Diversity of taxol producing endophytic fungi from *Taxus baccata* and process optimization for taxol production

A Thesis
Submitted in fulfillment of the requirement
for the award of the degree of

DOCTOR OF PHILOSOPHY

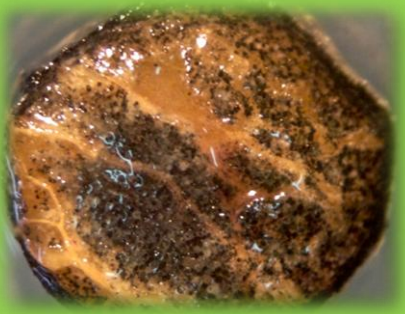
IN

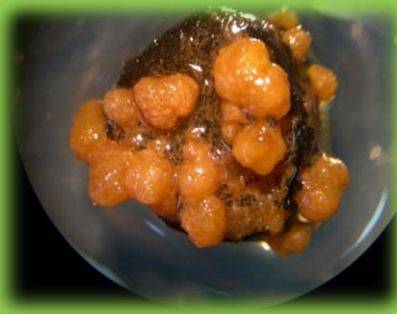
BIOTECHNOLOGY

By

Sanjog Garyali

(Reg. No. 900900003)





Department of Biotechnology

Thapar University, Patiala –147004

Punjab (India)

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CERTIFICATE

Certified that the thesis **"Diversity of taxol producing endophytic fungi from *Taxus baccata* and process optimization for taxol production"** which is submitted by **Mr. Sanjog Garyali**, in fulfillment of the requirement for the award of the degree of **Doctor of Philosophy** in the **Department of Biotechnology,** Thapar University, Patiala, is a record of the candidate's own independent and original research work carried out by him under our supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree.

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DECLARATION

I hereby declare that the work which is being presented in this thesis titled "Diversity of taxol producing endophytic fungi from *Taxus baccata* and process optimization for taxol production" submitted by me for the award of the degree of *Doctor of Philosophy* in the Department of Biotechnology, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervisions of Dr. M. Sudhakara Reddy, Professor, and Dr. Anil Kumar, Associate Professor, Department of Biotechnology, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or abroad.

(Sanjog Garyali)

ACKNOWLEDGEMENT

The completion of my dissertation and subsequent Ph.D. has been a long journey. It's true that "Life is what happens" when you are completing your dissertation. Life doesn't stand still, nor wait until you are finished and have time to manage it but one of the joys of completion is to look over the journey past and remember all the friends and family who have helped and supported me along this long but fulfilling road.

My dissertation has always been a priority. At any rate, I have finished, but not alone, and am elated. I could not have succeeded without the invaluable support of several people around me. Without these supporters, especially the selected few I'm about to mention, I may not have gotten to where I am today, at least not sanely.

First of all I bow my head to "**Almighty God**" to give me strength to complete this work.

I'd now like to give special thanks and gratitude to my major advisor **Dr. M. S. Reddy**, **Professor**, Department of Biotechnology, Thapar University, Patiala who stepped in as my supervisor and helped push me through all the phases of this beautiful journey. I could not have asked for a better role model, supportive & prudent advisor who was always ready to help me whenever I was struck in any doubt. His intelligent ideas, thought provoking discussions and pertinent guidance helped me sail forward in every obstacle during my work. I sincerely thank him for his confidence and faith on me throughout my research.

I am highly thankful to my second research advisor **Dr. Anil Kumar Dutta, Associate Professor,** Department of Biotechnology, Thapar University, Patiala for his guidance for successful completion of my work. I could not be prouder of my academic roots and hope that I can in turn pass on the research values and the dreams that he has given to me. His

dedication for research rendered to me during my work. I extend my heartfelt thanks to him forever.

I would like to express my deep appreciation to my committee members **Dr. Sanjai Saxena** and **Dr. M. Chhibber** for their valuable suggestions, patient advice and motivation during every progress presentation of my PhD course. I could always look back on them for any support during for my study.

I am also indebted to all faculty members of the Department of Biotechnology and Environmental Sciences for their help and suggestions during my experimental work.

I extend my sincere thanks to **Ms. M Vasundhara** for her constant help and guidance during the various stages of my work.

I extend my sincere word of thanks to **Dr. K. K. Raina**, Deputy Director, Thapar University, **Dr. P.K. Bajpai**, Dean (Research and Sponsored projects) and **Dr. Dinesh Goyal**, Professor, Department of Biotechnology and Environmental Sciences for their encouragement and support during the course of my PhD.

My sincere thanks are to my lab seniors Dr. Diwakar Aggarwal and Dr. Deepika Kumari for their guidance and support at every step I needed.

I would also like to thank my friends Dr. Puja Tandon, Ritam Garg, Gurpreet Kaur Khaira, Seema Bhanwar, Rahul Rathore, Tumul Shyam Verma, Gurudutt, Aman Sandher, Fatehbir Singh, Rishi, Neha Lohia, Taranpreet Kaur, Mahiti, Vineet, Prerna, Charu, Rajneesh Verma, Kunal Garg, Hiteshi and Swapnil for their support when I was low with tough times and fighting back to get back on the track. I really find myself lucky to have friends like them in my life.

My deepest gratitude goes to my lab mates Dr. Navdeep Kaur Dhami, Mahima Bansal, Balwant Verma, Dr. Gurdeep Kaur and Amanpreet Bath for providing me gracious support, love and enthusiastic encouragement throughout this period. The cooperation received through my juniors Barkha, Nishant, Sipla, Omika, Preeti, Yadvinder, Amrita, Gurvinder and Manpreet is thankfully acknowledged.

I am very thankful to Mr. Manmeet, Mr. Naminder, Mr. Jain, Mr. Bheem Singh and Mr. Chaudhary for their help.

Most of the work would have been incomplete without the sincere support of Lab assistants. So, I owe a word of thanks to Mr. Lallan Yadav, Mr. Joga, Mr. Babban (Ram Neval), Ms. Lalita, Mr.Gurmeet and Satish ji.

I would not have crossed this road if not for my parents, who instilled within me a love of creative pursuits, science and language, all of which finds a place in this thesis. To my parents, thank you. Sincere thanks are for my grandparents, Anita massi & Vinod uncle, Anjali massi & Vinod uncle, Nirmal bua & Naveen uncle, Kaki tayi & Ashok uncle, Vasundhra chachi & Vijay uncle and my beloved cousins. Thank you all!! I finish with a final silence of gratitude for my life.

Date:

Place: Patiala

(Sanjog Garyali)

I dedicate this thesis to my dadu

Late Sh. Janki Nath Garyali (Miss you always.....)

List of Publications

The following publications are the outcome of the present research work:

1. Garyali S, A Kumar, M S Reddy (2013). Taxol production by an endophytic fungus, *Fusarium redolens*, isolated from Himalayan yew. *Journal of Microbiology and Biotechnology* 23: 1372-1380.

2. Garyali S, A Kumar, M S Reddy (2013). Diversity and antimitotic activity of taxol-producing endophytic fungi isolated from Himalayan yew. *Annals of Microbiology* (DOI: 10.1007/s13213-013-0786-7).

3. Garyali S, A Kumar, M S Reddy (2014). Enhancement of taxol production from endophytic fungus *Fusarium redolens*. *Biotechnology and Bioprocess Engineering* (Accepted)

Papers presented in conferences

Garyali S, Kumar A, Reddy MS. Diversity of taxol producing endophytic fungi from *Taxus baccata* and process optimization for taxol production. *National Conference on Emerging Trends in Biopharmaceuticals: Relevance to Human Health, November 11-13, 2010,* Thapar University, Patiala, Punjab.

Garyali S, Kumar A, Reddy MS. Diversity of taxol producing endophytic fungi from *Taxus baccata* growing in Northern Himalaya. *National seminar on current perspectives of fungi in health care and environment, March 13-14, 2013,* Department of Biotechnology, Bangalore University, Bangalore.

Garyali S, Kumar A, Reddy MS. Screening of taxol producing endophytic fungi using molecular markers. *International conference on Plant Biotechnology, Molecular medicine and Human Health, October 18-20, 2013,* Department of Genetics, University of Delhi, South Campus New Delhi.

Garyali S, Bansal M, Dhami N, Kumar A, Reddy MS. Response surface methodology as a tool for enhanced production of secondary metabolites and extracellular enzymes. *National symposium on Emerging trends in botanical sciences, February 17-18, 2014,* Department of botany, Punjabi University, Patiala, Punjab.

