crude extract, researches prefer preparative chromatographic analysis followed by HPLC analysis [9, 18, 81]. Besides, LC-MS [82-83], Ion spray MS [84] and HPLC- tandem MS [85] has also frequently been tested. Taxol from both the medium and from cell lines are extracted after suspension culture, filtered, processed and purified and quantified by using UV detection using standard curve for Taxol.

12.3. Molecular Aspects

Genetic diversity using microsatellite markers help to describe the level of genetic variability of *Taxus* populations for better understanding of the genetic relationship among the Himalayan populations. DNA analysis helps to understand the quality of *Taxus* populations for commercial propagation.

Few studies on genetic variation in *T. baccata* are available to date done with concerning the genetic research of the genus (ITSs [86]; RAPD [87]; ISSR [88]). Zamani *et. al.* (2008) studied the genetic diversity among Yew (*T. baccata*) genotypes of Iran using RAPD markers [89], showed high levels of polymorphism confirming RAPD markers are suitable for genetic diversity study of Yew. More recent studies in *T. canadensis* and *T. brevifolia* have used isozymes, RAPDs, restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs) to elucidate clonal variation within populations, population dynamics, or meta-population structure.

Regional differentiation in Swiss populations of English yew was carried out using RAPD markers [87] and to assess the genomic diversity of individual plants within a *T. cuspidata* population [90]. Collins *et. al.* (2003) looked for DNA markers to distinguish species; *T. baccata*, *T. canadensis*, *T. cuspidata* and hybrids (*T. media*, *T. hunnawelliana*) [91]. Huang *et. al.* (2008) developed 12 microsatellite loci in *T. sumatrana* [92]. Mahmoodi *et. al.* (2009) reported isolation and characterization of 31 new polymorphic microsatellite loci from a repeat enriched genomic library of *T. baccata* [93].

RAPD markers are a modification of PCR introduced in the late 1980's [94]. The random amplified polymorphic DNA (RAPD) technique can reveal polymorphism between very closely related genotypes. Recently, RAPD markers have been widely accepted in gene mapping, population genetics, molecular evolutionary genetics and plant and animal breeding. This is mainly due to the speed, cost and efficiency of the RAPD technique to generate large numbers of markers in a short period compared with previous methods [95].

In 2009, Zarek used RAPD markers to generate genetic structure for natural *Taxus baccata* populations from southern Poland [96]. Recently, he successfully screened RAPD and ISSR primers to find molecular markers enabling sex determination of individuals of the European Yew [88].

13. Conclusion

Without any knowledge of its status in the wild and ample knowledge about its ecological requirements, the extensive use of the plant for commercial purposes is enough to drive the species towards its extinction. Populations may be vulnerable and factors such as population size, degree of isolation and fitness [97] can help predict their fate. Not yet known to be successfully regenerated by artificial means, and characterized by poor regeneration in nature [61], an insight into its microhabitat preferences will be useful for future assisted regeneration programs.

