STUDIES ON THE PRODUCTION OF TAXOL FROM ENDOPHYTIC FUNGI OF *TAXUS* TREE

By

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Department of Biological Sciences

Quaid-i-Azam University

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Philosophy in

(BIOTECHNOLOGY)

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

In The Name Of Allah The Most Beneficent & The Most Merciful

DEDICATED

TO

MY LOVING PARENTS

Declaration

The material contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

ASIF IQBAL

CERTIFICATE

This Thesis, submitted by **Asif Iqbal** is accepted in its present form by the Department of Biological Sciences, **Quaid-I-Azam University**, **Islamabad** as satisfying the thesis requirement for the degree of Master of Philosophy in Biotechnology.

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Chairperson: _____

Dated 15th Oct. 2005

(Dr. Samina Jalali)

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List of Abbreviations

°C	degree centigrade
µg	Microgram
ETMCM	Enhanced taxol microbial culture media
g	gram
L	Liter
mg	Milligram
ml	milliliter
nm	Nanometer
O.D.	Optical Density
PDA	Potato dextrose agar
PDB	Potato dextrose broth
rpm	Revolutions per minute
SDA	sabouraud dextrose agar
sp	specie
TMCM	taxol microbial culture media

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ABSTRACT

Taxol (paclitaxel) is an anticancerous agent used in the treatment of breast, ovarian and lungs cancer where other standard chemotherapies are failed. This drug is extracted from the bark and leaves of *taxus* species including *Taxus wallichiana* and *Taxus brevifolia*. Unfortunately the whole bark of 100 years old taxus tree yields only 4-5 gm of taxol. The present study is based on the biosynthesis of taxol from endophytic fungi associated in symbiotic relationship with the *Taxus wallichiana*. Different endophytic fungi were isolated from different parts of *Taxus wallichiana* including leaves, stem, tinny and large branches, fruits and seeds. Isolation was done by implanting, surface sterilized, inner tissues and plant parts in water agar plates. The incubation temperature was 20 ^{o}C. Pure culture of different isolated endophytic fungal strains were grown in different mycological medium in shake flask at 20 ^{o}C at 150 rpm for biosynthesis and estimation of taxol. Taxol produced in liquid mycological media was identified by bioassay, chromatographic and spectrophotometric techniques. Maximum taxol 46.54 µg/ml was produced in enhanced taxol microbial culture medium (ETMCM) after 4 weeks of incubation period at 20 ^{o}C in shake flask culture.

INTRODUCTION

INTRODUCTION

An increase in the number of people in the world having health problems caused by certain cancers, drug-resistant bacteria, parasitic protozoans, and fungi has caused alarm. An intensive search for newer and more effective agents to deal with these problems are now underway. Endophytes are a potential source of novel chemistry and biology to assist solving not only human health, but plant and animal health problems also. Endophytes reside in the tissues between living plant cells. The relationship that they establish with the plant varies from symbiotic to bordering on pathogenic. Of all of the world's plants, it seems that only a few grass species have had their complete complement of endophytes studied. As a result, the opportunity to find new and interesting endophytes among the myriad of plants is great. Sometimes extremely unusual and valuable organic substances are produced by these endophytes. These compounds may contribute to the host-microbe relationship. The initial step in dealing with endophytic microorganisms is their successful isolation from plant materials. Then, the isolation and characterization of bioactive substances from culture filtrates is done using bioassay guided by fractionation and spectroscopic methods. Some of the more interesting compounds produced by endophytic microbes with which we have dealt so far, are taxol, cryptocin, cryptocandin, jesterone, oocydin, isopestacin, the pseudomycins and ambuic acid (Strobel, 2002).

Alkaloids are a class of "secondary" plant metabolites that traditionally have been classified as basic compounds derived from amino acids that contain one or more heterocyclic nitrogen atoms. Although this definition holds for most known alkaloids; but now, any N containing secondary compound is considered an alkaloid if it cannot readily be classified otherwise i.e. not an amine, cyanogenic glycoside, glucosinolate, etc. The word alkaloid is derived from the Arabic *al-qali*, a plant from which soda was first isolated. The original definition for alkaloids is pharmacologically active, N-containing basic compounds of plant origin. Humans have been using alkaloids in the form of plant extracts for poisons, narcotics, stimulants and medicines for at least the past several thousand years. The antimalarial properties of quinine, an alkaloid extracted from the bark of *Cinchona* spp. trees have long been known. More than 10,000 alkaloids of

widely differing structures are now known from the small fraction of the planet's plants that have so far been examined. Most medicinal compounds have traditionally been extracted from plant tissues although modern synthetic chemistry has attempted to synthesize all important medicinal compounds. Taxol (paclitaxel), an effective antitumor compound, is an example of a steroid alkaloid.

Taxol, a highly functionalized diterpenoid, is found in each of the world's yew (*Taxus*) species. This compound is the world's first billion-dollar anticancer drug and is used to treat a number of other human tissue proliferating diseases as well. Its high cost makes it unavailable to many people in the world. Taxol is a microtubules agent isolated by Wall's group at research triangular institute, research triangular park, from the stem bark of *Taxus brevifolia,* the western pacific yew (Wani *et al.*, 1971). Unlike all other antimicrotubule agents, taxol acts by promoting the formation of unusually stable microtubules, inhibiting the normal dynamic reorganization of microtubules network required for the mitosis and cell prolifilration (Rowinsky *et al.*, 1990). Early development of this drug was hindered by the relative unavailability of the bark and difficult in solubilizing the compound. However, the novel mechanism of action and activity of taxol in the intra-peritoneally implanted B 16 melanoma murine tumor were impetuses for further development. Potential efficacy in the treatment of breast cancer was suggested by the activity of taxol in the subrenally implanted human MX 1 mammary tumor xenograft, although it was not effective in the subcutaneously implanted murine CD8F, mammary tumor (Taxol-NSC 125973, Md: NCI, 1990).

The natural product Taxol (generic name paclitaxel, Fig.1) is a structurally complex diterpenoid comprised of the tricyclic taxane core containing several acyloxy groups, including the 13-*O*-(*N*-benzoyl phenylisoserinoyl) side chain, which is a structural requirement for efficacy of the drug against several cancers (Kingston, 2000). In particular, the side-chain *N*-benzoyl function is a necessary structural component for the crucial Taxol bioactivity of binding to tubulin heterodimers and promoting the assembly and stability of microtubules that ultimately disrupts cell division (Kingston, 2001).

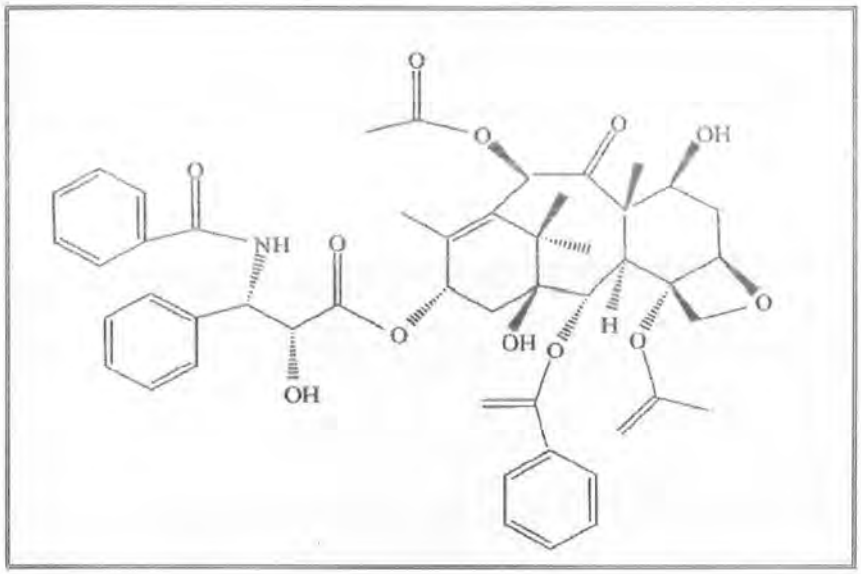


Fig. 1 Structure of Taxol (paclitaxel)

Currently, Taxol is manufactured semisynthetically by coupling the advanced naturally occurring taxoid 10-deacetylbaccatin III, isolated from the needles of *Taxus baccata*, to a synthetic side-chain precursor (Guenard, *et al.*, 1993, Georg et *al.,* 1994). The reliance on the isolation of natural products from *Taxus* species, or *Taxus* derived cell cultures, will continue for the foreseeable future because total synthesis of the drug is not commercially viable. However, isolation from natural sources will become limiting as the clinical applications and resulting demand for Taxol and other advanced, second-generation taxoid drugs increase. However, this approach requires, foremost, an understanding of the pathway(s) leading to not only the diterpenoid portion of Taxol but also to the *N*-benzoyl-phenylisoserinoyl side chain.

Previous biogenetic studies have indicated that benzamidation of *N*-debenzoyltaxol is the final step of the Taxol pathway (Floss and Mocek 1995) (Fig. 2). Isolation of *N*-benzoyltransferase gene required the functional screening of a previously acquired family of related acyl/ aroyltransferase cDNAs from *Taxus* (Walker *et al.*, 2000) by using benzoyl-CoA and a suitable taxoid cosubstrate. Syntheses of *N*-debenzoylated Taxol derivatives have been described (Georg et al., 1994), but require multistep synthesis of the side chain (Kingston, 2000) or use low-abundance taxoids as starting materials for sequential protection, deacylation, and deprotection to the target substrate. As an

semisynthesis of *N*-debenzoyl-(3́*RS*)-2́-deoxytaxol provided a productive surrogate substrate for screening the related (58–69% identity) set of recombinant *Taxus* acyltransferases (Walker et al., 2000).