Garyali S, Kumar A, Reddy MS. Optimization of taxol production from endophytic fungus *Fusarium redolens*. *National conference on: Fungal Diversity and biotechnology for food and chemicals*, *February 27-28, 2014*, Centre of advanced study in marine biology, Annamalai University, Tamil Nadu.

ABSTRACT

Endophytic fungi represent an under explored resource of novel lead compounds and have the capacity to produce diverse classes of plant secondary metabolites. In the present study, we investigated the diversity of taxol-producing endophytic fungi from *Taxus baccata* L. subsp. *wallichiana* (Zucc.) Pilger. A total of 60 fungal endophytes were isolated from the inner bark (phloem-cambium) of *T. baccata*, collected from different locations of the northern Himalayan region of India. Two key genes, *DBAT* (10-deacetylbaccatin III-10-O-acetyl transferase) and *BAPT* (C-13 phenylpropanoid side chain-CoA acyltransferase), involved in taxol biosynthesis were used as molecular markers for screening of the taxol producing strains. Five different endophytic species gave positive amplification hits by molecular marker screening with the *bapt* gene. These fungi were characterized based on morphological characters and internal transcribed spacer (ITS) sequence analysis and identified as: *Fusarium redolens* (TBPJ-B), *Gibberella avenacea* (C-1), *Paraconiothyrium brasiliense* (TBPJ-13), *Microdiplodia* sp. (TBPJ-A) and *Fusarium tricinctum* (B-7),

The taxol-producing capability of these endophytic fungi was validated by HPLC-MS. The highest yield of taxol was found to be 66.25 µg/L by *Fusarium redolens* compared to other four strains. The amount of taxol produced by other four strains in S-7 liquid medium are TBPJ-A (27.40 µg/L), B-7 (23.47 µg/L), TBPJ-13 (19.60 µg/L) and C-1 (11.03 µg/L). The antitumour activity of the fungal taxol was tested by potato disc tumor induction assay using *Agrobacterium tumefaciens* as the tumor induction agent. This assay depicted that fungal taxol from all the endophytes successfully inhibited tumor formation in potato discs just like authentic taxol while it did not affect bacterial viability

in any case. These results confirmed that all fungal extracts tested in this study had similar antineoplastic activity as that of paclitaxel.

The medium components and different growth parameters were optimized for production of taxol from the endophytic fungus *Fusarium redolens*. Optimization of medium components was performed using Plackett-Burman (PB) design and response surface methodology (RSM). Different carbon and nitrogen sources were compared and then PB design was employed for screening the important trace elements. Results showed that NH_4NO_3, $MgSO_4$ and NaOAc were the most important components which were further investigated by Box-Behnken design. Optimal concentrations of NH_4NO_3, $MgSO_4$ and NaOAc achieved for maximum taxol production were 6.25, 0.63 and 1.25 g/L, respectively. The predicted response in Box-Behnken experimental design for taxol production gave a value of 195 µg/L, while the actual experimental value was 198 µg/L, suggesting that experimental and predicted values were in good agreement. About three fold increase of taxol production was observed after optimization of fermentation conditions and medium components by RSM. These results suggested the success of RSM in enhancing the production of fungal taxol.

In the present investigation, potential of pharmaceutically important endophytes has been investigated. This study has shed light on endophyte biotechnology for its applications in production of anticancerous drug taxol. PCR amplification of genes involved in taxol biosynthesis (*DBAT* and *BAPT*) is an efficient and reliable method for pre-screening the taxol-producing fungi.

F. redolens and *F. verticillioides* are the first ever reports of endophytic fungi capable of taxol production obtained from *Taxus baccata* subsp. *wallichiana*. *Paraconiothyrium*

brasiliense, *Microdiplodia* sp. and *Phomopsis* sp. reported from other *Taxus* plants but not reported from the Himalayan yew. This study offers important information and a new source for the production of the important anticancer drug taxol by endophytic fungus fermentation. The endophytic taxol was found to be equally effective as that of authentic paclitaxel which paves way for exploitation of these potent endophytic fungi for industrial bioprocesses. Enhanced production of fungal taxol by RSM encouraged the use of these statistical tools for large scale production of this bioactive compound. Though further research at the molecular level is requisite for better understanding of the host endophyte interactions involved in production of this immensely demanding antitumour drug, the current work is a step forward to encourage the use of these fungal endophytes as alternative to the conventional sources.

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ABBREVIATIONS

Abbreviations	Description
µm	micrometer
aa	amino acid
amsl	above mean sea level
ACN	acetonitrile
AD	Anno Domini
AIDS	acquired immuno deficiency syndrome
BLAST	Basic Local Alignment Search Tool
bp	base pair
cDNA	complementary DNA
$CHCl_3$	chloroform
cm	centimeter
CTAB	cetyltrimethylammonium bromide
DAD	diode array detector
DCM	dichloromethane
dd	double distillation
DMSO	dimethyl sulfoxide
E. coli	*Escherichia coli*
et.al.	et alii
F	forward
FW	fresh weight
g	gram

Inc.	Incorporation
IPTG	isopropyl β-D-1-thiogalactopyranoside
kg	kilogram
kV	kilo volt
L	Litre
L.	*Linnaean*
M	molar
m/z	mass-to-charge ratio
MeOH	methanol
mg	milligram
min	minute
mM	millimolar
NCBI	National Center for Biotechnology Information
ng	nanogram
nm	nanometer
No.	number
ºC	degree celsius
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	negative decimal logarithm of the hydrogen ion activity in a solution
R	reverse
RNA	ribonucleic acid
rpm	revolutions per minute

SD	standard deviation
sec	seconds
sp.	specie
spp.	species
T. baccata	*Taxus baccata*
µg	micro gram
µL	micro liter
Tm	melting temperature
V	volt
V_m/V_f	medium-to-flask volume
v/v	volume per volume
w/v	weight per volume

CHAPTER 1
Introduction

1.1 General Introduction

Pharmaceutical biology perceives plants as 'bio-factories' of therapeutic compounds with high potential. Even in the present era of modern technology, it is not possible to estimate the impact of plant derived products as pharmaceutical relevant lead compounds. With expanding realization of the health hazards and toxicity related with the indiscriminate use of synthetic drugs and antibiotics, interest in the use of biogenic drugs has resuscitated throughout the world (Nalawade et al. 2003). Even the world's most universally used drug "Aspirin™" is derivative of salicylic acid which was originally obtained from white willow (*Salix alba*).

Plants, like all other organisms, live in thriving community of microbes and provide a unique environment for endophytes. Diverse fungal endophytes exist within plant tissues, with a global approximation of up to a million species (Petrini 1991). Endophytes (Gr. *endon*, within; *phyton*, plant), the microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects have attracted considerable attention of many scientists worldwide as potential producers of novel and biologically active metabolites (Cai et al. 2004; Strobel et al. 2004; Cragg and Newman 2006). The term *Endophyte* was first coined by de Bary in 1866 and has become deeply embedded in the literature ever since.

Endophytic fungi delineate an important and quantifiable component of fungal biodiversity and are well known to affect plant community diversity and structure (Manoharachary et al. 2005; Krings 2007). Endophytes are the important components of plant micro-ecosystems (Tan et al. 2001; Zhang et al. 2006; Rodrigues et al. 2009; Suryanarayanan et al. 2011) and it has been suggested that fungi can influence the distribution, ecology, physiology, and biochemistry of the host plants (Sridhar 1995; Ramesh et al. 2009). To

endophytes, land plants present a complex, spatially and temporally diverse ecological habitat. Of nearly 3,00,000 plant species that inhibit our planet, each individual one is host to several to hundreds of endophytic fungi (Tan and Zou 2001; Damon et al. 2012), creating a vast biodiversity: a myriad of undescribed species, a rich source of novel natural products there from and an unknown genetic background of all the affiliations thus implied.