14. References

[1] IUCN Red List, 2016-3, http://www.iucnredlist.org/details/summary/46171879/0 (March 27, 2017).
[2] Joshi, K. (2009). *Taxus wallichina* (Himalayan Yew; Loth salla) in Nepal: Indigenous Uses, Conservation and Agenda for Sustainable Management. *Ethnobotanical Leaflets*, 13, 1504-1508.
[3] Nhut, D. T., Hien, N. T. T., Don, N. T. and Khiem, D. V. (2007). *In vitro* shoot development of *Taxus wallichiana* Zucc., a Valuable Medicinal Plant.. *Protocols for Micropropagation of Woody Trees and Fruits*. S. M. Jain, and H. Häggman, editors. Springer Netherlands, 107-116.
[4] Rikhari, H. C., Palni, L. M. S., Sharma, S., Nandi, S. K. (1998). Himalayan Yew: stand structure, canopy damage, regeneration and conservation strategy. *Environmental Conservation*, 25,334-341.
[5] Wani, M.C., Taylor, H. L., Wall, M. E., Coggon, P. and McPhail, A. T. (1971). Plant antitumor agents. VI. The isolation and structure of Taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *Journal of the American Chemical Society*, *93*, 2325–2327.
[6] Sahni, K. C. (1990). Gymnosperms of India & adjacent countries, *BSMPS Publishers*, Dehradun.
[7] Spjut, R. W. (2007). Taxonomy and nomenclature of *Taxus* (Taxaceae). *Journal of the Botanical Research Institute of Texas*, 1(1), 203–289.
[8] Wallich, N. (1826). *An illustrated flora nepalensis.* Calcutta and Serampore, 2.
[9] Fu, L. (1992). China Plant Red Data Book: Rare and Endangered Plants. *Science Press*, Beijing, China and New York, USA.
[10] Collin, H. A. (2001). Secondary product formation in plant tissue cultures. *Plant Growth Regulation*, 34, 119-134.
[11] Frense, D. (2007). Taxanes: perspectives for biotechnological production. *Applied Microbiology and Biotechnology*, *73*, *1233-1240*.
[12] Mohammad, N., Kumar, A., Rikhari, H. C. and Nandi, S. K. (2002). Taxol Content in the Bark of Himalayan Yew in Relation to Tree Age and Sex. *Phytochemistry*, 60(6), 627-631.
[13] Krauze-Baranowska, M. (2004). Flavonoids from the Genus *Taxus. Zeitschrift fur Naturforschung C*, DOI: 10.1515/znc-2004-1-210.
[14] Holmes, F. A., Walters, R. S., Theriault, R. L., Forman, A. D., Newton, L. K., Raber, M. N., Buzdar, A. U., Frye, D. K. and Hortobaygi, G. N. (1991). Phase II trial of Taxol: An active drug in the treatment of metastatic breast cancer. *Journal of the National Cancer Institute*, 83, 1797.
[15] Skeel, R. T. (1999). *Handbook of Chemotherapy*, 5th Ed., ed. Skeel, R. T. (Lippincott Williams & Wilkins, Baltimore), 63-144.
[16] Ehrlich, A., Booher, S., Becerra, Y., Borris, D. L., Figg, W. D., Turner, M. L. and Blauvelt, A. (2004). Micellar paclitaxel improves severe psoriasis in a prospective phase II pilot study. *Journal of the American Academy of Dermatology*, 50(4), 533-540.
[17] Woo, D. D. L., Miao, S. Y. P., Pelayo, J. C. and Woolf, A. S. (1994). Taxol inhibits progression of congenital polycystic kidney disease. *Nature*, 368, 750-753.
[18] Theodoridis, G., Laskaris, G., Bounkhay, M., Heijden, R. V. D., Verpoorte, R. and Jaziri, M. (1999). Induction of geranylgeranyl diphosphate synthase activity and taxane accumulation in *Taxus* baccata cell cultures after elicitation by methyl jasmonate. *Plant Science*, 147(1), 1-8.
[19] Zhang, C. H., Mei, X. G., Liu, L. and Yu, L. J. (2000). Enhanced paclitaxel production induced by the combination of elicitors in cell suspension cultures of *Taxus chinensis*. *Biotechnology Letters*, Kluwer Academic Publishers, Netherlands, 22, 1561-1564.
[20] Ettinger, D. S. (1992). Taxol in the treatment of lung cancer. *Second National Cancer Institute Workshop on Taxol and Taxus*, September 23-24, 1992, Alexandria, Virginia.
[21] Forastiere, A. A., Neuberg, D., Taylor, S.G., DeConti, R., Adams, G. (1993). Phase II evaluation of Taxol in advanced head and neck cancer: an Eastern Cooperative Oncology Group Trial. *Monograph of National Cancer Institute*, 15, 181-184.
[22] Einzig, A. I., Wiemik, P. H., Schwartz, E. L. (1991). Taxol: a new agent active in melanoma and ovarian cancer. *Cancer Treatment and Research*, 58, 89-100.
[23] Arbuck, S. G., Christian, M. C., Fisherman, J. S., Cazenave, L. A., Sarosy, G., Suffness, M., Adams, J., Canetta, R., Cole, K. E., Friedman, M. A. (1993). Clinical development of Taxol. *Monograph of National Cancer Institute*, 15, 11-24.
[24] Roth, B. J., Yep, B. Y., Wilding, G., Kasimes, B., McLeod, D., Loehrer, P. J. (1993). Taxol in advanced hormone refractory carcinoma of the prostrate: a phase II trial of the Eastern Cooperative Oncology Group. *Cancer*, 72, 2457.
[25] Jha, S. and Majumder, A. (2009). Characterization of Podophyllotoxin Yielding Cell Lines of *Podophyllum hexandrum*. *Caryologia*, 62(3), 220-235.