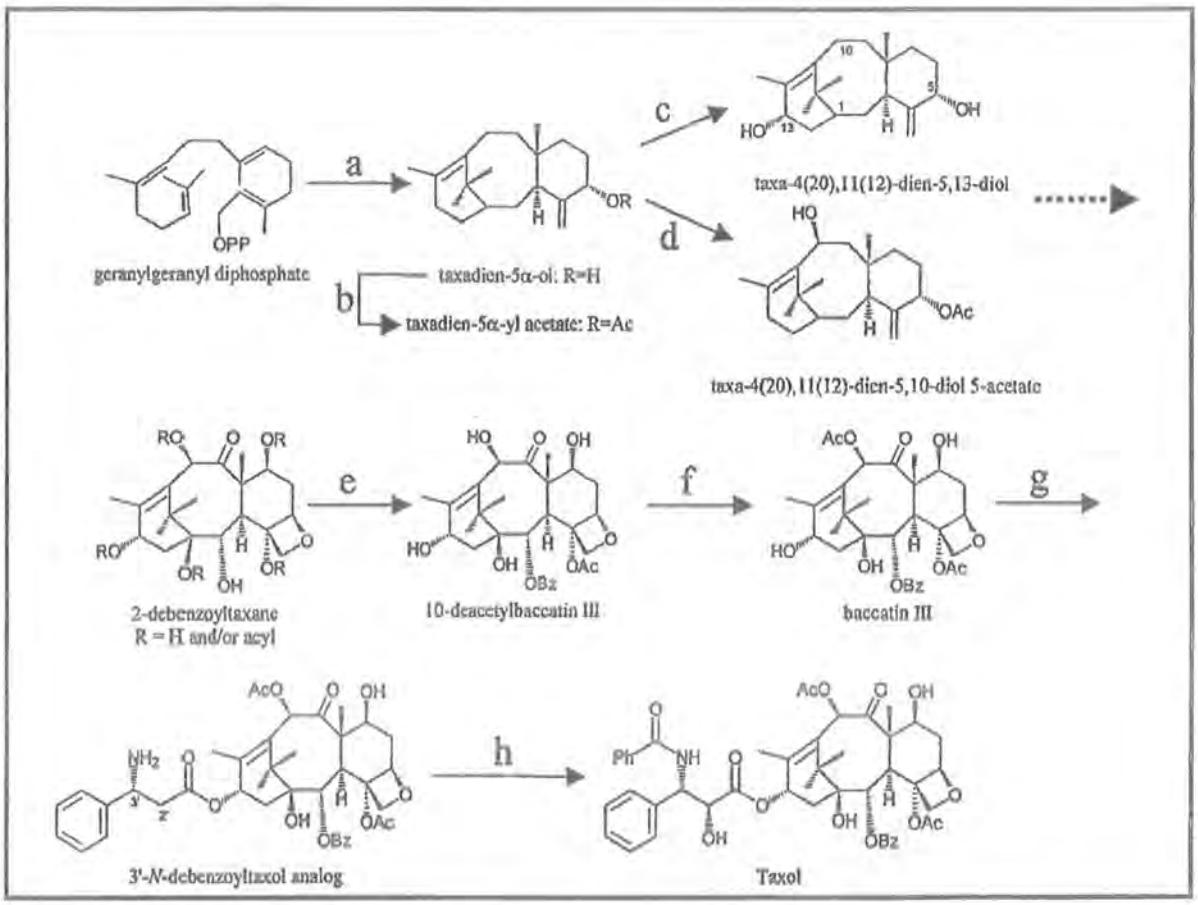


Fig. 2 Outline of the Taxol biosynthetic pathway. are the cyclization of geranylgeranyl diphosphate to taxadiene by taxadiene synthase and the hydroxylation to taxadien-5α-ol by taxadiene 5α-hydroxylase (*a*), the acetylation of taxadien-5α-ol by taxadien-5α-ol acetyltransferase (*b*), the conversion of taxadien-5α-ol to 5,13-diol by a 13α-hydroxylase (*c*), the hydroxylation of taxadien-5α-yl acetate by a 10β-hydroxylase (*d*), the formation of a 2-benzoxy taxoid by a taxane 2α-*O*-benzoyltransferase (*e*), the conversion of 10-deacetylbaccatin III to baccatin III by a 10-*O*-acetyltransferase (*f*), side-chain attachment by the phenylpropanoyltransferase (*g*), and side-chain benzamidation by DBTNBT to form Taxol (*h*). The broken arrow indicates multiple convergent steps (Walker et al., 2002).

Taxus belong to the family Taxaceae. Different *Taxus* species have been found in rain forests throughout the world. This tree has been known for ages from religious and medical point of view. Early it was known as poisonous tree but after the discovery of Taxol (Paclitaxel) an anticancerous agent, it become very important for researchers and pharmaceutical companies. Different species of taxus, which are available around the world, are shown in table 1.

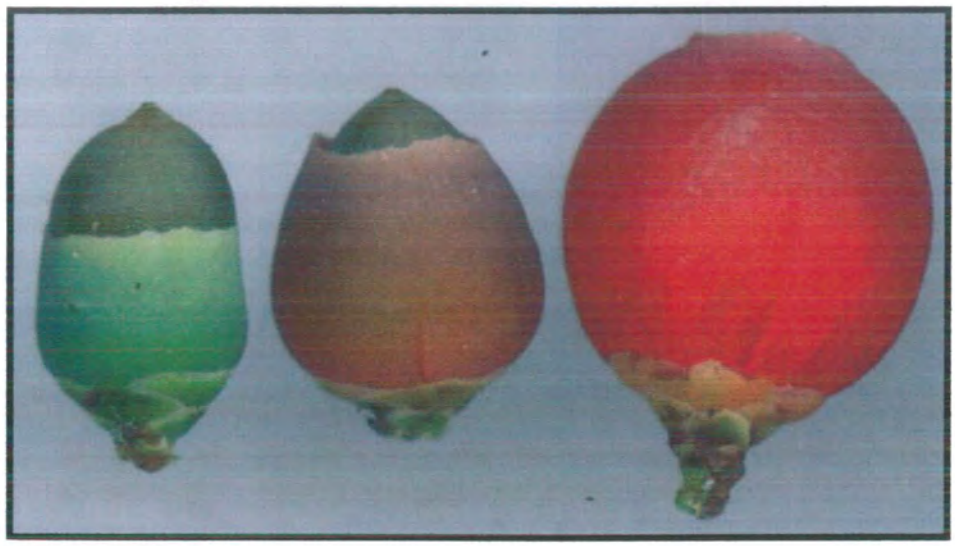
Table 1 Different species of *Taxus* and their common names

Scientific name	Common Name
Taxus wallichiana	Himalayan yew
Taxus baccata	European yew
Taxus brevifolia	Pacific yew
Taxus Canadensis	Canadian yew
Taxus caspidata	Japanese yew
Taxus floridena	Florida yew
Taxus globosa	Mexican yew
Taxus sumatrana	Sumtran yew
Taxus mairi	Chinese yew

Taxus wallichiana (HIMALAYAN YEW)

Taxus wallichiana (a Himalayan yew), locally known as *Burmi*, is widely distributed in Pakistan throughout Azad Kashmir (Poonch), Nothern Areas, NWFP (Hazara, Swat, Chitral, Kurram, Dir) and Punjab (Murree). Its different parts (Leaves, stem, bark and fruit) have many medicinal uses, e.g., its bark is used in lungs, breast and ovarian cancer, ripened fruit and bark is used in bronchitis and high cough, powder of dried ground leaves is applied on wounds, wood is used for cabinet making, furniture, poles and excels of carts, foliage is fed to cattle and fleshy fruits are edible. Tree is also used for fuel.

a



b

Fig. 3 *Taxus wallichiana* (Himalayan Yew) a. Leaves and branches b. ripening stages of fruit

TAXOL PRODUCING ENDOPHYTIC FUNGI

Over the years, a great deal of scientific attention has been given to economically important plants suffering from disease distress. However, it is now known that plants serve as a reservoir for an untold number of microbes known as endophytes (Bacon and White 2000; Ellis and Ellis 1985). Some of these endophytes produce various useful bioactive molecules, which has encouraged a worldwide scientific effort to isolate and study them. While there is a myriad of epiphytic microbes associated with plants, the endophytic ones seem to be more attracting possibly because of their close biological associations with taxus spp. The types of biological associations that endophytic microbes have developed with higher plants range from borderline pathogenic to commensal, and to symbiotic relation. In any of these situations, it is obvious that the minimum contribution of the plant to the endophyte is one of providing nutrition. However, it is also possible that the plant provides, to the endophyte, compounds critical for the completion of their life cycle or essential for growth or self-defense. In addition, one of the least studied, yet imaginable, roles of endophytic fungi is to initiate the biological degradation of the dead or dying host plant, which begins the critical processes of nutrient recycling. On still another, more molecular biological front, it is likely that mechanisms exist for the transfer of nucleic acids from plants to endophytes and vice versa, since some of the same relatively rare bioorganic molecules made by specific higher plants can be produced by certain endophytes as well. The study of plant-associated microbes, in general, may offer opportunities for discoveries relating to agriculture, industry, and medicine.

SPECIFIC ENDOPHYTIC FUNGI

Pestalotiopsis spp. are some of most commonly isolated endophytic fungi of rain forest plants (Raj 1993). In fact, it is rare to isolate one of these species from any tropical plant. Thus, this fungus could be considered as the *"Escherichia coli* of the rain forest." However, its role in the plant, and to the ecosystem in general, is only beginning to be understood. One of the most common species of *Pestalotiopsis* is *P. microspora* (Speg.) (Sutton 1980) (Figs.4). Organisms virtually identical to the taxonomic description of *P.*

microspora are numerous and have usually been isolated as leaf and stem pathogens of economically important tropical plants, such as the palms, pines, loquats, guavas, mangoes, and a large number of ornamental plants (Raj 1993). Generally, this fungus is considered a relatively weak plant pathogen, which, at times, acts in a more severe manner to cause major plant loss. The widely held view that this is a relatively obscure fungal group of interest only to tropical pathologists should undoubtedly be revisited. It seems that this fungus and its close relatives are not as important as plant pathogens as they are playing some role as endophytic fungi, living in symbiotic relationships with plants of the world's temperate and tropical rain forests. From a global perspective, it probably represents one of the largest biomasses of any plant-associated endophytic fungus in the world.

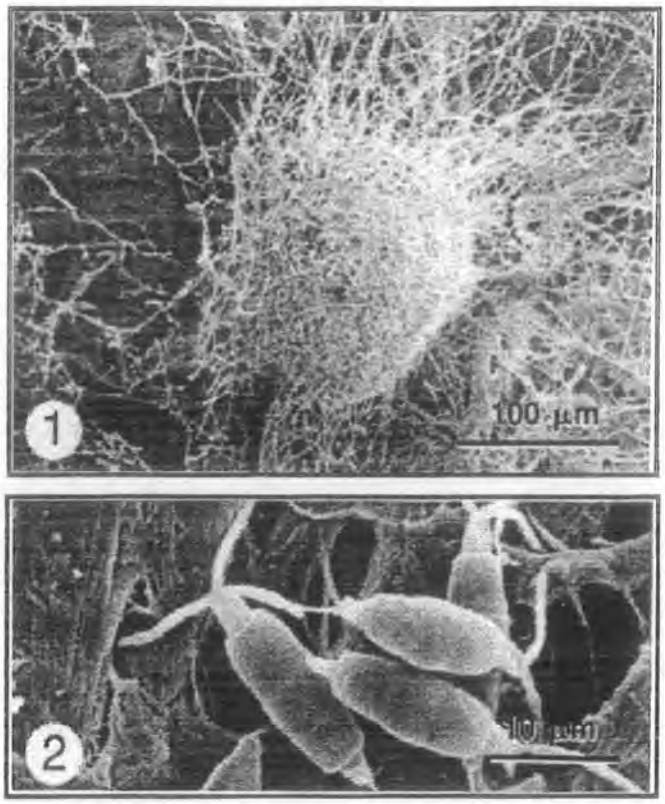


Fig. 4 Endophytic fungi. **(1).** Representative acervulus of *Pestalotiopsis microspora*, a major endophytic fungus found in the world's rain forests taxus species. **(2).** Conidiospores of *Pestalotiopsis microspora* with their characteristic appendages (Strobel, 2002).