Single endophyte can invade a wide host range and can be isolated from different plants belonging to different families and classes growing under different ecological and geographical conditions (Petrini 1996). These endophytes are found virtually in every plant on earth. These endophytic fungi reside in the living tissue of host plant and do so in a variety of relationships ranging from symbiotic to pathogenic (Perotto et al. 2002; Strobel et al. 2004: Deshmukh and Verekar 2008; Verekar et al. 2014). They receive nutrition and protection from the host plant, while the host plant may benefit from enhanced competitive abilities and increased resistance to herbivores, pathogens, and various abiotic stresses by attaining the metabolic substances of endophytes (Saikkonen et al. 1998; Tan and Zou 2001; Zhang et al. 2006; Daghino et al. 2009; Girlanda et al. 2009; Achal et al. 2011). The endophytes have been investigated to be very rich source of novel biological active secondary metabolites (Strobel et al. 2004; Deshmukh and Verekar 2008; Jagadish et al. 2009; Verma et al. 2009; Suryanarayanan et al. 2009; Kharwar et al. 2011; Verekar et al. 2014). In 2008, Moricca and Ragazzi showed that the type of interaction between an endophyte and a plant is administered by the genes of both organisms and modulated by the environment. Endophytes can be transmitted from one generation to the next through the tissue of host seed or vegetative propagules (Carroll 1988). Apparently, acquired chemical defense appears to be a common basis for endophytic association between a plant and its particular endosymbiont (Carroll 1988; Clay 1988). These endophytes are metabolically more active than their free analogues due to their specific functions in nature and activation of various metabolic pathways to

survive in the host tissue (Strobel and Daisy 2003). In particular, the capability to produce a large number of chemically different secondary metabolites is related mainly with the filamentous fungi for the eukaryotes (Donadio et al. 2002).

1.2 Endophytes as biological factories of functional metabolites

Drug discovery and development has a long history and dates back to the early days of human civilization. The Mayans used endophytic fungi grown on roasted green corn to treat intestinal sickness (Buss and Hayes 2000). Later, the Benedictine monks (800 AD) began to apply *Papaver somniferum* belonging to the poppy family (*Papaveraceae*) as an anesthetic and analgesic agent. In 1928 Alexander Fleming, a Scottish scientist found penicillin from the mold *Penicillium notatum*. Although this result was the milestone of the beginning of the antibiotic era, the importance of Alexander Fleming's discovery was not known (Bellis 2012). Ever since the discovery of penicillin, the attention of pharmaceutical companies and research laboratories was focused more on fungi as a source of lead compounds. Since then, people have been engaged to search and discover new microbial metabolites with activity against plant, animals and human pathogens.

Endophytes play a significant role in the daily life of human being besides they may produce over abundance of compounds with a wide range of properties, including antibiotics, antivirals, antimycotics, immunosuppressant, antidiabetic agents, antioxidants, insecticidal products and anticancer agents (Demain 1999; Strobel and Daisy 2003; Strobel et al. 2004; Keller et al. 2005; Strobel 2006; Mitchell 2008; Verma et al. 2009; Aly et al. 2010 and 2011; Porras-Alfaro and Bayman 2011). The various secondary metabolites including active principles produced by endophytes possess unique structures and great bioactivities, representing a huge reservoir which offers an immense potential for exploitation for medicinal, agricultural and industrial uses (Tan 2001; Zhang 2006).

The new technologies offer distinctive opportunities in screening of the natural products which serves as a major source for drug discovery. Microorganisms act as absolute, readily renewable, reproducible and inexhaustible source of enduring pharmaceutical potential. It is hypothesized that many microorganisms (99%) have yet to be discovered (Davis et al. 2005). In the recent past, endophytes have been enormously studied for their potential as novel sources for various bioactive compounds. The diversity and specialized habituation makes these endophytes an exhilarating field of study in the search for new medicines or novel drugs.

1.3 Taxol - history, clinical impact

Functional metabolites of endophytic origin have displayed a considerable potential to impact the pharmaceutical sector (Tan and Zou 2001; Strobel 2003; Strobel and Daisy 2003; Strobel et al. 2004; Gunatilaka 2006). Among the bioactive compounds of endophytic fungi origin described to date, the ones notable for their antitumour activity have drawn special attention, with paclitaxel (Taxol) as the most striking example (Figure 1). It is one of the most successful and widely used anticancer drugs developed in the past 50 years. This anticancer drug was first found in the bark of Pacific yew tree; *Taxus brevifolia* (Wani et al. 1971). All the species of *Taxus* are known to produce taxol; a chemical substance of tetracyclic diterpene lactam which is highly efficient, has low toxicity and broad-spectrum natural anticancer drug (Georg et al. 1995). In India it is represented by *Taxus baccata* subsp. *wallichiana* (Himalayan yew).

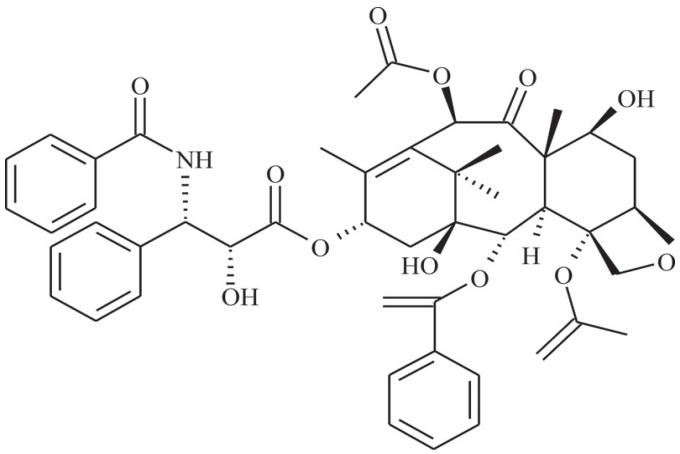


Fig. 1 Structure of Paclitaxel (Taxol)

Paclitaxel represents a new class of antineoplastic agent as it has a distinctive mode of action. Unlike other antimicrotubule agents like podophyllotoxin, colchicine, vinca alkaloids, and combretastatin which impede microtubule assembly, paclitaxel stabilizes microtubules against depolymerization; it promotes the polymerization of microtubules but inhibits depolymerization (Schiff et al. 1978; Horowitz et al. 1986). This atypical stability blocks the cells ability to disassemble the mitotic spindle during cell division; cells are blocked in the G2/M phase of the cell cycle (Schiff et al. 1978, 1980) and this finally leads to cell death. Taxol underwent clinical trials in the 1980s and was approved by the Food and Drug Administration (FDA) in 1992 as a very important chemotherapeutic agent for treatment of a variety of cancers (Suffness and Wall 1995). While the number of cancers treated by taxol is expanding, to date it has been chiefly used to treat metastatic carcinomas of the ovary, cell lung cancer and metastatic breast cancer as well as in second-line treatment of AIDS-related Kaposi's sarcoma (McGuire et al. 1989; Rowinsky et al.1990; Holmes et al. 1991; Yuan et al. 2000). It has also been investigated for the treatment of diseases not related with cancer such as Alzheimer's or Parkinsonism (Zhang 2005).

1.4 Gap in studies

Since the discovery of taxol, substantial energy has been invested in trying to increase its extraction, but supply has been a crucial challenge throughout the clinical development of this drug. Taxol makes up only a minor proportion of the total taxoid content of *Taxus* trees. The conventional source of taxol relies mainly on Yew trees as the most reliable source but most of these species are rare, endangered and slow growing. Even the amount of taxol produced in them is comparatively low in relation to other taxoids; 0.001-0.05% of taxol found even in the most productive species *Taxus brevifolia* (Wheeler et al. 1992). The natural sources for taxol do not represent reliable production system therefore. The commercial isolation of 1 kg of taxol from *T. brevifolia* requires 10,000 kg of *Taxus* bark or 2000 - 3000 very slow-growing yew trees (Hartzell 1991; Croom 1995; Suffness and Wall 1995; Schippmann 2001). Normal cancer patient needs approximately 2.5 - 3.0 gm of Paclitaxel for a full regimen of antitumor treatment (Bedi 1996). Furthermore, the yield of taxol is highly dependent on the *Taxus* species. While species such as *Taxus baccata* (European yew tree) produce scarcely any taxol at all (Nadeem et al. 2002). Additionally, extraction of taxol from yew trees requires a complex system and purification techniques are very expensive. So, considering these facts, together with the high demand for the drug, there is an urgent need to find other alternative sources and new ways of taxol production. One of the ways is to develop sustainable harvesting protocols of yews in natural stands, transforming elite cultivars of the wild species into a commercially reared crop (Smith 2002). Other way is to generate taxol by chemical synthesis which was achieved by Holton and Nicolau in 1994. However, the complexity of the biosynthetic pathway and low yield limit its applicability. An alternative approach is production by semisynthesis, which requires intermediates such as baccatin III or 10-deacetylbaccatin III, extracted from renewable needles of *Taxus* without

destroying the trees (Holton et al. 1995). This production system still relies on yew trees for precursor molecules and therefore depends on epigenetic and environmental factors.