[26] Kayastha, B. P. (2002). *A Handbook of Trees of Nepal*. S. D. Kayastha, Gongabu, Kathmandu, Nepal.
[27] Poudel, R. C., Gao, L-M., Moller, M., Baral, S. R., Uprety, Y., Liu, J. and Li, D-Z. (2013). Yews (*Taxus*) along the Hindu Kush-Himalayan region: Exploring the ethno-pharmacological relevance among communities of Mongol and Caucasian origins. *Journal of Ethnopharmacology*, 147(1), 190-203.
[28] Schiff, P. B., Fant, J. and Horowitz, S. B. (1979). Promotion of microtubule assembly *in vitro* by Taxol. *Nature*, 277, 665-667.
[29] Ho, C. K., Chang, S. H., Chen, Z. Z. (1997). Content variation of taxanes in needles and stems of *Taxus* mairei trees naturally distributed in Taiwan (in Chinese with English Summary). *Taiwan Journal for Science*, 12, 23-37.
[30] Kingston, D. G. I. (2000). Recent advances in the chemistry of Taxol. *Journal of Natural Products*, 63, 726-734.
[31] Jennewein, S. and Croteau, R. (2001). Taxol: biosynthesis, molecular genetics, and biotechnoogical applications. *Applied Microbiology and Biotechnology*, 57, 13-19.
[32] Pazdur, R., Newman, R. A., Newman, B. M., Fuentes, A., Benvenuto, J., Bready, B., Moore, D., Jaiyesimi, I., Vreeland, F., Bayssas, M. M. and Raber, M. N. (1992). Phase I trial of Taxotere: five-day schedule. *Journal of the National Cancer Institute*, 84, 1781-1788.
[33] Gibson, D. M., Ketchum, R. E. B., Vance, N. C. and Christen, A. A. (1993). Initiation and growth of cell lines of *Taxus brevfolia* (Pacific Yew). *Plant Cell Reports*, 12, 479-482.
[34] Banerjee, S., Upadhyay, N., Kukreja, A. K., Ahuja, P. S., Kumar, S., Saha, G. C., Sharma, R. P. and Chattopadhyay, S. K. (1996). Taxanes from *in vitro* cultures of the Himalayan yew, *Taxus wallichiana*. *Planta Medica*, 62(4), 329-331.
[35] Schippmann, U. (2001). *CITES medicinal plants significant trade study*. Project S 109, German Federal Agency for Nature Conservation, Bonn.
[36] Bedi, Y. S., Ogra, R. K., Koul, K., Kaul, B. L. and Kapil, R. S. (1996). Yew (*Taxus* spp.), A new look on utilization, cultivation and conservation. *Supplement to Cultivation and Utilization of Medicinal Plants* (Handa SS & Kaul MK, eds), 443–456. Regional Research Laboratory, Jammu-Tawi, India.
[37] Nicolaou, K. C., Yang, Z., Liu, J. J., Ueno, H., Nantermet, P. G., Guy, R. K., Claiborne, C. F., Renaud, J., Couladouros, E. A., Paulvannan, K. and Sorensen, E. J. (1994). Total synthesis of Taxol. *Nature*, 367, 630-634.