A background understanding of the presence of endophytic fungi in higher plants helped researchers to develop a drug discovery program. Taxol, a highly functionalized diterpenoid, is found in each of the world's yew (*Taxus*) species. This compound is the world's first billion-dollar anticancer drug and is used to treat a number of other human tissue proliferating diseases as well. Its high cost makes it unavailable to many people in the world. Therefore, alternative sources are needed because organic synthesis is not economically feasible yet (Nicolaou *et al.*, 1994). A few years ago, it was reasoned that yew trees may support certain endophytic microbes that also make taxol. Thus, if a microbial source of the drug would be available (Strobel, 2002), it would eliminate the need to harvest and extract the slow growing and relatively rare yew trees for this drug. The price for the drug would then be reduced, since taxol would conceivably be produced via fermentation in much the same way that penicillin is produced. It also speculated that the ability of any endophyte to make taxol might have arisen from the exchange of genetic material from the yew tree to one or more microbes living in close association with it. At the time, however, no endophytic fungi had been isolated or were even known from any of the world's representative yew species. After several years of effort, a taxol-producing endophytic fungus, *Taxomyces andreanae* Strobel, Stierle, & Hess, was discovered (Strobel *et al.*, 1993; Stierle *et al.*, 1993, 1995). Efforts of several pharmaceutical companies are now underway to determine the feasibility of making microbial taxol a commercial reality.

AIMS AND OBJECTIVES

The aims and objectives of the present study are:

- Isolation of the endophytic fungi from Taxus tree
- Identification and characterization of isolated fungi.
- Production of metabolites from these fungi.
- Characterization of the metabolites to produce and their antibacterial activity.
- Optimization of conditions like pH and temperature for the production of taxol by the endophytic fungi.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Vidensek *et al.*, (1990) studied taxol content in bark, wood, root, leaf, twig, and seedling from several Taxus species. Taxol content in various parts of several *Taxus* species have been determined. The weight percent ranged from 0.00003 to 0.069. Fett Neto,. and DiCosmo (1992) analysed for taxol concentration from extract of different fresh shoot parts of male and female plants of *Taxus cuspidata* by high performance liquid chromatography (HPLC). Extracted parts included: young needles (first 10 top needle pairs of 30 cm long branches), old needles (last 10 needle pairs of 30 cm long branches), green bark, dark bark (with intense secondary growth), young wood (originally surrounded by green bark), wood (originally surrounded by dark bark), young stems (surrounded by green bark and devoid of needles), and mature male cones. All parts except male cones had measurable amounts of taxol; no effect of plant sex on taxol levels of the plant parts analysed was observed. Results indicated that the bark accounted for almost all the taxol present in stems devoid of needles. Needles showed the highest levels of taxol (overall average of 0.035 +/- 0.006% of the extracted dry weight), significantly higher than those displayed by dark bark samples (0.012 +/- 0.001% of the extracted dry weight). Different needle post-harvesting procedures were evaluated in relation to taxol yields, 96 h dark incubation at –12 °C and 96 h dark incubation at 25 °C under vacuum gave taxol yields equivalent to those of freshly extracted samples. However, results obtained for 96 h dark incubation at 60 °C indicated some extent of taxol degradation.

Wheeler *et al.*, (1992) analytical determined taxol, cephalomannine, and baccatin III in more than 200 trees representing several populations of *T. brevifolia* and other yew taxa indicate that significant variation in taxane content exists among and within populations and species, taxol levels exceeding those reported for *T. brevifolia* bark were found in shoots of individual trees from most taxa studied, and the season in which samples are collected and handling procedures can influence taxane content.

Keisey and Vance (1992) measured taxol and cephalomannine concentrations in the bark and foliage of Pacific yew trees growing in the shade of a forest canopy and at a site nearby where trees had been exposed to full sunlight for *6* year. Bark was the only tissue showing concentration differences due to light, with significantly greater quantities of both compounds in the bark of shaded trees than in the bark of sun-exposed trees. In

either light regimen taxol concentrations were greater in the bark than in the needles or twigs. Taxol concentrations were higher in older needles than in younger needles, and the reverse was observed for twigs. Cephalomannine was substantially lower than taxol in concentration. Cephalomannine varied similarly to taxol among tissues of shade trees but not among tissues of sun-exposed trees.

Rao (1993) published procedures for the isolation of taxol from the Pacific yew (*Taxus brevifolia*) and other species of *Taxus* are cumbersome, and the yields of taxol are in the range of 0.0075-0.01%. This describes a simple and efficient procedure for the isolation of taxol and its major natural analogues from the bark of *T. brevifolia* consisting of a single chromatographic column (using silica gel, Florisil, or a reverse-phase C18-silica), followed by crystallization. Isolated yields of taxol from five "pooled" bark samples (blended from many different batches by the supplier) were in the range of 0.02-0.04%, and from bark collected from a more restricted locale, yields reached 0.06%. The procedure also yielded taxol analogues, such as 10-deacetylbaccatin III (0.02-0.04%), 10-deacetyltaxol-7-xyloside (0.06-0.1%), taxol-7-xyloside (0.005-0.01%), 10-deacetyltaxol (0.01-0.02%), 10-deacetylcephalomannine-7-xyloside (0.006-0.01%), and cephalomannine (0.005-0.007%). Of these, 10-deacetyltaxol-7-xyloside is the most abundant taxane in the Pacific yew bark.

Han *et al.*, (1994) reported Genetic transformation of mature *Taxus* This report demonstrates genetic transformation of two *Taxus* species. *Taxus brevifolia* and *Taxus baccata*, and expression of bacterial genes transferred into the plant genome by *Agrobacterium tumefaciens*. They used two strains of *Agrobacterium tumefaciens* (Bo542 and C58) to inoculate shoot segments of mature yew trees. The highest gall formation frequency (28.3%) was achieved with *Taxus baccata* using the Bo542 strain. *Agrobacterium tumefaciens* strain Bo542 induced significantly more galls (24%) than strin C58 (4%). Taxol and related taxane produced by the transgenic callus cultures were identified by mass spectrometry and immunoassay with monoclonal antibodies specific for taxol. Suffness and Arbuck (1994) discover that the extract of the bark of the Pacific yew tree contained cytotoxic principles.

Das *et al.*, (1995) studied 13-Acetyl-13-decinnamoyltaxchinin B, a taxoid having a rearranged 11(15 → 1)*abeo*-taxane skeleton, had been isolated from the needles of the

Himalayan yew, *Taxus baccata*. The compound has not previously been encountered in nature. Its structure was established by 1- and 2D NMR techniques. The conversion of 13-decinnamoyltaxchinin B, a known taxoid, to 13-acetyl 13-decinnamoyltaxchinin B confirmed the structure of the latter.

ElSohly *et al.,* (1995) studied the taxane content of the needles of 57 cultivars obtained from five major nurseries in the United States presented. The analysis of taxol, and five other related taxanes, 10-desacetyl-7-epi-taxol, cephalomannine, 10-desacetyl taxol, baccatin III and 10-desacetyl baccatin III was carried out using a high performance liquid chromatographic method. The total 'useful' taxanes varied between 0.0232%-0.113%, with 1 and 6 being the most abundant taxanes. The survey identified 11 cultivars with a content of 1 ranging from 0.028% to 0.062%, and ten cultivars with a content of 6 ranging from 0.028% to 0.066%; 3 and 4 each represented approximately 30%-50% of the concentration of taxol in most cultivars. Therefore, the needles of ornamental Taxus represent a significant, rich and renewable source for taxol and other related taxanes.

Choi *et al.*, (1995) reported the concentrations of taxol and related compounds in the bark and needles of *Taxus cuspidata* grown on Mt. Jiri, Mt. Sobaek, and Cheju Island, and *T. cuspidata* var. *latifolia* on Ullung Island in Korea were determined by high performance liquid chromatography (HPLC). The taxane content significantly varied with the location and plant part. The taxol content in the bark of native yews from Mt. Jiri and Mt. Sobaek was high when compared to that reported for Pacific yew *(T. brevifolia*), whereas bark from trees on Cheju and Ullung islands contained a much lower level. Surprisingly, the needles from Cheju and Ullung islands contained a much higher level of taxol (0.022% and 0.0173%, respectively) than those of intermountain locations (0.0058% to 0.0085%), on the basis of dry weight. The bark and needles of *T. cuspidata* var. *latifolia* on Ullung Island also contained relatively high concentrations of 10-deacetylbaccatin III, 0.0497% and 0.0545%, respectively, and indicated that environmental factors may affect the quantity. Taxol in the needles was confirmed by electrospray mass spectrometry. These results suggested that foliage from yew trees growing in their natural habitats on Cheju and Ullung islands might provide a renewable source for taxol.

Rikhari *et al.*, (2000) investigated that the *Taxus baccata* L. subsp. *wallichiana* (Zucc.) Pilger has come into prominence in recent times due to its uncontrolled harvesting from

the Himalayan wilds for the extraction of the anti-cancer drug Taxol™. It is a very slow growing tree with poor regeneration, and the extent of canopy damage is likely to have serious consequences on biomass yield, plant survival and natural regeneration by affecting 'seed' output. The study in the Jageshwar area of the Central Himalaya aimed to determine the stand and canopy structure, microsite characteristics, extent of canopy removal, and regeneration in human-disturbed and undisturbed sites. The number of trees, saplings and seedlings varied amongst plots. Leaf area index and canopy volume increased with increasing circumference at breast height. Of the total canopy volume, 57.4% was found to have been removed from the study area (9.54 ha; representing about 8% of the total *T. baccata* habitat). Regeneration of the species was found to be better in moist and shady microsites at undisturbed locations than in disturbed sites.

Tsumura *et al.,* (1999) reported that two taxa of *Taxodium,* bald cypress and pond cypress, occur in the southeastern United States. The ranges of these taxa overlap in the southeastern Coastal Plain, with the range of the latter being more restricted. Although these taxa co-occur throughout a portion of the more expansive range of bald cypress *Taxodium distichum* (L.) Rich. The habitats of the two taxa appear to differ. Consequently, considerable debate has occurred regarding the taxonomic status of pond cypress. Some authors recognize pond cypress as a distinct species (*Taxodium ascendens* Brongn.), whereas others recognize it as a variety/ecotype (*Taxodium distichum* var. imbricarium (Nutt.) Croom). In their study, Tsumura et al., (1999) reported the genetic diversity of these two taxa was investigated using 10 DNA markers based on sequences from cDNA clones of *Cryptomeria japonica* Results of DNA analysis using cleaved amplified polymorphic sequences (CAPS) in this study did not suggest that pond cypress was a species distinct from bald cypress. So they concluded that the two taxa of *Taxodium* should be given varietal status.

Rikhari, *et al.,* (1998) gave an idea that *Taxus baccata* L. subsp. wallichiana (Zucc.) Pilger has come into prominence in recent years because of its over exploitation from the Himalayan forests for pharmaceutical drugs. Despite wide elevational distribution (1770-3400 m elevation), it never forms extensive stretches and commonly occurs as undercanopy species. Further, it is an extremely slow-growing tree with poor seed germination. Along the disturbance gradient *Taxus* shows different population patterns,

Least disturbed mixed broadleaf forest association shows stable population. The number of seedlings was related to crown cover and soil pH.