An alternative production strategy is the use of *Taxus* cell suspension cultures, acquired from the species *T. brevifolia* (Gibson et al. 1993), *T. baccata* (Srinivasan et al. 1995) and *T. canadensis* (Ketchum et al. 1999). These cell cultures yield biomass faster than *Taxus* trees and can be grown under reproducible environments. Under optimized culture conditions and induction of production with methyl jasmonate, it is possible to generate up to 23 mg/L/d of taxanes with a taxol content of 13-20% (Ketchum et al. 1999). These yields signify the majestic biosynthetic capacity of *Taxus* cell cultures. However, upholding such high rates of secondary metabolite production in plant cell culture is very strenuous (Deus-Neumann and Zenk 1984; Hall and Yeoman 1987; Morris et al. 1989; Parr et al. 1990; Schripsema and Verpoorte 1992). Additionally several total synthesis routes have been developed too, however at best providing a maximum yield of 2% of taxol, hence not representing a useful alternative production platform (Holton et al. 1994a; Holton et al. 1994b; Nicolaou et al. 1994; Danishefsky et al. 1996; Xiao et al. 2003).

All together the production methods used today have improved a lot over the times, still are very difficult and costly regarding not only the production itself but also with concern of purification of either taxol or late precursors (Baccatin III) from complex taxane mixtures. This discrepancy between demand and supply is the biggest confrontation in clinical application of this drug. It has propelled research into new production strategies, such as metabolic engineering of the yeast *Saccharomyces cerevisiae* (Jennewein et al. 2005; Dejong et al. 2006; Engels et al. 2008), *Escherichia coli* (Huang et al. 1998; Ajikumar et al. 2010) and different plant systems like *Arabidopsis thaliana* (Besumbes et al. 2004) and the moss *Physcomitrella patens* (Anterola et al. 2009). However, metabolic engineering of yeast for the total biosynthesis of taxol or other advanced taxoids is extremely complicated and still in

its infancy. Today, the total fermentation of taxadiene has been attained in significant amounts in *Saccharomyces cerevisiae* (Engels et al. 2008) and *E. coli* (Ajikumar et al. 2010). Hence, the venture of recombinant microorganisms, like yeast or bacteria, offers great prospects not only for the production of taxol but also for other complex natural products and derivatives thereof (Chang and Keasling 2006).

Ultimately to lower the price of taxol and make it more widely available, an extensive search for alternative sources for taxol and related taxanes was initiated. This approach led to the isolation of endophytic fungi, surprisingly having been shown to contain the identical natural products after cultivation independently from their plant host. The discovery of novel taxol-producing endophytes: *Taxomyces andreanae* and *Pestalotiopsis microspora*, demonstrated that organisms other than *Taxus* spp. could produce taxol (Stierle 1993 and Strobel 1996). Since then scientists throughout the world have isolated and identified a number of "taxol-synthesizing" endophytes from various sources (Li et al. 1996; Li et al. 1998; Kim et al. 1999; Wang et al. 2000; Huang et al. 2001; Guo et al. 2006; Sun et al. 2008; Kumaran et al. 2010; Wang and Tang 2011). It is remarkable that taxol produced by these endophytes is identical to that produced by *Taxus* spp., both chemically and biologically (Stierle et al. 1993). Presently, isolation and utilization of taxol-producing fungi have made significant progress worldwide (Lin et al. 2003; Zhao et al. 2008). It is generally agreed that endophytic fungi grow rapidly and are easy to culture (Lin et al. 2003). So, biotechnological methods could be used to improve taxol-producing capability of fungi and eventually industrial production of taxol would be possible. But there is an urgent need to develop a reliable screening system for the identification of endophytes capable of taxol production based on the presence of important genes involved in taxol biosynthesis. Such screening system might facilitate the handling of large number of samples altogether and help in identification of taxol producing endophytes. Optimizing fermentation conditions may also

increase the yield of taxol by endophytic fungi. Taking into account the above possibility the available diversity of endophytic fungi from the *Taxus baccata* needs to be investigated and their potential for the production of the drug needs to be assessed.

1.5 Present study

In the present work, we investigated the taxol producing endophytic fungal diversity of *Taxus baccata* L. subsp. *wallichiana* (Zucc.) Pilger (Himalayan yew) as no work to study the diversity of taxol-producing endophytic fungi from this yew species growing in the northern Himalayan region of India has been reported to date. *Taxus baccata* subsp. *wallichiana* is the only species of *Taxus* which is found in the temperate Himalayas and in the hills of Meghalaya, Nagaland and Manipur at altitudes of 1,800-3,300 m amsl. It is a medium-sized, slow-growing, nonresinous, evergreen conifer that undergoes cross-pollination and has been found to grow best in well-drained moist areas, in cool temperate to sub-tropical climates.

Common names for *Taxus baccata* L. subsp. *wallichiana* (Zucc.) Pilger (Himalayan yew)

Dogri	Birmi, Brammi, Postul, Thuneer
Hindi	Gallu
Marathi	Barmi
Sanskrit	Manduparni

Scientific Classification	
Kingdom	Plantae
Division	Pinophyta
Class	Pinopsida
Order	Taxales
Family	Taxaceae
Genus	*Taxus*
Species	*baccata*
Sub-species	L. *wallichiana*

Traditional method for screening the taxol producing capability of endophytic fungi include thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and liquid chromatography mass spectroscopy (LC-MS). Undoubtedly these chromatographic techniques are a necessary to authenticate that compound produced by endophytic isolates is exactly the same that of our interest, but on the same context screening all the isolated endophytes biochemically is uneconomical, cumbersome and sometimes it may even be impossible. Thus, the primary separation of endophytic fungi from the plant material was comparatively simple operational process, but the detection process of taxol-producing endophytic fungi was laborious and uneconomical. Consequently, the objective of this study was to develop an efficient protocol for screening taxol producing fungal isolates from diverse endophytic fungi with the ultimate purpose of simplifying the screening process and reduce workload. So, in the present study to explore a simple and efficient protocol for screening of taxol generating endophytes, key genes involved in the taxol biosynthesis were brought into consideration i.e. *DBAT* and *BAPT* genes (Zhang et al. 2008). DBAT (10-

deacetylbaccatin III-10-O-acetly transferase) catalyzes the formation of baccatin III, which is the immediate diterpenoid precursor of taxol (Walker and Croteau 2000) and BAPT (C-13 phenylpropanoid side chain-CoA acyltransferase) as the acyl donor, to form N-debenzoyl-2´-deoxytaxol, that is, it catalyzes the attachment of the biologically important taxol side chain precursor (Walker et al. 2002). Gene specific PCR primers were used to screen the isolated endophytic isolates harbouring the key genes involved in taxol biosynthesis. This PCR amplification based protocol is an efficient, reliable and economical method for pre-screening taxol-producing fungi.

Verification of any claimed biological compound requires testing in bioassay to validate its activity before undergoing clinical testing. Bioassay methods used to assess the antitumor activity of various extracts have varied over years and have led to discoveries of important compounds. In the present work, potato disc tumor induction assay was used to validate the antineoplastic activity of fungal taxol using *Agrobacterium tumefaciens* as tumor inducing agent. This bioassay is a simple, inexpensive and fast screen for antitumor compounds and validates the antitumor activity of test compound regardless of mode of action on tumor formation.