[38] Cameron, S. I. and Smith, R. F. (2008). Seasonal changes in the concentration of major taxanes in the biomass of wild Canada yew (*Taxus canadensis* Marsh.). *Pharmaceutical Biology*, 46, 35-40.
[39] Suffness, M. and Wall, M. E. (1995). Discovery and development of Taxol. *Taxol – Science and Applications.* In: Suffness M, editor, CRC Press; Boca Raton, FL, USA, 3–25.
[40] Ahuja, M. R. (1993). *Micropropagation of woody plants*. Kluwer Academic publishers.
[41] Chang, S. H., Ho, C. K., Chen, Z. Z. and Tsay, J. Y. (2001). Micropropagation of *Taxus mairei* from mature trees. *Plant Cell Reports*, 20, 496-502.
[42] Chee, P. P. (1995). Organogenesis in *Taxus brevifolia* tissue cultures. *Plant Cell Reports*, 14, 560-565.
[43] Brunakova, K., Babincovaz, Z., Takac, M. and Cellarova, E. (2004). Selection of callus cultures of *Taxus baccata* L. as a potential source of paclitaxel production. *Engineering in Life sciences*, 4, 465–469.
[44] Ramachandra Rao, S. and Ravishankar, G. A. (2002). Plant cell cultures: Chemical factories of secondary metabolites. *Biotechnology Advances*, 20, 101-153.
[45] Jha, S., Sanyal, D., Ghosh, B. and Jha, T. B. (1998). Improved Taxol yield in cell suspension culture of *Taxus wallichiana* (Himalayan Yew). *Planta Medica*, 64, 270-272.
[46] Datta, M. M., Majumder, A. and Jha, S. (2006). Organogenesis and plant regeneration in *Taxus wallichiana* (Zucc.). *Plant Cell Rep*orts, 25, 11-18.
[47] Lambardi, M., Capuana, M., Sozzi, L. and Giannini, R. (1995). Factors affecting *in vitro* adventitious bud induction from excised embryos of Swiss Stone Pine (*Pinus cembra* L.). *Forest Genetics*, 2, 49-58.
[48] Datta, M. M. and Jha, S. (2008). Plant Regeneration through Somatic Embryogenesis in *Taxus wallichiana*. *Journal of Plant Biochemistry and Biotechnology*, 17(1).
[49] Le Page-Degivry, M. T. (1973). An embryo culture study of embryo dormancy in *Taxus baccata*. *Biologia Plantarum*, 15, 264-269.
[50] Ewald, D., Weckwerth, W., Naujoks, G. and Zocher, R. (1995). Formation of embryo-like structures in tissue cultures of different yew species. *Journal of Plant Physiology*, 147, 139-143.
[51] Qiu, D., Li, R. and Li, L. (1998). Studies on the somatic embryogenesis of *Taxus chinensis* and *Taxus chinensis* var. Mairei. *Scientia Silvae Sinicae*, 34, 50-54.
[52] Ewald, D. (2007). Micropropagation of Yew (*Taxus baccata* L.). *Protocols for*