Nadeem, *et al.*, (2002) studied Taxol content in the bark of Himalayan Yew in relation to tree age and sex. In this study Taxol content in the bark of Taxus baccata trees growing in a homogenous (uniform) environment at Jageshwar, District Almora in Central Himalaya has been quantified. The average taxol concentration in the bark of sampled trees was 0.0558+/-0.008% (of dry wt.) and was about 64% higher for male plants (averaged across tree age) in comparison to female trees. Maximum taxol content was recorded in the bark samples collected from trees of >110 yrs age. The total taxol content of the bark of Taxus trees across an age series was found to range between 0.064 to 8.032 g/tree, and a tree of about 100 yrs age can yield 5.74 kg dry bark.

Luo, *et al.*, (2002) studied paclitaxel production by *Taxus chinensis* in 5 L bioreactors. Paclitaxel accumulation was doubled by the cultivation of cells initially with dissolved O_2 tension at 60% for 20 days followed by being at 20% for another 12 days in the bioreactor. Combination of these two strategies gave maximum paclitaxel production of 19 mg/L after 32 days.

Mukherjee *et al.*, in 2002 worked on Extraction and analysis of paclitaxel and other taxanes in bark, needle leaves and stem segments of male and female plants of *Taxus wallichiana*, representing several populations, indicated that significant variation in taxane content exists within the population. Bark accumulated maximum amount of paclitaxel in almost all plants. Populations located at higher altitude tended to accumulate more paclitaxel than lower altitude plants. Seasons in which samples were collected and plant age had also been shown to affect paclitaxel accumulation. No effects of plant sex on paclitaxel content of the plants analyzed were observed in that study but significant differences in baccatin-III and 10-deacetylbaccatin III content were found to exist in the analyzed trees.

Deutsch, *et al.*, (1989) reported that taxol had shown good in vivo antitumor activity in a number of test systems. The formulation of taxol for antitumor testing has been difficult. Esterification at either (2-2' or C-7) resulted in loss of in vitro tubulin assembly activity but not cytotoxicity. These observations suggested that esters a t C-2' and/or C-7, which

would tend to promote water solubility, might serve as useful prodrugs of taxol. The reaction of taxol with either succinic anhydride or glutaric anhydride in pyridine solution at room temperature gave the crystalline mono 2' adducta l b and lf, respectively.

McGuire *et al.*, (1989) assist the activity of taxol in patients with advanced, progressive, and drug-refractory ovarian cancer and to delineate more clearly the toxicity of taxol in this patient population. Forty-seven patients with drug-refractory epithelial ovarian cancer who had one or more lesions measurable in perpendicular diameters. Of these patients, 45 were evaluable for toxicity and 40 were evaluable for response. Twelve patients (30%; CI, 16% to 44%) responded to taxol for periods lasting from 3 to 15 months. The dose-limiting toxicity was myelosuppression with leukocytes affected more severely and commonly than thrombocytes or reticulocytes. Leukopenia was usually brief in duration but was associated with sepsis in 3 cases (2 fatal). Other adverse effects included myalgias, arthralgias, alopecia, diarrhea, nausea, vomiting, mucositis, and peripheral neuropathy. Rare cases of cardiac and central neurotoxicity were also noted. So Taxol was an active agent in drug-refractory ovarian cancer and deserves further study in combination with other active drugs in previously untreated patients with advanced ovarian cancer.

Leu *et al.*, (1993) developed a mouse monoclonal anti-taxol antibody (69E4A8E) and rabbit polyclonal anti-taxol antiserums, which were used to measure taxol levels in plant extracts in a double-blind experiment in conjunction with assays by HPLC. The antiserum, on the other hand, was, by radioimmunoassay (RIA), essentially equally reactive with taxol and cephalomannine. Immunoassays of the plant extracts gave results in agreement with that found by HPLC, suggesting that the antibodies can be used in simple routine procedures for the quantification of taxol or taxol-like compounds in extracts of plants or other potential natural sources.

Arbuck *et al.*, (1993) first noted cardiac toxicity in patients receiving Taxol during continuous cardiac monitoring, which was performed because of the high incidence of serious hypersensitivity reactions noted early in phase I trials. After cardiac events were documented, patients with cardiac disease and those on medications known to alter cardiac conduction were excluded from most trials. Adverse cardiac events were reviewed in four clinical databases: 1) the Cancer Therapy Evaluation Program's Adverse

Drug Reaction database following treatment of more than 3400 patients; 2) all cardiac toxicities in patients on GOG-111 who were randomized to cisplatin plus either Taxol or cyclophosphamide; 3) cardiac toxicity in 198 patients who received 618 courses of Taxol with or without cisplatin during continuous cardiac monitoring; and 4) cardiac toxicities reported for the first 696 patients on NCI TRC-9103 for ovarian cancer. Published reports of studies of taxine's cardiac effects, and of cardiac toxicity associated with yew poisoning, Cremophor EL, and H1 and H2 antagonists, are also reviewed. In patients without significant cardiac risk factors, asymptomatic sinus bradycardia is frequent (approximately 30%). To maximize patient safety and the clinical database, physicians who administer Taxol should continue to be alert to potential cardiac toxicities associated with Taxol.

Arbuck *et al.*, (1993a) reported that taxol's unique effects include its ability to polymerize tubulin into stable microtubules in the absence of cofactors and to induce the formation of stable microtubule bundles. During its development, formidable challenges were overcome: a suitable formulation was developed, an adequate supply was ensured, severe hypersensitivity reactions were diminished in incidence and severity, and clinical efficacy was demonstrated. In December 1992, Food and Drug Administration approval was granted for use of Taxol as second-line therapy in ovarian cancer patients.

Suffness, (1994) reported that taxol is well recognized as an antitumor agent and a biochemical tool for studies of microtubules. A proposal was made that taxol is a surrogate for a key endogenous regulator of microtubules, which has the particular function of stabilizing the mitotic spindle. The proposal is based on evidence from the breadth of taxol activity across organisms, data supporting a highly conserved binding site for taxol, low-dose effects of taxol targeting the mitotic spindle, the restriction of the binding sit to a highly conserved segment of B-tubulin, data on the biosynthesis and distribution of taxol, and the recent discovery of an anti-idiotype antibody with taxol-like activity.

Valeriote, *et al.*, (1995) discoved anticancer agents from natural products. They developed and employed Cellular, in vitro assays for new, solid tumor specific anticancer agents. Parallel in vivo models are then used to assess the therapeutic efficacy of candidates selected by the in vitro assays. Nemeth-Kiss *et al.*, (1996) reported that the

different Taxus species by high-performance liquid chromatography to prove the presence of taxol in Hungarian Taxus species. Taxol was established as being present in measurable amounts in each Hungarian Taxus species. According to the results bark was richer in taxol than foliage. It could also be observed that the older the bark or foliage, the more taxol it contained. The validation process proved that the method is reliable and can be used for the separation and quantitative determination of taxol in both the bark and foliage of Taxus species grown in Hungary.

Ramesh (1998) developed paclitaxel for treatment of various cancers; the Food and Drug Administration have approved it for the treatment of ovarian and metastatic breast cancer. Originally paclitaxel was isolated and purified from the bark of Pacific yew trees. This source of paclitaxel was considered to be economically and ecologically unsuitable, as it required the destruction of the yew trees. The alternate methods for the production of paclitaxel, specifically, a semisynthetic approach and the application of biocatalysis in enabling the semisynthesis of paclitaxel.

Mu *et al.*, (1999) reported that the anti-cancer drug taxol binds to beta-tubulin in assembled microtubules and causes cell cycle arrest in animal cells; in contrast, in fungi, the effect of taxol varies. For instance, the taxol-producer *Pestalotiopsis microspora* Ne32, an ascomycete, is resistant to taxol (IC50 greater than 11.7 microM), whereas *Pythium ultimum*, an oomycete, is sensitive to taxol (IC50 0.1 microM). The results suggested efficient binding of taxol to microtubules in *P. ultimum*, but not in *P. microspora,* and are consistent with the differential taxol sensitivity of these two organisms. A comparison of previously characterized taxol binding sites in various beta-tubulin sequences showed that beta-tubulins of taxol-sensitive organisms, including *P. ultimum*, contain Thr219, but beta-tubulins of resistant organisms, including *P. microspora,* contain Asn or Gln at this position, suggesting an important role for residue 219 in the interaction between taxol and beta-tubulin.

A randomized, noncomparative, parallel-group study was designed to evaluate the pathologic complete response (pCR) rate of combined doxorubicin plus paclitaxel (AP) and doxorubicin plus cyclophosphamide (AC) as neoadjuvant chemotherapy in patients with previously untreated breast cancer who were unsuitable for conservative surgery (Véronique *et al.*, 1999).

Yuan *et al.*, (2000) investigated the effects of taxol on SMMC-7721 human hepatoma and its mechanisms. In vitro cell growth was assessed by trypan blue exclusion method. Experimental hepatoma model was established by seeding SMMC-7721 cells subcutaneously into Balb/c (nu/nu) nude mice. In vivo tumor growth was determined by measurement of tumor diameter with Vernier calipers. The syntheses of DNA, RNA and protein were analyzed by incorporation of ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-leucine respectively. Using light and electron microscopes to observe the morphological changes of cells including mitosis and apoptosis. Taxol was effective against SMMC-7721 human hepatoma cell growth in the ranges of 2.5 nmol/L - 10 nmol/L with mitotic arrest and apoptosis in vitro. DNA, RNA and protein syntheses in cells were also obviously suppressed by in vitro treatment of taxol for 72 h. In vivo, taxol significantly inhibited SMMC-7721 tumor growth at 10 mg/kg, i.p., once daily for 10 d. A more than 90% decrease in tumor volume was observed by day 11 ($P<0.01$) similarly with mitotic arrest and cell apoptosis. They concluded Taxol has a marked anticancer activity in SMMC-7721 human hepatoma both in vitro and in nude mice. Its mechanisms might be associated with mitotic arrest, subsequently, apoptosis of the hepatoma cells. No obvious toxicity was observed with in vivo administration of taxol.

Hezari and Croteau (1997) reported that the novel diterpenoid taxol (paclitaxel) is now well-established as a potent chemotherapeutic agent. Total synthesis of the drug is not commercially feasible and, in the foreseeable future, the supply of taxol and its synthetically useful progenitors must rely on biological methods of production. The first three steps of taxol biosynthesis have been defined and the responsible enzymes were described. These are the cyclization of the universal diterpenoid precursor geranylgeranyl diphosphate to taxa-4(5),11(12)-diene, the cytochrome P450-catalyzed hydroxylation of this olefin to taxa-4(20),11(12)-dien-5α-ol, and the acetyl CoA-dependent conversion of the alcohol to the corresponding acetate ester. Demonstration of these early steps of taxol biosynthesis suggests that the complete pathway can be defined by a systematic, stepwise approach at the cell-free enzyme level. When combined with in vivo studies to determine contribution to pathway flux, slow steps can be targeted for gene isolation and subsequent overexpression in Taxus to improve the yield of taxol and related compounds

Long *et al.*, (1998) studied the transformation of the taxol-producing filamentous fungus *Pestalotiopsis microspora* with a plasmid containing the bacterial hygromycin resistance gene fused to *Aspergillus* regulatory sequences resulted in the *in vivo* formation of extrachromosomal DNAs with telomeric repeats in the majority of transformants. Repeats of the telomeric sequence 5′-TTAGGG-3′ were appended to nontelomeric transforming DNA termini. No fungal sequences other than telomeric repeats were detected in extrachromosomal DNAs. Transformants contained three to six different sizes or conformational forms of extrachromosomal DNAs. The DNAs showed no change in size or internal structure during 6 months of growth with selection, but were lost after 20 days of growth without selection. Transformation of wild-type *P. microspora* with a PCR-amplified extrachromosomal DNA having terminal telomeric repeats produced up to 50-fold more transformants than the original transformation vector. The addition of telomeric repeats to foreign DNA is unusual among fungi and may have important adaptive or developmental implications.