Optimization of medium components is regarded as the most effective measure to improve fermentation productivity of secondary metabolites i.e., designing an appropriate medium and investigating the most suitable conditions such as pH, temperature, incubation time, medium-to-flask volume ratio etc. This operation relates to several methods of statistical experimental design. The traditional method of optimization is based on one factor at a time (OFAT) approach. Plackett-Burman design and Response surface methodology are found to be most efficient tools for process optimization. These statistical tools have been widely and successfully used in optimizing the critical factors affecting secondary metabolite production in different organisms and systems (Xu et al. 2006; Luo et al. 2004;

Saravanakumar and Kaviyarasan 2010; Srivastava et al. 2012; Wang et al. 2013). Present study was aimed to optimize the fermentation medium by Plackett-Burman (PB) design and response surface methodology (RSM) for the enhanced production of taxol by *Fusarium redolens.*

Present study will shed light on diversity of taxol producing fungi from different locations. Taxol production by different fungi will be screened by biochemical as well as molecular methods based on *DBAT* and *BAPT* gene. Anti tumour effect of the fungal taxol will be assessed by potato disc tumour induction assay and an attempt will be made to enhance the production of taxol by novel endophytic fungus with aid of statistical tools based on RSM. The objectives of the present work aim to isolate and characterize taxol producing endophytic fungi which will be screened for efficient taxol production so as to harness it for various pharmaceutical applications.

1.6 Specific objectives of the present investigations

1. Isolation and characterization of endophytic fungi from *Taxus baccata* subsp. *wallichiana* growing at different locations of Indian Himalayan region (IHR)
2. Molecular screening of endophytic isolates to screen and select capable taxol producing endophytes based on PCR amplification of *DBAT* and *BAPT* gene
3. Biochemical screening of taxol produced by endophytic fungi
4. Evaluating the antitumorous efficacy of fungal taxol
5. Optimization of parameters and media components for efficient production of fungal taxol by application of Response surface methodology

Chapter 2
Review of literature

Endophytes are the organisms which spend whole or part of their life cycle colonizing inter and/or intracellulary within tissues of host plant (Wilson 1995; Sturza et al. 2000). As per Petrini (1991), these are "the organisms which inhibit plant organs that at some time in their life colonize internal plant tissues without causing apparent harm to the host". The term "endophyte" was introduced by De Bary (de Bary 1866) and included "Fungi and bacteria which, for all or part of their life cycle, invade the tissues of living plants and cause unapparent, asymptomatic infections entirely within the plant tissues but cause no symptoms of disease" (Wilson 1995). In literal translation, the word endophyte is derived from Greek 'endon' meaning within and 'phyton' meaning plant. These endophytes are found to be very important components of the plant micro-ecosystems (Zhao et al. 2010). They have been found to influence distribution, ecology, physiology and biochemistry of host plants (Sridhar and Raviraja 1995).

The plant endophytes spend the whole or part of their life cycle inside the healthy tissues of the host plants (Stone et al. 2000; Tan and Zou 2001) (Fig. 2.1). The first report describing these microbes antecede to the turn of 19^{th} and 20^{th} century (Freeman 1904). Most oftenly encountered endophytes are from fungi; however, the existence of many endophytic bacteria has also been been documented. Plant endophytic fungi have been found in each plant species examined and it is estimated that there are over one million fungal endophytes in nature (Petrini 1991). Endophytes represent vast diversity of microbial adaptations which have developed in special environments. A variety of associations exist between fungal endophytes and their host plants, varying from mutualistic or symbiotic to antagonistic or slightly pathogenic (Perotto et al. 2002; Schulz 2005; Arnold 2007; Cingeetham et al. 2014).

The diversity and distinctive habituation makes these organisms important in the search for novel molecules. Endophytic fungi have been acknowledged as important and novel resource of natural bioactive products. These endophytes produce a number of bioactive compounds for helping the host plant to endure external biotic and abiotic stresses, and benefit the host growth in return (Silvia et al. 2007; Rodriguez et al. 2009). Some endophytic fungi have developed the potential to produce the same/similar bioactive compounds as those generated from the host plans, and to develop a substitutable approach for efficiently synthesizing these scarce and valuable bioactive compounds (Gunatilaka 2006; Zhou et al. 2009). Many medicinal plants have been reported to be storehouse of fungal endophytes with metabolites of pharmaceutical importance (Padhi et al. 2013). Several reviewers have shed light onto natural bioactive products produced by these endophytes with potential applications in agriculture, medicine and food industry (Tan and Zou 2001; Strobel and Daisy 2003; Owen & Hundley 2004; Schulz and Boyle 2005; Gunatilaka 2006).

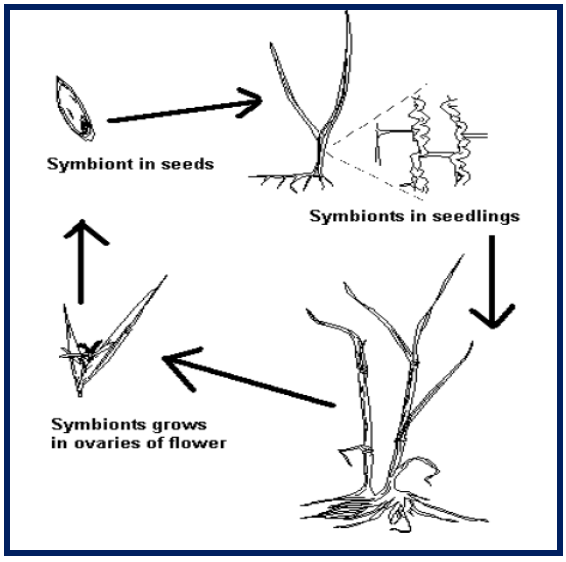


Fig. 2.1 Endophyte asexual life cycle (Tan and Zou, 2001)

The aim of the present study is to deliver a comprehensive overview of the current knowledge on the subject and considerable emphasis is put on exploiting the astounding diversity of the endophytic world for its pharmaceutical potential.

2.1 Endophyte vs. host: the relationship between endophytic fungi and host plant

Endophytes develop special mechanisms to penetrate and inhibit the host tissues in close association. They possess the exoenzymes compulsory to colonize their hosts and grow well in the apoplastic washing fluid of the host. To truly define the interaction between endophyte and the host plant seems to be quite a task (Kusari et al. 2012; Padhi et al. 2013). Though a variety of relationships have been studied to exist between fungal endophytes and their host plants, varying from mutualistic or symbiotic to antagonistic or slightly pathogenic, based on a fine-tuned equilibrium between the demands of the invader and the plant response (Arnold 2007; Schulz and Boyle 2005; Kogel et al. 2006). It has been found that the concentrations of some plant defense metabolites are lower than in the control when the host is infected with a pathogen than with an endophyte (Schulz et al. 2002). There exists equilibrium between fungal virulence and plant defense. Schulz and co-workers, documented an elegant hypothesis postulating this relationship to be a 'balanced antagonism' (Figure 2.2) (Schulz and Boyle 2005).

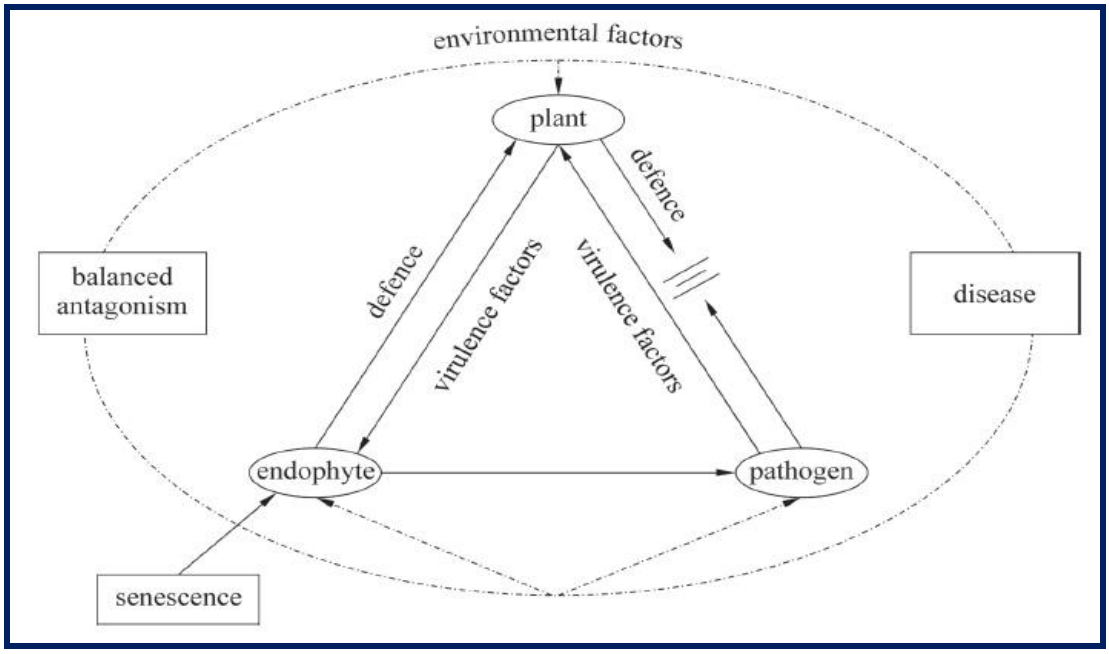


Fig. 2.2 Balanced antagonism hypothesis of endophyte vs. host plant relationship (Schulz and Boyle 2005)