Micropropagation of woody trees and fruits. S. M. Jain and H. Haggman eds, 117-123.
[53] Stull, D. P. (1992). *Current* Taxol *production from yew bark and future production strategies.* Second National Cancer Institute Workshop on Taxol and *Taxus*, September 23-24, Alexandria, Virginia.
[54] Flores, H. E. and Sgrignoli, P. J. (1991). *In vitro* culture and precocious germination of *Taxus* embryos. *In vitro Cell Development Biology - Plant*, 27, 139-142.
[55] Flores, T., Wagner, L. J. and Flores, H. E. (1993). Embryo culture and taxane production in *Taxus* spp. *In vitro Cell Development Biology - Plant*, 29, 160-165.
[56] Deyu, X. and Zhongchen, G. (1999). Zygotic embryo culture of *Taxus chinensis* var *mairei* and plant regeneration through organogenesis. *Israel Journal of Plant Science*, 47, 287-290.
[57] Wickremesinhe, E. R. M. and Arteca, R. N. (1993) *Taxus* callus cultures: Initiation, growth optimization, characterization and Taxol production. *Plant Cell, Tissue and Organ Culture*, 35, 181-193.
[58] Chee, P. P. (1994). *In vitro* culture of Zygotic embryos of *Taxus* Species. *HortScience*, 29 (6), 695-697.
[59] Xie, D. and Guo, Z. C. (1999). Zygotic embryo culture of *Taxus chinensis* var. *Mairei* and plant regeneration through organogenesis. *Israel Journal of Plant Science*, 47, 287-290.
[60] Zarek, M. (2007). A practical method for overcoming the dormancy of *Taxus* baccata isolated embryos under *in vitro* conditions. *In vitro Cell Development Biology - Plant*, 43, 623-630.
[61] Svenning, J-C. and Magard, E. (1999). Population Ecology and Conservation Status of the last natural Population of English Yew, *Taxus baccata* in Denmark. *Biological Conservation*, 88(2), 173-182.
[62] Garcia, D. and Lavabre, J. E. (2015). Geographic consistency in the seed dispersal patterns of *Taxus baccata* L. In the Iberian Peninsula. *Forest Systems*, 24(3).
[63] Lee, D-H., Kim, S-G., Mun, S. and Kim, J-H. (2010). Evaluation of feeding and mixing conditions for fractional precipitation of paclitaxel from plant cell cultures. *Process Biochemistry*, 45(7), 1134-1140.
[64] Dornenburg, H. and Knorr, D. (1995). Strategies for the improvement of secondary metabolite production in plant cell cultures. *Enzyme and Microbial Technology*, 17, 674-684.
[65] Cusido, R. M., Palazon, J., Bonfill, M., Navia-Osorio, A., Morales, C. and Pinol, M. T. (2002). Improved paclitaxel and baccatin III production in suspension cultures of *Taxus media*. *Biotechnology Progress*, 18, 418-23.
[66] Brincat, M. C., Gibson, D. M. and Shuler, M. L. (2002). Alterations in Taxol production in plant cell culture via manipulation of the phenylalanine ammonia lyase pathway. *Biotechnology Progress*, 18, 1149-56.
[67] Zhang, C. H., Wu, J. Y. and He, G. Y. (2002) Effects of inoculum size and age on biomass growth and paclitaxel production of elicitor-treated *Taxus yunnanensis* cell cultures. *Applied Microbiology and Biotechnology*, 60, 396-402.
[68] Syklowska-Baranek, K. and Furmanowa, M. (2005). Taxane production in suspension culture of *Taxus media* var. Hicksii carried out in flasks and bioreactor. *Biotechnology Letters*, 27, 1301-1304.
[69] Fett-Neto, A. G., Melanson, S. J., Nicholson, S. A., Pennington, J. J. and DiCosmo, F. (1994). Improved Taxol yield by aromatic carboxylic acid and amino acid feeding to cell cultures of *Taxus cuspidata*. *Biotechnology and Bioengineering*, 44, 967-971.
[70] Yuan, Y. J., Wei, Z. J., Wu, Z. L. and Wu, J. C. (2001). Improved Taxol production in suspension cultures of *Taxus chinensis* var. Mairei by in situ extraction combined with precursor feeding and additional carbon source introduction in airlift loop reactor. *Biotechnology Letters*, 23, 1659-1662.
[71] Luo, J. and He, G. Y. (2004). Optimization of elicitors and precursors for paclitaxel production in cell suspension culture of *Taxus chinensis* in the presence of nutrient feeding. *Process Biochemistry*, 39, 1073-1079.
[72] Zhang, C. H. and Wu, J. Y. (2003). Ethylene inhibitors enhance elicitor-induced paclitaxel production in suspension cultures of *Taxus* spp. cells. *Enzyme and Microbial Technology*, 32, 71-77.
[73] Linden, J. C. and Phisalaphong, M. (2000). Oligosaccharides potentiate methyl jasmonate induced production of paclitaxel in *Taxus canadensis*. *Plant Science*, 158, 41-51.
[74] Ketchum, R. E. B., Gibson, D. M., Croteau, R. B. and Shuler, M. L. (1999). The kinetics of taxoid accumulation in cell suspension cultures of *Taxus* following elicitation with methyl jasmonate. *Biotechnology and Bioengineering*, 62, 97-105.
[75] Mirjalili, N. and Linden, J. C. (1996). Methyl jasmonate induced production of Taxol in suspension cultures of *Taxus cuspidata*; ethylene interaction and induction models. *Biotechnology Progress*, 12, 110-118.
[76] Yukimune, Y., Tabata, H., Higashi, H. and Hara, Y. (1996). Methyl jasmonate induced overproduction of paclitaxel and baccatin III in