Furmanowa and Syklowska-Baranek (2000) established Hairy root cultures of *Taxus media* var. *Hicksii* by infection of the plantlets with the *Agrobacterium rhizogenes* strain LBA 9402. The paclitaxel accumulation in hairy root cultures increased after 100 μM methyl jasmonate treatment from 69 to 210 $\mu g\ g^{-1}$ dry wt while the 10-deacetybaccatin III content was not affected by the elicitor.

Walker and Croteau (2001) studied in the function and properties of heterologously expressed full-length cDNA clones, isolated from a Taxus cDNA library and specific to Taxol biosynthesis, Recombinant enzymes were described that catalyze early steps of the pathway, including taxadiene synthase, taxadien-5alpha-ol-O-acetyltransferase and taxadien-5alpha-yl acetate 10beta-hydroxylase, and that catalyze late steps, including 10-deacetylbaccatin III-10beta-O-acetyltransferase and taxane 2alpha-O-benzoyltransferase.

Stierle *et al.*, (1993) isolated *Taxomyces andreanae*, a fungal endophyte, from the phloem (inner bark) of the Pacific yew, *Taxus brevifolia*. The fungus is hyphomyceteous and when grown in a semi-synthetic liquid medium, produced taxol and related compounds. Taxol was identified by mass spectrometry, chromatography, and reactivity with monoclonal antibodies specific for taxol. Both acetic acid and phenylalanine served as

precursors of taxol in fungal cultures. No taxol was detected in zero-time cultures or in the small agar plugs used to inoculate the culture flasks.

Stierle *et al.*, (1995) reported that Endophytic microbes associated with the Pacific yew tree, *Taxus brevifolia,* were examined as potential sources of the anticancer drug taxol, a secondary metabolite of the host organism. The first promising organism found was the novel fungus, *Taxomyces andreanae,* which was isolated from the inner bark of a yew tree growing in northwestern Montana. It appeared to produce taxol and other taxanes in de novo fashion when grown in semi-synthetic liquid media.

Lee *et al.*, (1995) studied The Florida torreya (*Torreya taxifolia*) began a catastrophic decline in the late 1950s and is the rarest tree in North America for which a full species designation has been established. The trees have common plant disease symptoms, but the reason for the decline has never been identified. *T. taxifolia*'s imminent extinction gains special poignancy through its close relationship to the Pacific yew (*Taxus brevifolia*), which produces the potent anticancer agent, taxol. An examination of the endophytic fungal communities of wild *torreyas* consistently found a filamentous fungus, *Pestalotiopsis microspora,* associated with diseased trees and also with most symptomless trees. *P. microspora* can be cultured in the laboratory, and when it is introduced into greenhouse-grown *torreyas*, it causes disease symptoms similar to those seen in the field. The fungus can then be reisolated from these deliberately infected trees. The *phytotoxins pestalopyrone, hydroxypestalopyrone* and *pestaloside* have been isolated and characterized from axenic fungal cultures, and both *pestalopyrone* and *hydroxypestalopyrone* can be isolated from artificially infected *torreyas*. In addition, *pestaloside* has antifungal activity against other fungal endophytes of *T. taxifolia*. The filamentous fungus, *P. microspora*, had an endophytic-pathologic relationship with *T. taxifolia*. The fungus resides in the inner bark of symptomless trees, and physiological or environmental factors could trigger its pathological activity. *P. microspora* produced the phytotoxins pestalopyrone, hydroxypestalopyrone, and pestaloside which give rise to the disease. *Pestaloside*, which also showed antifungal activity, could reduce competition from other fungal endophytes within the host.

Li *et al.*, (1996) studied the *Pestalotiopsis microspora* and reported that it occurs as a range of strains in bald cypress, *Taxodium distichum*. The organisms live as endophytes

in the bark, phloem and xylem, and isolates show differences in cultural and microscopic characteristics on common laboratory media. Many of these fungi make taxol as determined by the reactivity of partially purified culture extracts with specific monoclonal antibodies against taxol. In the case of one strain of *P. microspora* (CP-4), taxol was isolated from culture medium and was shown to be identical to authentic Taxol by chromatographic and spectroscopic means.

In 1996 Strobel *et al.*, isolated *Pestalotiopsis microspora* from the inner bark of a small limb of Himalayan yew, *Taxus wallachiana,* and was shown to produce taxol in mycelial culture. Taxol was identified by spectroscopic and chromatographic comparisons with authentic taxol. Optimal taxol production occurred after 2-3 weeks in still culture at 23 °C. Acetate and phenylalanine served as precursors for fungal taxol. These observations on *P. microspora* were discussed in relation to the biological importance of taxol production by fungi in general.

Itokawa *et al.*, (1998) reported that a lot of anticancer agents have been isolated from natural sources, especially from microorganisms and plants. However, there is no special type of compounds for cancer therapy. Various types of substances are effective for various types of cancers and tumors: for instance, alkaloids, lignans, terpenes and steroids, etc.

Li, *et al.*, (1998 a) reported that a *Periconiasp* was isolated from *Torreya grandifolia* (a relative of yew that does not synthesize taxol) near Huangshan National Park in the People's Republic of China. That fungus, not previously known as a tree endophyte, was isolated from the inner bark of a small lower limb. When freshly isolated from the tree and placed in a semi-synthetic medium, the fungus produced readily detectable quantities of the anticancer drug taxol. Other taxol producing endophytes were also isolated from this source. The production of taxol by *Periconia sp.* was demonstrated unequivocally via spectroscopic and immunological methods. However, successive transfers of the fungus in semi-synthetic medium resulted in gradual attenuation until low production occurred even though fungal growth was relatively unaffected. Several compounds, known previously as activators of microbial metabolism, including serinol, p-hydroxybenzoic acid, and a mixture of phenolic acids, were capable of fully or partially restoring taxol production to otherwise taxol-attenuated cultures. The compound with the most

impressive ability to activate taxol production was benzoic acid at 0.01 mM. Benzoic acid was not a taxol precursor.

Li *et al.*, (1998 b) reported that *Pestalotiopsis microspora* is an endophyte associated with many plants including *Taxus wallachiana*. Isolates commonly produce taxol in liquid culture. Defining culture amendments to optimize taxol production by *P. microspora* is a critical step toward the realization of fungal taxol for treating human cancers. The lowering of phosphate and the addition of sodium benzoate in the medium increased taxol production. Sterol biosynthesis inhibitors, such as tebuconazole and triadimefon, dramatically increased taxol yields.

Wang *et al.*, (2000) studied Taxol from *Tubercularia* sp. strain TF5, an endophytic fungus of *Taxus mairei*. The diterpenoid taxol is an important anticancer agent used widely in the clinic. Strain TF5 was identified as a *Tubercularia* sp. according to the morphology of the fungal culture, the mechanism of spore production and the characteristics of the spores. Strain TF5 produced taxol, when grown in potato dextrose liquid medium and analyzed by thin layer chromatography, high performance liquid chromatography, ultraviolet and mass spectrometry. The fungal taxol, which was isolated from the organic extract of the TF5 culture, had strong cytotoxic activity towards KB and P388 cancer cells in vitro, tested by the MTT assay. Observed with immunofluorescence and electron microscopy, the fungal taxol enhanced microtubule stability and bundling in culture cells and induced tubulin polymerization in vitro similar to the authentic taxol.

Shao, *et al.*, (2001) isolated different kind of endophytic fungus from the bark of *Taxus Cuspidata Sieb et Zucc* by aseptic techniques, which can produce taxol compounds, and the analysis of the culture fluid from the fermentation of fungus was carried out by high performance liquid chromatography (HPLC). The chemical analysis showed that the culture of fungus contain taxol compounds.

Wang *et al.*, (2001) reported an endophytic fungus, *Aspergillus niger*, isolated from the inner bark of a *Taxus chinensis* tree, was used as an elicitor to stimulate the Taxol (paclitaxel) production in a *Taxus chinensis* cell suspension culture. Different elicitor doses and elicitation times were tested in a batch culture; and the highest volumetric taxol yield was achieved when 40 mg of the fungal elicitor (carbohydrate equivalent) l^{-1} was added to the culture during the late exponential-growth phase. The elicitation resulted in a

more than two-fold increase in the Taxol yield and about a six-fold increase in total secretion. The Taxol yield was further improved substantially by applying medium renewal and re-elicitation to the culture. In particular, with repeated medium renewal (in a way similar to medium perfusion) and a second elicitation of the culture, the volumetric Taxol yield was increased to 67.1±7.5 mg l^{-1}, which was about seven times the amount obtained in the non-elicited batch culture. The Taxol productivity of the perfusion-like culture with repeated fungal elicitation was 1.5 mg l^{-1} day^{-1}, which was about 40% higher than that of the elicitor-treated batch culture and three times the productivity of the non-elicited batch culture.

MATERIALS & METHODS

MATERIALS AND METHODS

Present study was carried out in Microbiology Research Lab, Department of Biological Sciences, Quaid-i-Azam University, Islamabad. The materials and methods employed in the present study are described as:

Materials:

The plant material of *Taxus wallichiana* (A Himalayan yew) consisting of leaves, tinny branches bark and fruits were obtained from Ayubia National Park, Abbotabad. All other chemicals and media components used were of analytical grade.

Microorganisms:

Endophytic fungal strains used in this study were isolated from different collected plant parts of *Taxus wallichiana.* These strains were identified on the basis of their vegetative characteristics from Pakistan Museum of Natural History, Islamabad. For screening purpose, two human pathogenic fungi were obtained from Shifa International Hospital, Islamabad, other fungi were obtained from the Microbiology Research Lab.

Isolation of Endophytic Fungi from *Taxus wallichiana*:

- For obtaining a taxol producing endophytic fungi, *Taxus wallichiana* (A Himalayan yew) was selected.
- Small pieces of different parts of *T. wallichiana* were cut from the tree and placed in sterile plastic bags.
- The plant material i.e. leaves, bark, stem parts, seed and branches were stored at 4 °C, until isolation procedure was accomplished.
- Plant material was thoroughly surface treated with 70% ethanol, under a laminar flow cabinet until dried.
- The outer tissue was removed from different plant parts, with sterile scalpel previously autoclaved, and inner tissue were carefully dissected and placed on water agar plates and incubated at 20 °C for five days.