The said preception can be interpreted as equilibrium under environmental, physiological and genetic control, resulting in fitness benefits for both partners. If this balance is interrupted by either a decrease in plant defense or an increase in fungal virulence, disease develops. On one hand, the theory portrays fungal endophytes as masters of phenotypic plasticity, capable of infecting as endosymbionts, colonize cryptically and finally sporulate as pathogens or saprophytes. This creative variability insinuates evolutionary potential. On the other hand, it does not exclude the possibility of secondary metabolites being a contribution of endophytic partner to a mutualistic relationship. There is always a conflict of interests at all stages of relationships between endophytes and plant partners (Smith and Read 1997). However, the development of tools for non-invasive monitoring of sub-cellular activities during the establishment of mutualistic interactions will contribute to a deeper understanding of the mechanism that balances virulence against defence; hostility against hospitality (Kogel et al. 2006). Endophytes synthesize metabolites in order to compete with epiphytes and/or pathogens to colonize the host and to regulate host metabolism in balanced association. Selection of host plant, screening, and utilization of potential endophytes involves studies on plant diversity, ethnobotany and fungal taxonomy. The metabolic interactions of the endophyte with its host through mutualism may favor the synthesis of some similar secondary metabolites (Preeti et al. 2009; Kusari et al. 2013). Endophytes undergo long-term symbiotic relationships with their host plants and many of them may produce bioactive substances as part of these relationships. They exist in the same habitat, through long-term coexistence and through direct contact, they might exchange genetic material (Wang and Dai 2011; Nadeem et al. 2012).

In order to adjust to the ecological environment, plants have developed several mechanisms to overcome microbial diseases including production of several toxic substances. Some are available in healthy plants and some are synthesized during pathogenesis.

Endophytes have a sturdy tolerance toward host's unique metabolites. The detoxification of these highly bioactive defense compounds is an important transformation ability of many endophytes which to a certain extent decides the colonization span of their hosts (Wang and Dai 2011). Biotransformation abilities of endophytes help in detoxification effects towards toxic metabolites produced by host plant and production of some novel bioactive secondary metabolites (Zikmundova et al. 2002; Saunders and Kohn 2009). Only with excellent biotransformation abilities, they can face the external environments directly. It is believed that the types of active compounds produced by endophytes have been much more than those produced by their host plants (Wang and Dai 2011). The former have become an important origin of novel biologically active secondary metabolites with potential in different sectors of agriculture, medicine and food. As several microorganisms have developed resistance to some of current drugs, there has been a hunt for new drugs and endophytes have been found to produce several novel drug like molecules. Improvement of existing drugs by modifying them with endophytes is one of the novel ways of exploiting these bioactive metabolites. Because of their effective biotransformation enzymes, endophytic fungi have been employed to change the three dimensional conformation of compounds. Some researchers have attempted to use endophytes to obtain more active substances. Studies of Borges et al. (2008), Agusta et al. (2005) and Verza et al. (2009) showed that divergent metabolites could be obtained by using different types of fungi and those metabolite productions were stereoselective. Utilization of endophytes for region and stereoselective production of novel products allows us to procure novel compounds which cannot be synthesized by chemical ways. So, natural product drugs generated as microbial secondary metabolites exhibit a number of properties that make them excellent candidates for industrial processes (Tejesvi et al. 2007). The endophytes in culture also offer higher yield of secondary metabolites upon subjecting to strain improvement program (Penalva et al. 1998). These factors encourage us

to explore and study different groups of fungi from different biotopes in order to utilize their biotechnological promise. Documented plant species should also be evaluated from the point of their distribution and taxonomy and also for their chemical and microbial profile.

2.2 The plausibility of Horizontal gene transfer (HGT) hypothesis

The presence of bacterial genes in phagotrophic eukaryotes was initially described by the 'you are what you eat hypothesis' (Doolittle 1998). However, the presence of bacterial genes in nonphagotrophic organisms (including members of the fungal kingdom) has exhibited that mechanisms other than phagocytosis are responsible. While enthusiasts call it 'the essence of the phylogenetic process and the driving force in a new paradigm for evolution' (Doolittle 1999), sceptics delineate it as no more than one of many phylogenetic anomalies (Kurland et al. 2003). Horizontal (or lateral) gene transfer is defined as the exchange and stable integration of genetic material between different strains or species (Doolittle 1999). Horizontal gene transfer (HGT) differs from vertical gene transfer, which is the normal transmission of genetic material from parent to offspring. HGT is oftenly observed in prokaryotes and until recently was supposed to be of limited importance to eukaryotes. However, there is an increasing body of evidence to suggest that HGT is an important mechanism in eukaryotic genome evolution, particularly in unicellular organisms. The transfer of individual genes, gene clusters or entire chromosomes can have significant influence on niche specification, disease emergence or shift in metabolic capabilities. In terms of genomic sequencing, the fungal kingdom is one of the most densely sampled eukaryotic lineages and is at the forefront of eukaryote comparative genomics and equips us to use fungi to study eukaryotic evolutionary mechanisms including HGT. Though highly controversial, the hypothesis of horizontal gene transfer (HGT) seems quite fascinating. Two intriguing examples from endophyties have been well studied. Both instances deal with the occurrence of identical natural products in unrelated taxa, namely: the host and the invader.

The potent cytotoxic agents, maytansinoids were first detected in the Ethiopian shrub, *Maytenus serrata* (Kupchan et al. 1972). Further, investigations revealed occurrence of maytansinoids not only from higher plants (Wani et al. 1973; Ahmed et al. 1981; Powel et al. 1982), but also in mosses (Sakai et al. 1988; Suwanborirux et al. 1990) and in Gram positive *Actinomycetes* (Higashide et al. 1977; Asai et al. 1978). One can assume that the biosynthesis of these natural products has been repeatedly developed during evolution. However, the fact that approximately 48 genes are involved in the bacterial synthesis of maytansinoids makes it highly unlikely (Yu et al. 2002). Similarly, the aforementioned ubiquity of paclitaxel manifestation in yews as well as in taxonomically distant fungi raises questions. Therefore, it seems possible that in the course of evolution a horizontal gene transfer took place between different, taxonomically unrelated species, thus describing the distant distribution of the antineoplastic secondary metabolites.

Nevertheless, before supplicating horizontal gene transfer, alternative and equally possible explanations need to be thoroughly considered. In case of maytansinoids, all affirmations seem to suggest them that plant associated microorganisms are being ultimately producing these compounds. Maytansine, the unique parent compound, was found neither in cell suspension cultures from *Maytenus buchananii* (Kutney et al. 1981) nor in callus cultures raised from *Maytenus wallichiana* (Dymowski and Furmanowa 1990) and *Putterlickia verrucosa* (Pullen et al. 2003). This is in line with the result of an in-depth search for unique gene involved in maytansinoid biosynthesis, encoding for 3-amino-5-hydroxybenzoic acid (AHBA) synthase, in *Putterlickia verrucosa* cell cultures. A comprehensive PCR based homology screen gave negative results (Pullen et al. 2003). These observations indicate that plants do not produce maytansinoids *ab initio*. However, an active role of the plant in an overall biosynthesis can not be eliminated as the host converts a bacterially synthesized precursor into the final biologically active compound. Secondly, maytansine is only produced

as a consequence of a pathogen attack on the plant. Hence the plants may contain a biologically inactive bacterially produced precursor, which is converted into the potent final product only in response to a signal resulting from the pathogen attack. Alternatively, the bacterial production of the maytansinoid precursor could be activated by a plant signal in response to the pathogen aggression (Cassady et al. 2004).