Taxus cell suspension cultures. *Nature Biotechnology*, 14, 1129-1132.
[77] Bonfill, M., Expósito, O., Moyano, E., Cusidó, R.M., Palazón, J. and Piñol, M.T. (2006). Manipulation by culture mixing and elicitation of paclitaxel and baccatin III production in *Taxus*. *In vitro Cellular and Developmental Biology – Plant*, 42, 422–426.
[78] Wang, C., Wu, J. and Mei, X. (2001). Enhanced Taxol production and release in *Taxus chinensis* cell suspension cultures with selected organic solvents and sucrose feeding. *Biotechnology Progress*, 17, 89-94.
[79] Khosroushahi, A. Y., Valizadeh, M., Ghasempour, A., Khosrowshahli, M., Naghdibadi, H., Dadpour, M. R. and Omidi, Y. (2006). Improved Taxol production by combination of inducing factors in suspension cell culture of *Taxus baccata*. *Cell Biology International*, 30, 262-269.
[80] Kajani, A. A., Moghim, S. and Mofid, M. R. (2012). Enhanced taxane production and secretion from *Taxus baccata* cell culture by adding dimethylsulfoxide. *Biotechnology and Applied Biochemistry*. (wileyonlinelibrary.com).
[81] Li, Y. C., Tao, W. Y. and Cheng, L. (2009). Paclitaxel production using co-culture of *Taxus* suspension cells and paclitaxel-producing endophytic fungi in a co-bioreactor. *Applied Microbiology Biotechnology*, 83(2), 233-239.
[82] Bitsch, F., Ma, W., Macdonald, F., Nieder, M., and Shackleton, C.H.L. (1993). Analysis of Taxol and related diterpenoids from cell cultures by liquid chromatography-electrospray mass spectrometry. *Journal of Chromatography*, 615, 273–280.
[83] Sadghi-Aliabadi, H., Asghari, G., Mostafavi, S. A. and Esmaeili, A. (2009). Solvent optimization on Taxol extraction from *Taxus baccata* L., using HPLC and LC-MS. *Daru - Journal of Faculty of Pharmacy*, 17(3), 192-198.
[84] Li, N. and Fu, L.K. (1997). Notes on gymnosperms I. Taxonomic treatments of some Chinese conifers. *Novon*, 7, 261–264.
[85] Cass, B. J., Scott, D. S. and Legge, R. L. (1999). Determination of taxane concentrations in *Taxus canadensis* clippings using high performance liquid chromatographic analysis with an internal standard. *Phytochemical Analysis*, 10(2), 88-92.
[86] Spanu, I., Emiliani, G., Paffetti, D. and Giannini, R. (2006). *Proceedings of the 50th Italian Society of Agricultural Genetics Annual Congress*, Ischia, Italy.
[87] Hilfiker, K., Holderegger, R., Rotach, P. and Gugerli, F. (2004). Dynamics of genetic variation in *Taxus baccata*: local versus regional perspectives. *Canadian Journal of Botany*, 82, 219-227.
[88] Zarek, M. (2016). Preliminary studies on the molecular identification of sex in *Taxus baccata* L. *Forest Research Papers*, 77(1), 68-75.
[89] Zamani, S., Abbasian, Z., Khaksar, G., Movahedi, S., Talebi, M. and Tabatabaei, B.E.S. (2008). Genomic diversity among yew (*Taxus* baccata L), Genotypes of Iran Revealed by Random Amplified Polymorphism DNA Markers. *International Journal of Agriculture and Biology*, 10, 648-652.
[90] Li, X. L., Yu, X. M., Guo, W. L., Li, Y. D., Liu, X. D., Wang, N. N. and Liu, B. (2006). Genomic diversity within *Taxus cuspidata* var. nana revealed by random amplified polymorphic DNA markers. *Russian Journal of Plant Physiology*, 53, 684-688.
[91] Collins, D., Mill, R. R. and Moller, M. (2003). Species separation of *Taxus* baccata, *T. canadensis* and *T. cuspidata* (Taxaceae) and origins of their reputed hybrids interfered from RAPD and cp-DNA data. *American Journal of Botany*, 90, 175-182.
[92] Huang, C. C., Chiang, T-Y. and Hsu, T-W. (2008). Isolation and characterization of microsatellite loci in *Taxus sumatrana* (Taxaceae) using PCR-based isolation of microsatellite arrays (PIMA). *Conservation Genetics*, 9(2), 471-473.
[93] Mahmoodi, P., Nekouei, S. M. K., Mardi, M., Pirseyedi, S. M., Ghaffari, M. R., Ramroodi, M. and Siahsar, B. A. (2010). Isolation and characterization of new microsatellite marker in *Taxus baccata* L. *Conservation Genetics Resources*, 2, 195-199.
[94] Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., Tingy, S. V. (1990). DNA polymorphisms amplified byarbitrary primers are useful as genetic markers. *Nucleic Acids Research*, 18, 6531–6535.
[95] Bardakci F. 2001. Random Amplified Polymorphic DNA (RAPD) Markers. *Turkish Journal of Biology*, 25, 185-196.
[96] Zarek, M. (2009). RAPD Analysis of genetic structure in four natural populations of *Taxus* baccata from southern Poland. *Acta Biologica Cracoviensia Series Botanica*, 51(2), 67-75.
[97] Ellstrand, N. C. and Diane, R. E. (1993). Population Genetic Consequences of Small Population Size: Implications for Plant Conservation. *Annual Review of Ecology and Systematics*, 24, 217-242.