- Hyphal tips of the endophytic fungi were removed and transferred to potato dextrose agar plate and was again incubated for 5 days at 20 °C
- The fungi were also encouraged to grow on the original plant material to which it has symbiotic relationship, when these endophytic fungi were grown on plant material, was very helpful for identification.

Description of endophytic fungi:

Endophytic fungi isolated from *T. wallichiana* leaves, stems, seed, tinny and large branches, fruit and bark were named as TLBF, TS, Tst, TB, TL, Ts1, Tst1 and Tl1.

Identification of endophytic fungi:

For identification purposes, all these fungal strains were grown on the original *T. wallichiana* plant material by the following method:

The plant material including the leaves and bark were first dried at 70 °C in oven for 10 hours. The dried plant material was blended and wrapped in aluminum foil and autoclaved, so that to avoid contamination.

The composition of media was prepared as

Agar	15g
Plant material	15g
Distilled water	1000 ml

After 4 weeks of incubation period at room temperature for 12 hours day light, fruiting bodies were observed only in endophytic fungal strains isolated from Taxus large branches denoted by TLBF, which were similar fruiting bodies as *Pestalotiopsis microspora,* and this was identified as *Pestalotiopsis microspora*, other fungal strains did not produced fruiting bodies even after 2 months of incubation at the same temperature and day light duration.

Screening of fungal strains for taxol production:

- Fungal strains were screened by keeping in mind that taxol,an anticancer agent also posses antimytotic activity especially against oomycetes, plant saprophytic fungi and human pathogenic fungi. For this purpose, different strains of plant saprophytic, oomycetes and human pathogenic fungi were used as a first screening tools, for this purpose the following procedure was carried out:
- All the fungal strains, named TS, Tst, TL, TB, TLBF were grown in potato dextrose broth containing phylalaine and benzoic acid as taxol enhancer in the following ratio:

Potato dextrose	40 g/L	
Glucose	20 g/L	
Phenylalaine	0.005 g/L	
Sodium benzoate	0.1 g/L	
Distilled Water	1000 ml	pH 5.6 ± 2

- All the five fungal strains were grown in 500 ml shake flask contain 250 ml broth medium at 150 rpm at room temperature for 3 weeks.
- The mycelial culture of the fungi in the broth was grinded and the broth was filtered and centrifuged at 10,000 rpm at 4 °C for 20 minutes. The extract was obtained, which was expected to contain taxol.
- The four fungal strains i.e. *Rhizoctonia,* Oomycetes, *Penicillium* (U549) and *Candida albican* abbreviated as A, B C and D respectively were tested against taxol as sensitive test strains.
- SDA medium was prepared and autoclaved at 121 °C for 20 min. then cooled and 5 ml of the endophytic fungi extract was spreaded by means of a sterile spreading glass rod and left for an hour so that the sample extracts of the endophytic fungi denoted by TL, Tst, TB, TLBF and TS diffused into the SDA media. Different testing fungi were inoculated from their pure culture and incubated at 30 °C for 1 week.
- The testing fungal strains were named as A, B, C and D while the broth extract of endophytic fungi were named as 1,2,3,4 and 5 to TLBF, TL, TS, TB and Tst

respectively. Control plate was also prepared, which did not contain any sample extract of endophytic fungi.

- After 7 days of incubation, sample results were noted as growth inhibition of the fungi.

Well diffusion method:

By well diffusion method different test fungal isolates were tested for growth inhibition by the endophytic fungi broth extract. The broth extract used, was 4 weeks old incubated at room temperature at 150 rpm in 500 ml shake flask. The endophytic fungal strains were named as 1,2,3 and 4 while, the nine fungal strains which were used, as a screening tool against this broth were named as A, B, C, D, E, F, G, H and I. where as the abbrevation stand for,

1: TLBF

2: TL

3: TS

4: TB

and alphabets stands for testing fungi were as follows:

A: *Rhizectonia*

B: Oomycetes

C: *Penicillium* (U549)

D: *Candida albican*

E: *T. viride*

F: *A. alternata*

G: *T. harzianum*

H: Kanamycetes

I: *F. oxysporium*

Production of taxol by endophytic fungal isolates:

The broth was composed of the following ingredients gm/L the pH was adjusted at 5.6 ± 0.2 with 0.1N NaOH and 0.1N HCl.

Potatoes extract	4
Glucose	20
Phenylalanine	0.005
Sodium benzoate	0.1
Distilled Water	1000 ml

All the strains were cultured in the culture media in 500 ml shake flask at 20 °C at 150 rpm. After 4 weeks of incubation period, the mycelial cultures of all the 5 isolated fungi were grinded and filtered through Whatman No. 1 and finally centrifuge at 10,000 rpm. As taxol is soluble in chloroform, therefore it was extracted from the centrifuged broth media with three portion of 20 ml chloroform by taking 20 ml of broth samples of different strains in separating funnels and shaken vigorously for 10 minutes for each interval; the bottom layer was separated and screened for taxol concentration.

Estimation of taxol:

For the estimation of taxol, HPLC and spectrophotometric methods were used by using authentic taxol, taxol obtained from Pharmadic Pharmaceutical Company LHR in injection form

For analysis High performance Liquid Chromatography (HPLC) was used as an initial chromatographic techniques. Taxol standard dilution was prepared by dissolving 3mg of the reference taxol in chloroform and further dilutions were made up to 100 ml with same solvent (equivalent to 0.03 mg/ml). Analysis was done on Sykam Isocretic HPLC system with hyper sil (R) BDS C-18 column (250 x 4.6 mm). Taxol was eluted with chloroform at flow rate of 1.5 ml/min. detection was done at λ 227 nm. Under these conditions taxol appeared with a retention time 2.6 min.

Sample preparation:

The samples, which were extracted with chloroform, were used as such without any dilution. Sample chromatogram was compared to the reference chromatogram of authentic taxol, obtained from Pharmadic Pharmaceutical.

Taxol in the sample culture of TLBF, TS, TST, TB and TL was also observed and estimated by using single beam spectrophotometer on λ max 254 nm. Estimation of taxol was carried out by following the *Beer's Lambart's law*, which states that Absorption is directly proportional to concentration.

Enhancement of taxol production:

Five isolated fungal strains were further cultured in two different media for the enhancement of taxol production, and incubated for 4 week at 20 °C in shake flask experiment. Extraction was carried out by the same way as earlier. Concentrations of taxol were noted in different culture media after 4 weeks time.

Composition of Taxol Microbial Culture Medium (g/L):

Glucose	1	
Fructose	3	
Sucrose	6	
$MgSO_4$	0.36	
Ca++	0.65	
Yeast extract	0.5	
$ZnSO_4$	2.5	
$FeCl_2$	2	
Leucine	0.1 mm	
Phenylalanine	0.005	
Sodium acetate	1	(pH 5.6 ± 0.2)

Composition of Enhanced Taxol Microbial Medium (g/L):

Glucose	1
Fructose	3

Sucrose	6	
$KHPO_4$	1 ml (1M)	
Ca++	0.65	
Yeast extract	0.5	
$ZnSO_4$	2.5	
$FeCl_2$	2	
$MnCl_2$	0.5	
Phenylalanine	0.005	
Sodium acetate	1	
Sodium benzoate	0.1	(pH 5.6 ± 0.2)

Effect of pH on taxol production:

Effect of pH on production of taxol by the isolates was done. Three pH range were used including 5, 5.6 and 6.0 by using enhanced taxol microbial culture medium (ETMCM) after 4 week of incubation period results were obtained by spectrophotmeteric techniques.

Effect of temperature on taxol production:

Three temperatures range such as 20, 25 and 30 °C were used all the strains were cultured in static flask but occasionally shaked. Potato dextrose broth was used containing all the ingredients of the enhanced taxol microbial culture medium. Analysis of taxol was done after 4 weeks in the chloroform extract of the broth spectrophotometrically.

RESULTS

RESULTS

Present study was carried out for exploration of Taxol biosynthesis from different strains of endophytic fungi isolated from different part of a yew tree *(T. wallichiana)* For this purpose the present study was focused on isolation of different fungal strains from *T. wallichiana* (A Himalayan yew) located in Aayubia National Park District Abbotabad, Pakistan and checking their ability for taxol production.

Isolation of endophytic fungal strains from *T. wallichiana*

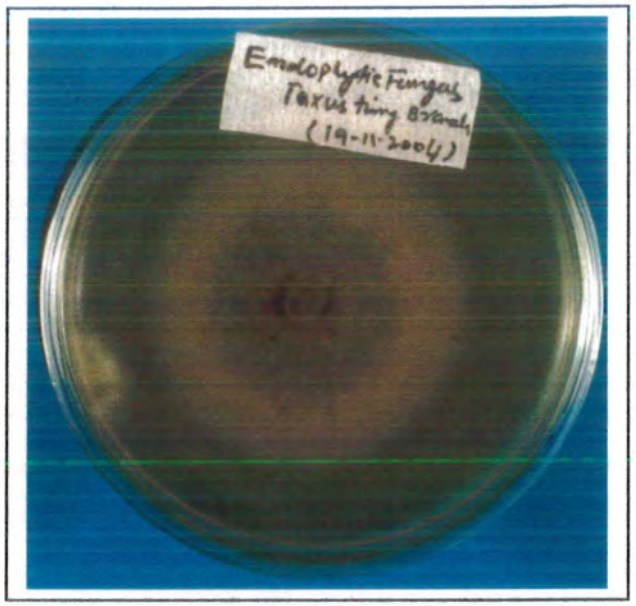
Endophytic fungal strains were isolated from different parts of *T. wallichiana* i.e. leaves, stem parts, bark, seed, tinny and large branches and fruit. Total 10 isolates were obtained which were named as TL, Tst, TS, TB, TLBF, TF, Ts1, Tst1 and Tl1, out of which five strains were selected in initial screening for taxol biosynthesis.

Identification of endophytic fungal strains

These strains were processed for identification on the basis of their vegetative characteristics from Pakistan Museums of Natural History, Islamabad. Only one fungal strain was identified which has similar fruiting bodies and hyphal morphology with *Pestalotiopsis microspora.* Fruiting bodies of these fungal strains were obtained when grown on actual plant material i.e. taxus leaves and bark. Other fungal strains could not identified their growth pattern is shown below in fig 5.