On the contrary, the synthesis of paclitaxel appears to be a genuine feature of the yew host, as adequate evidence supporting the production of the diterpenoid by sterile cell suspension cultures of *Taxus* species (e.g. Ketchum and Gibson 1996; Ketchum and Croteau 1998; Yukimune et al. 2000; Wu and Lin 2003; Naill and Roberts 2005; Khosroushahi et al. 2006; Vongpaseuth and Roberts 2007). This is further validated by the above mentioned work of Croteau and his associates who succeefully isolated paclitaxel biosynthetic genes of plant origin (Hezari et al. 1995). The taxadiene synthase (*ts*) gene which has a long N-terminal targeting sequence play a role in localization and processing in the plastids, indicating that this gene is plant derived rather than of a fungal origin (Koepp et al. 1995; Walker and Croteau 2001). Accordingly, an extensive PCR based screening for microbial *ts*, *dbat* (encoding 10-deacetylbaccatin III-10-O-acetyltransferase) and *bapt* (encoding C-13 phenylpropanoyl side chain-CoA acyltransferase) using the designed PCR primer based on the conserved regions of these key genes of taxol biosynthetic pathway in yew provides essential evidence for the molecular blueprint of taxol biosynthesis being an inherent genetic trait of endophytic fungi (Flores-Bustamante et al. 2010; Zhou et al. 2007; Zhang et al. 2008; Staniek et al. 2009). Moreover, the biochemical detection of taxol production affords definitive proof for the presence of taxol biosynthetic pathway in endophytic fungi, supporting the fact that evolutionary trajectory of taxol gene cluster between microbial and plant origin might be coexisting.

Very meager incidences of eukaryote (nonfungal) to fungal HGT have been located and current evidence suggests that rates of HGT into and between fungi are relatively low; therefore, reconstructing the fungal tree of life (FTOL) is a viable endeavour (Fitzpatrick 2012). Furthermore, there is not much evidence yet to suggest that fungal HGT has been so rampant that it undermines a tree of life outlook, replacing it with a web of life hierarchy similar to what is observed in prokaryotes. To recapitulate the evidence for lateral gene transfer in eukaryotes remains largely anecdotal (Rosewich and Kistler 2000). Although tempting and attractive, the HGT hypothesis has yet to give route to a more plausible alternative to postulate the endophyte-host co-evolution.

2.3 Biodiversity of endophytes

Many theoretical models and experimental tests described the important functions of diversity (Naeem 2002) including the enhancement of primary productivity (Tilman et al. 1997a), nutrient retention (Tilman et al. 1997), nutrient flow (Cardinale et al. 2002), water availability and resistance to pathogen invasion (Levine et al. 1999). The diversity of endophytes is exhibited not only in the specificity of the hosts and their morphology, but also the type of benefits, which they provide to the host (Bacon and White 2000). Nowadays, endophytes have been isolated from diverse groups of plants ranging from large trees (Gonthier et al. 2006; Oses et al. 2008), palms (Taylor et al. 1999; Frohlich et al. 2000), sea grasses (Alva et al. 2002) and even from lichens (Lie et al. 2007). Most endophytes isolated till date belongs to ascomycetes and their anamorphs; however, Rungjindamai et al. (2008) reported several endophytes of basidiomycetes. However, the colonization rate and the isolation rate of these fungi from plants vary considerably; some medicinal plants harbour more endophytes than others (Huang et al. 2008). Some of the common endophytes not only prevailed in more plant hosts but also with high relative frequencies within host. In contrast,

some other endophytic fungi were perceived in only one given plant host (Arnold et al. 2001; Bettucci et al. 2004; Huang et al. 2008; Kusari et al. 2012).

2.4 Isolation and identification of endophytic fungi

(a) Isolation of endophytic fungi

Endophytic fungi colonize in living, internal tissues of plants without inducing any immediate, overtly negative effects (Hirsch and Braun, 1992). These are mainly composed of sac fungi in its anamorphic state and also small numbers of basidiomycetes and zygomycetes. There are two main procedures of isolating endophytic fungi from host plants. The frequently used method is to surface sterilize the plant tissue and plant materials are carefully inoculated in water agar medium. Individual hyphal tips emerging from the tissue are excised and placed on potato dextrose agar (PDA) and the growth of mycelium is observed (Strobel et al. 1996; Li et al. 1996). In the second method, the outer bark of each sample is removed and cut into pieces under sterile conditions. Then, bark pieces of each sample are ground into paste and bark paste is added to melted PDA medium, poured into Petri plate and cultured at 25°C. After hypha grows from the medium, individual hyphal tips of the various fungi are transferred to new PDA plates and incubated at 25°C for at least 2 weeks. Each fungal culture is then checked for purity and transferred to another agar plate by the hyphal tip method (Huang et al. 2001).

The plant materials used in separation of endophytic fungi include different tissues and organs of yew trees, such as roots, stems, leaves, and fruits (Zhu et al. 2008; Venkatachalam et al. 2008). The initial separation of endophytic fungi from plant materials is a comparatively simple operational process, but screening is laborious and time consuming (Zhou et al. 2007). Compared to biochemical screening methods (traditional screening), molecular marker screening is a rapid and efficient alternative method for the detection of endophytes capable

of taxol production. Primers based on two key genes of the taxol biosynthetic pathway, 10-deacetylbaccatin III-10-Oacetyl transferase (*DBAT*) and C-13 phenylpropanoid side chain-CoA acyltransferase (*BAPT*), have been applied in the primary screening of taxol-producing endophytic fungi (Zhang et al. 2008). The molecular screening results of taxol producing isolates can further be authenticated by various chromatographic techniques (TLC, HPLC, LC-MS) (Cheng et al. 2007; Dai and Tao 2008; Zhang et al. 2009). In the past two decades, several endophytic microorganisms isolated from different geographical settings have been reported to produce taxol through biochemical or molecular marker screening (Flores-Bustamante et al. 2010). By now, at least 19 genera of endophytic fungi (i.e. *Alternaria*, *Aspergillus*, *Botryodiplodia*, *Botrytis*, *Cladosporium*, *Ectostroma*, *Fusarium*, *Metarhizium*, *Monochaetia*, *Mucor*, *Ozonium*, *Papulaspora*, *Periconia*, *Pestalotia*, *Pestalotiopsis*, *Phyllosticta*, *Pithomyces*, *Taxomyces*, *Tubercularia*) have been screened, which have the ability to produce paclitaxel and its analogues (i.e. baccatin III, 10-deacetylbaccatin III) (Stierle et al. 1993; Gangadevi and Muthumary 2008; Zhao et al. 2008, 2009; Zhou et al. 2010). The hosts of paclitaxel-producing fungi predominantly include *Taxus* (i.e. *T. baccata*, *T. cuspidata*, *T. media*, and *T. yunnanensis*) and non-*Taxus* species (i.e. *Cardiospermum helicacabum*, *Citrus medica*, *Cupressus* sp., *Ginkgo biloba*, *Hibiscus rosa-sinensis*, *Podocarpus* sp., *Taxodium distichum*, *Terminalia arjuna*, *Torreya grandifolia*, and *Wollemia nobilis*). Such a prominent number and wide range implies that both paclitaxel-producing fungi and their hosts have biological diversity. These outcomes showed a promising way that the endophytic fungi would be an alternative paclitaxel-producing resource. Although the amount of taxol found in most of the *Taxus*-associated endophytic fungi is small compared to that of trees, the short generation time and high growth rate of the fungi make it worthwhile to investigate these species for taxol production (Liu et al. 2009).

(b) Identification of endophyte fungi

Identification of endophyte fungi is a task that requires judging the taxonomic status of the endophyte by its morphological or molecular characteristics (Lin et al. 2003; Zhou et al. 2009). Therefore, the classification of fungi is convoluted and chaotic (Miu and Hong 2007). Like other biological classification systems, morphological identification of endophytic fungal strains is established on the morphology of the fungal culture colony (or hyphae), the characteristic of the spore and whether the reproductive structures of these features are distinguishable (Wei 1979; Carrnichael et al. 1980, Barnett and Hunter 1998). Specialized skills are needed to accurately classify fungi at the species level in the conventional manner. To overcome this limitation, it is effective to use the comparatively conservative 18S ribosomal DNA (rDNA) and internal-transcribed spacer (ITS) sequence to conduct phylogenetic clustering analysis and to research fungi diversity. Some researchers classify and identify fungi by analyzing 18S ribosomal RNA gene homology (Si et al. 2008).