Endophytic Fungus
Taxus Fruit
(19-11-2004)

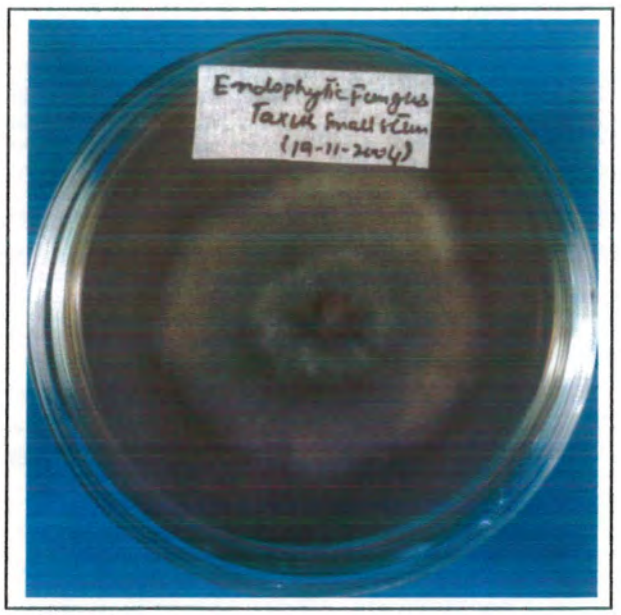
a


Endophytic Fungus
Taxus Stem Branch
(19-11-2004)

b


Endophytic Fungus
Taxus main stem
refreshed (Broth)
(23-11-2004)

c


Endophytic Fungus
Taxus Small Stem
(19-11-2004)

d

e

Fig. 5 Growth pattern of selected Endophytic fungal strains: a) TS, b) TB, c) TLBF (*Pestalotiopsis microspora*), d) Tst, e) TL

Screening of Selected Fungal Strains for Taxol Production

On the basis of antimycotic activity:

Antimycotic activity of selected fungal strains were checked against O*omycetes*, plant saprophytes and human pathogenic fungi i.e. *Rhizoctania solani*, *Oomycetes*, *candida albican* and *Penecillium* (U549). Two methods were employed which are given below.

Plate diffusion assay:

The broth extract of selected endophytic fungi was used to check the antimytotic activity against the above-mentioned fungal strains in plate diffusion manner. TLBF and TB showed maximum inhibitory action against all fungal strains i.e. no fungal growth was observed, while TS showed maximum activity against *C. albican* and *R. solani* while very minute activity against *Oomycetes* and *Penecillium* (U549) strains. strain TL only

showed best activity against *C. albican* while no activity was observed against rest of the test fungal strains. (Table 2)

Table 2 Plate diffusion assay: antifungal activity of the four endophytic fungal isolates against different fungi

Fungal Broth	A *(R. solani)*	B *(Oomycetes)*	C (*Penicillium* U549)	D *(C. albican)*
1(TLBF)	++++	++++	++++	++++
2(TL)	+	-	-	++++
3(TS)	++++	+	+	++++
4(TB)	++++	++++	++++	++++

++++ = Excellent inhibitory action
+++ = Good inhibition
++ = Average inhibition
- = No inhibition

Well diffusion assay

The broth extract of selected endophytic fungi was used to check the antimytotic activity against the nine fungal strains including oomycets, plant saprophytes and human pathogenic fungi i.e. *R. solani, Oomyctes, Penicillium (U549), C. albican, T. viride, A. alternata, T. harzianum, kanamycetes and F. oxysporium.* TL showed maximum inhibitory action against *R. solani*, *Oomyctes*, *Penicillium (U549)* and *T. viride* i.e. no fungal growth was observed, while TB showed maximum activity against *R. solani*, *Penicillium (U549)*, *T. viride* and *T. harzianum*, TS showed activity against C and E while very minute activity of TLBF against above mentioned fungal strains except C and E (Table 3)

Table 3 well diffusion methods: Antifungal activity of the four endophytic fungal isolates against different fungi

Fungal strain	1 (TLBF)	2 (TL)	3 (TS)	4 (TB)
A *(R. solani)*	-	+++	-	+++
B *(Oomyctes)*	++	+++	+	-
C *(Penicillium (U549))*	+++	+++	+++	+++
D *(C. albican)*	-	-	-	-
E *(T. viride)*	+++	+++	+++	+++
F *(A. alternata)*	-	+	-	+
G *(T. harzianum)*	-	+	-	+++
H *(kanamycetes)*	-	++	-	++
I *(F. oxysporium)*	-	-	-	-

++++ = Excellent inhibitory action
+++ = Good inhibition
++ = Average inhibition
- = No inhibition

Production of Taxol by Endophytic Fungal Strains:

Estimation of taxol synthesis by selected endophytic fungal strains was checked by Spectrophotometric method and these results were confirmed by HPLC analysis. For this purposes different cultural media were used i.e. potato dextrose (PDA) medium with phenylalanine (0.005 g/L) and PDA medium with benzoic acid (0.1 g/L) similarly Taxol microbial culture medium (TMCM) and Enhanced Taxol microbial culture medium (ETMCM).

Production of Taxol in PDA medium containing phenylalanine 0.005 g/L

Estimation of selected endophytic fungal strains on PDA medium with phenylalanine (0.005 g/L) was done. In this regard TLBF, TS, TL, TB and Tst gave estimated concentration of 33.90, 35.79, 9.26, 27.63 and 36.90 µg/ml respectively by spectrophotometric method and 8.09, 8.18, 2.85, 7.24 and 8.72 respectively by HPLC analysis (Table 4).

Table 4 Estimation of taxol in PDA medium containing phenylalanine 0.005 g/L by spectrophotometric method and HPLC analysis

Fungal Strain	Concentration (µg/ml) estimated by Spectrophotometer	Concentration (µg/ml) estimated by HPLC
TLBF	10.17	8.09
TS	10.73	8.18
TL	2.77	2.85
TB	8.28	7.24
Tst	11.07	8.72

Production of taxol in PDA medium containing phenylalanine (0.005 g/L) and benzoic acid (0.1g/L)

When production of taxol by selected endophytic fungal strains on PDA medium with phenylalanine (0.005 g/L) and benzoic acid (0.1 g/L) was checked. Strains TLBF, TS, TL, TB and Tst gave estimated concentration of 42.68, 46.38, 44.78, 22.23 and 38.04 µg/ml respectively by spectrophotometric method and 41.46, 42.40, 41.92, 33.51 and 37.61 respectively by HPLC analysis (Table 5).

Table 5 Estimation of taxol in PDA medium containing phenylalanine (0.005 g/L) and benzoic acid (0.1g/L) by spectrophotometric method and HPLC analysis

Fungal Strain	Concentration (µg/ml) estimated by Spectrophotometer	Concentration (µg/ml) estimated by HPLC
TLBF	42.68	41.46
TS	46.38	42.40
TL	44.78	41.92
TB	22.23	33.51
Tst	38.04	37.61

Production of Taxol in Taxol microbial culture medium (TMCM)

Estimation of taxol production by selected endophytic fungal strains on taxol microbial culture medium (TMCM) was done. The results showed that strains TLBF, TS, TL, TB and Tst gave estimated concentration of 36.60, 39.23, 37.77, 32.98 and 36 µg/ml respectively by spectrophotometric method and 33.97, 34.46, 34.76, 30.84 and 33.42 respectively by HPLC analysis (Table 6).

Table 6 Estimation of taxol in Taxol microbial culture medium (TMCM) by spectrophotometric method and HPLC analysis

Fungal Strain	Concentration (µg/ml) estimated by Spectrophotometer	Concentration (µg/ml) estimated by HPLC
TLBF	36.60	33.97
TS	39.23	34.46
TL	37.77	34.76
TB	32.98	30.84
Tst	36.00	33.42

Production of Taxol in Enhanced Taxol microbial culture medium (ETMCM)

Broth extract of fungal isolates TLBF, TS, TL, TB and Tst gave estimated concentration of 43.78, 46.54, 45.49, 29 and 38.07 µg/ml respectively by spectrophotometric method and 41.90, 44.21, 43.35, 28.08 and 39.66 respectively by HPLC analysis when grown on enhanced taxol microbial culture medium (Table7, Fig.6).

Table 7 Estimation of taxol in Enhanced Taxol microbial culture medium (ETMCM) by spectrophotometric method and HPLC analysis

Fungal Strain	Concentration (µg/ml) estimated by Spectrophotometer	Concentration (µg/ml) estimated by HPLC
TLBF	43.78	41.90
TS	46.54	44.21
TL	45.49	43.35
TB	29.00	28.08
Tst	38.07	39.66

Effect of pH on Taxol biosynthesis by Isolated Endophytic Fungal Strains:

Production in enhanced taxol microbial culture medium at different pH values i.e. 5, 5.6 and 6 was checked. Results showed that pH value 5.6 gave maximum concentration of taxol; therefore all isolated fungal strains were cultured at this pH value. Concentration of taxol produced by isolated endophytic fungal strain at different pH values is shown in Fig. 7.

Effect of different Temperatures on Taxol biosynthesis by Isolated Endophytic Fungal Strains:

When taxol production by fungal stains were checked at different temperatures i.e. 20, 25 and 30 °C were studied. Maximum concentration was observed at temperature 20 °C. Concentration of taxol produced by isolated endophytic fungal strain at different temperature values is shown in Fig. 8.

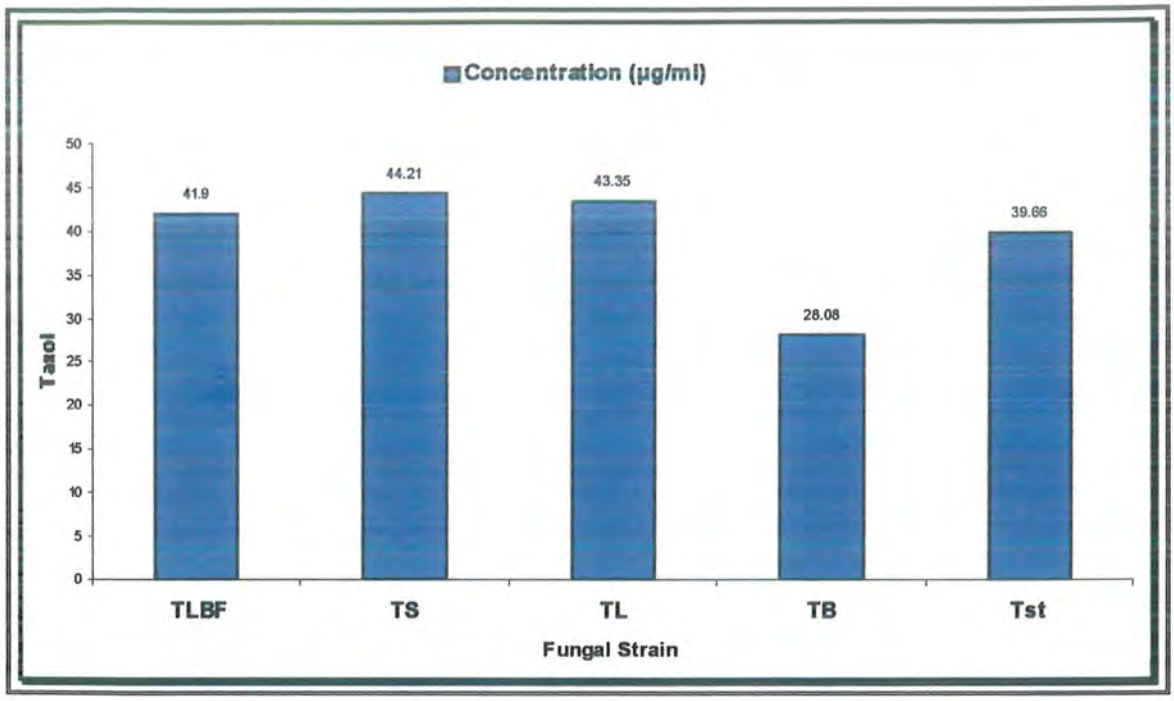


Fig 6. Production of taxol in Enhanced Taxol microbial culture medium (ETMCM) by selected fungal stains.

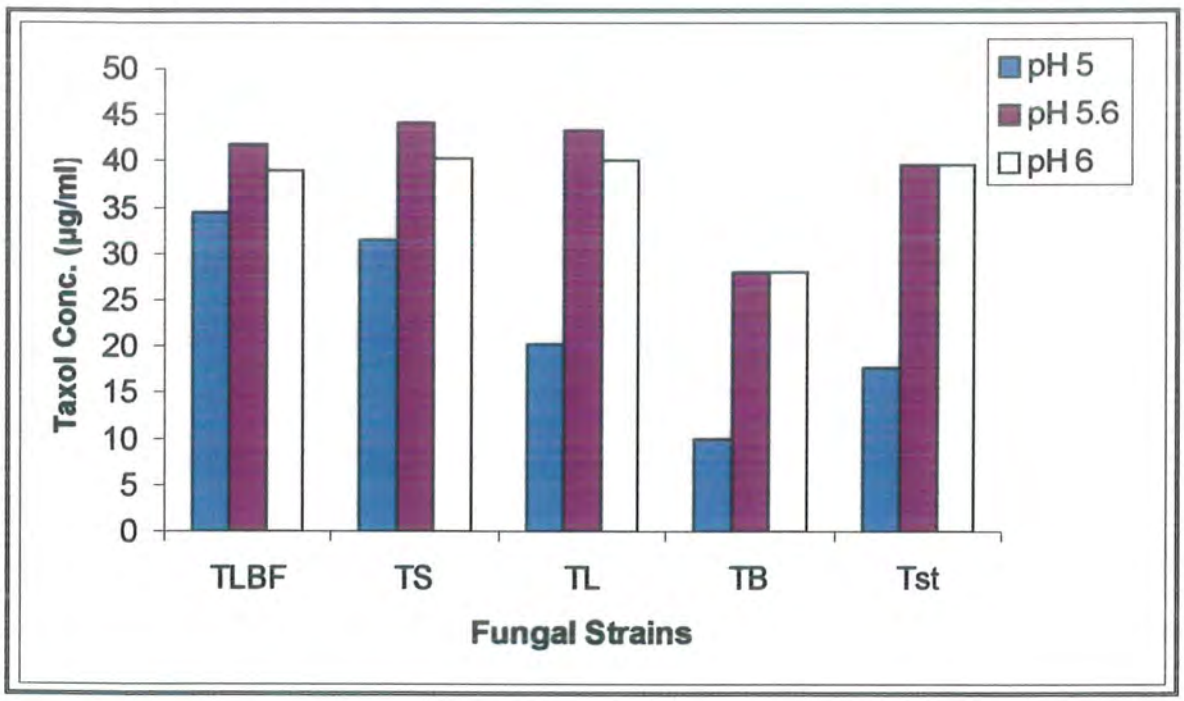


Fig. 7. Production of taxol at different pH values by endophytic fungal isolates.

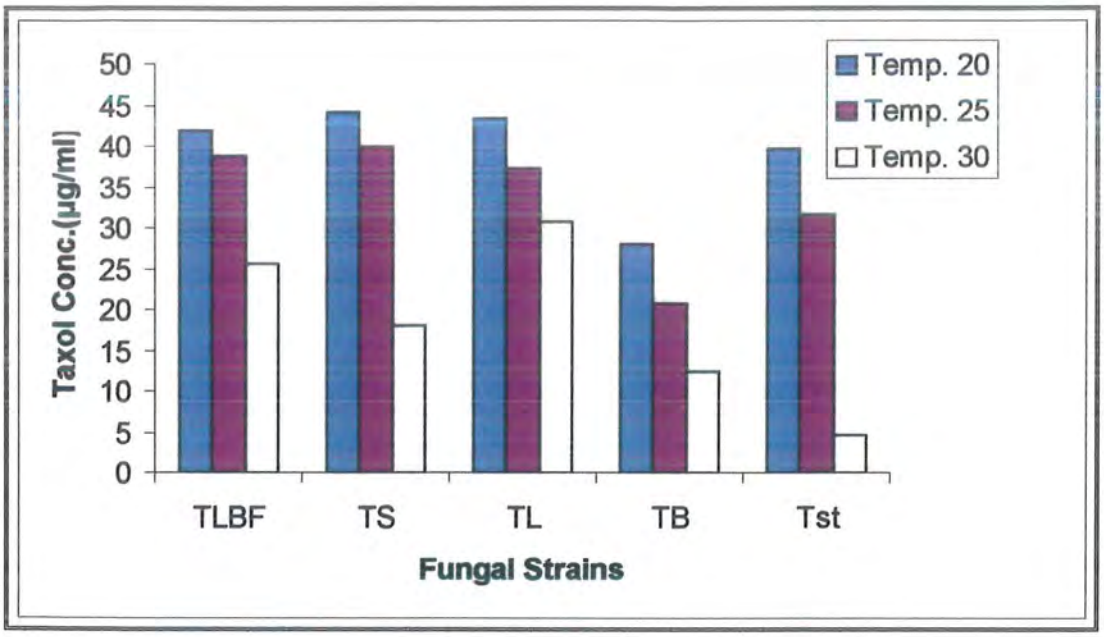


Fig. 8. Taxol produced at different temperatures by fungal isolates.

DISCUSSION

DISCUSSION

In the present study endophytic fungi were isolated from the plant *Taxus wallichiana* (a Himalayan yew) and their potential for the production of taxol was studied.

Stierle *et al.*, (1995) reported that Endophytic microbes associated with the Pacific yew tree, *Taxus brevifolia*, were examined as potential sources of the anticancer drug taxol, a secondary metabolite of the host organism. The first promising organism found was the novel fungus, *Taxomyces andreanae,* which was isolated from the inner bark of a yew tree growing in northwestern Montana. It appeared to produce taxol and other taxanes in de novo fashion when grown in semi-synthetic liquid media. An examination of the endophytic fungal communities of wild *torreyas* consistently found a filamentous fungus, *Pestalotiopsis microspora,* associated with diseased trees and also with most symptomless trees. *P. microspora* can be cultured in the laboratory, and when it was introduced into greenhouse-grown *torreyas*, it caused disease symptoms similar to those seen in the field. The fungus was then being reisolated from these deliberately infected trees. The *phytotoxins pestalopyrone, hydroxypestalopyrone* and *pestaloside* have been isolated and characterized from axenic fungal cultures, and both *pestalopyrone* and *hydroxypestalopyrone* could be isolated from artificially infected *torreyas*. In addition, *pestaloside* had antifungal activity against other fungal endophytes of *T. taxifolia*. The filamentous fungus, *P. microspora*, had an endophytic-pathologic relationship with *T. taxifolia*. The fungus resides in the inner bark of symptomless trees, and physiological or environmental factors could trigger its pathological activity. *P. microspora* produced the phytotoxins pestalopyrone, hydroxypestalopyrone, and pestaloside which gave rise to the disease. *Pestaloside*, which also showed antifungal activity, could reduce competition from other fungal endophytes within the host.

Li *et al.*, (1998 b) reported that *Pestalotiopsis microspora* is an endophyte associated with many plants including *Taxus wallachiana*. Isolates commonly produce taxol in liquid culture. Defining culture amendments to optimize taxol production by *P. microspora* was a critical step toward the realization of fungal taxol for treating human cancers. The lowering of phosphate and the addition of sodium benzoate in the medium increased taxol production. Sterol biosynthesis inhibitors, such as tebuconazole and triadimefon, dramatically increased taxol yields.

Luo et al., (2002) reported that taxol produced by isolated endophytic fungal strains in shake flask culture by using enhanced taxol microbial culture media was greater than the cultured cells of taxus chinensis having sucrose 20 gm/L in the medium which was estimated only 10 μg/ml.

Taxol production in shake flask culture study showed good yield in enhanced taxol microbial culture medium (ETMCM) at pH 5.6 the taxol produced in PDB containing both phenylalanine and benzoic acid was greater than the taxol microbial culture media. Where as taxol produced in enhanced taxol microbial culture media was greater than all other medias.

It is known that taxol and other related compounds produce in the broth culture of endophytic fungi has also antimycotic (anti Oomycetes) activity (Strobel 2002). Therefore when isolted fungal strains were tested for their antimycotic activity different levels of inhibition was observed, all the fungal strains showed antifungal activity against tested pathogenic fungi (table 2 & 3).

In 1996 Strobel *et al.*, isolated *Pestalotiopsis microspora* from the inner bark of a small limb of Himalayan yew, *Taxus wallachiana*, and was shown to produce taxol in mycelial culture. Taxol was identified by spectroscopic and chromatographic comparisons with authentic taxol. Optimal taxol production occurred after 2-3 weeks in still culture at 23 ^{o}C. Acetate and phenylalanine served as precursors for fungal taxol. Therefore taxol

production by fungal isolates TS, TB, TLBF, Tst, TL in our study was the best at 20 °C when fermentation was carried out in enhanced taxol microbial culture media (ETMC) at 20, 25 and 30 °C (Fig. 8).

Taxol produced by the endophytic fungal strains from the *Taxus wallichiana* was greater in concentration than the taxol obtained from the bark of *Taxus wallichiana*, which contained only 0.0558 ± 0.008 % of dry weight of the bark. The total taxol contents of the bark of taxus trees across in age series was found to the range between 0.064 to 8.032 g/tree of about 100 year old which can yield only 5.74 kg of dry bark reported by Nadeem et al., (2002). This indicates that the average taxol contents in the taxus wallichiana whole bark is only 4 gram per tree, which seems to be very minute quantity by comparing taxol biosynthesis from endophytic fungi. Wang et al., (2001) used an endophytic fungal strain *Aspergillus nigar* as taxol elicitor isolated from the inner bark of taxus chinansis. The heighest taxol yield was achieved when 40 mg of the elicitor (carbohydrate) /L was added to the culture during the late exponential growth phase. The elicitation resulted in a more than two fold increase in taxol yield and six fold increase in total secretion by the *Taxus chinansis* cell culture. This means that endophytic fungi have the ability to produce taxol, which also enhanced the taxol production in *Taxus chinansis* cell culture by using endophytic fungi as an elicitor.

CONCLUSIONS

CONCLUSIONS

From the present study it was concluded that

- Endophytic fungal strains isolated from *T. wallichiana* are capable of producing taxol.
- Biosynthesis of taxol from endophytic fungi is most feasible and economical.
- Endophytic fungal stains can be isolated from different parts of the plant.
- Best medium for taxol production by fungal isolates is the enhanced taxol microbial culture medium.
- Optimum pH for the production of taxol is 5.6.
- Optimum temperature for the production of taxol is 20 ^{o}C

FUTURE PROSPECTS

- Techniques for the isolation and purification of taxol from endophytic fungal strains could be developed.
- Process could be developed for scaling up the production of taxol
- More study needed for exploration of endophytic microorganism including fungi and bacteria for novel bioactive compounds used in pharmaceutical and agriculture.

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