Internal transcribed spacers (ITS): The most favoured locus for DNA-based mycological studies at the subgeneric level for species identification is the internal transcribed spacer (ITS) region of the nuclear ribosomal repeat unit (Horton and Bruns 2001; Pandey et al. 2003; Bridge et al. 2005). The nucleotide sequence of Ribosomal DNA (rDNA) changes very slowly and in eukaryotes it is arranged in tandemly repeated units containing the coding regions for highly conserved regions and variable regions such as internal transcribed spacer (ITS) regions and 18S, 5.8S, and 28S ribosomal RNA separated by spacers (Fig. 2.3). Fungal rRNA operons contain two ITS regions (Fig. 2.4). One is located between the 18S and 5.8S rRNA genes (ITS1) and the other exists between the 5.8S and 28S rRNA genes (ITS2). The sequence of the two ITS regions accumulate mutations at a faster rate than the 5.8S, 18S, and 28S rRNA genes because the two ITS sequences are excised and not required for any functional purpose after the transcription of rRNA operon. Hence analysis of ITS regions

(variation in the spacers) has proven useful for distinguishing among a wide diversity of difficult-to-identify taxa. The ITS region is now conceivably the most widely sequenced DNA region in fungi. It has typically been most functional for molecular systematics at the species level, and even within species. Gardes and Bruns (1993) designed two taxon selective primers, ITS1-F and ITS4-R, intended to be specific for fungi and basidiomycetes, respectively.

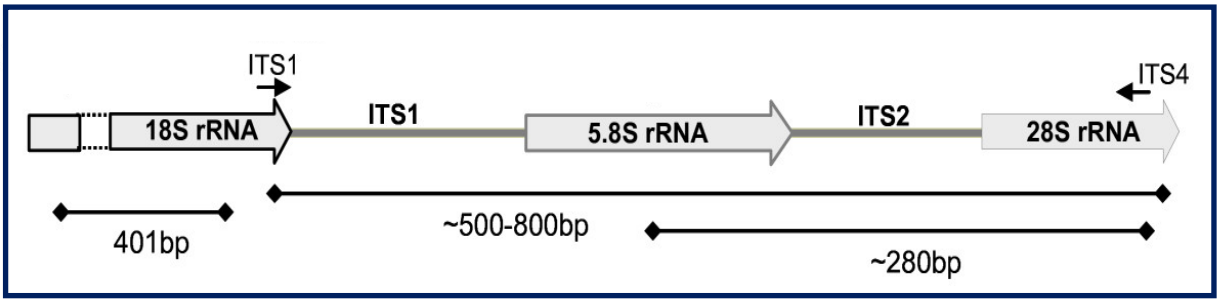


Fig. 2.3 Schematic representation of the fungal ribosomal 18S rRNA gene and ITS regions with primer binding locations (Embong et al. 2008)

With frequent improvements and better access to technology, molecular biology procedures are introduced to identify taxol-producing endophytic fungi. For example, in the process of classifying *Fusarium* species, Yli-Mattila et al. (2004) reported that it is difficult to distinguish *Fusarium* species and its varieties because the divergence among the interspecific modalities within the *Fusarium* species are tiny (Yli-Mattila et al. 2004; Konstantinova and Yli-Mattila 2004; Seifert and Lvesque 2004; Guadet et al. 1989). Such classifications should be augmented by molecular biology methods. Cheng et al. (2007) used not only traditional morphological methods but also molecular biology methods, such as sequence alignment of 18S rDNA, ITS, and β-tubulin gene (TB), to identify Y1117 as a new endophytic fungus of *Fusarium*, a taxol-producing fungus. While molecular biology method shares high

specificity, high accuracy and simplicity, they cannot completely substitute conventional methods of fungi identification (Yang and Zhou 2004).

Morphological identification: It is based on observing the fungal growth conditions in the culture media. Selected hyphae are put through liquid culture or solid culture and observing the mycelium under the microscope. The fungus is identified by the mycelium, spore structure, hygiene conditions of the conidiophore, spore morphology, color and other characteristics in culture. The examination of the colony include its shape and height, growth rate, surface characteristics, edge characteristics, texture, color, medium color, smell, and so on (Wei 1979).

2.5 Endophytes as biological factories of functional metabolites

Diverse endophytic fungi inhabit the plants, representing abundant resource of bioactive natural compounds with potential for exploitation in pharmaceutical and agricultural field (Schulz et al. 2002). However, it is observed that most of the endophytic fungal diversity remains uncovered (Huang et al. 2008). Many of compounds produced by these fungi are biologically active and comprises of alkaloids, flavonoids, steroids, terpenoids, peptides, polyketones, quinols and phenols as well as some chlorinated compounds. Until 2003, approximately 4,000 secondary metabolites with biological activity have been reported from fungi (Dreyfuss et al. 1994). Most of these metabolites are produced by species of *Acremonium*, *Aspergillus*, *Fusarium* and *Penicillium*. Schulz et al. (2002) isolated around 6,500 endophytic fungi and tested their biological potential. They analyzed 135 secondary metabolites and found that 51% of bioactive compounds (38% for soil isolates) isolated from endophytic fungi were new natural products. These workers deduced that endophytic fungi are a good source of novel compounds and that "screening is not a random walk though a forest".

A large number of secondary metabolites have been extracted and characterized from different endophytic microbes (Dreyfuss et al. 1994; Tan and Zou 2001; Kumar et al. 2004; Strobel et al. 2004; Tejesvi et al. 2007; Suryanarayanan et al. 2012). In some cases, plant-associated fungi are able to make the identical bioactive metabolites as the host plant itself. One of the best examples of this is the discovery of phytohormones "gibberellins" in *Fusarium fujikuroi* in the early 1930s (Kharwar et al. 2008). Another crucial example is that of taxol (currently used as an anti-cancer agent) which was first found in the bark of the Pacific yew tree (Wani et al. 1971). The discovery that taxol could be produced by endophytes of the Yew tree (*Taxus* sp.) by Strobel et al. (1996) lead to an outburst of endophyte studies worldwide on a number of other medicinal plants (Tan and Zou 2001; Strobel et al. 2004; Tejesvi et al. 2007; Huang et al. 2008).

Fungal endophytes render important roles in the biosynthesis of secondary metabolites. Combination of inducing factors, including both plants and endophytic fungi increased the accumulation of secondary metabolites in plants and fungi, respectively (Zhang et al. 2009; Li et al. 2009). Biosynthetic pathway studies unveil that plants and endophytic fungi have similar but distinct metabolic pathways for production of secondary metabolites (Jennewein et al. 2001). Independent production of taxol by endophytic fungi has been shown by the isolation of the gene 10-deacetylbaccatin-III-10-O-acetyl transferase from the endophytic fungus *Clasdosporium cladosporiodes* MD2 isolated from *Taxus media* (Zhang et al. 2009). This gene is involved in the taxol biosynthetic pathway and shares 99 % identity with *T. media* and 97 % identity with *Taxus wallichiana* var. *marirei*. A few studies showed that endophytes associated with non taxol producing plants (Yew species) have also been found to produce taxol. An endophytic taxol-producing fungus *Colletotrichum gloeosporioides* isolated from the leaves of a medicinal plant, *Justicia gendarussa*, produced high amount of taxol (Gangadevi and Muthumary 2008). While studies of Wang et al. (2008) revealed the

endophytic association of *Colletotrichum* species as endophytes most frequently isolated from *T. mairei*, have not yet been reported as endophytes of *Taxus* though they have been reported as usual endophytes from other plants (Frohlich et al. 2000; Larran et al. 2001; Photita et al. 2001; Cannon and Simmons 2002; Arnold et al. 2003). Several other bioactive natural products of industrial importance have been found to be produced by different endophytic fungi (Wang et al. 2001, 2002; Zhin-Lin et al. 2007; Debbab et al. 2009).

2.5.1 Endophytic natural products as drugs and novel drug leads

Over 20 natural-product-derived drugs have been flinged onto the worldwide market from 2001 to 2005 and approximately 140 have undergone various stages of clinical trials (Butler 2005; Lam 2007). Functional metabolites of endophytic origin have already exhibited considerable potential to influence the pharmaceutical arena (Tan and Zou 2001; Strobel 2003; Strobel and Daisy 2003; Strobel et al. 2004; Gunatilaka 2006). A few examples are mentioned below with the focus on their presumed therapeutic significance.

(a) Endophytes as producers of antibiotics

A number of antibacterial and antifungal compounds have been isolated from several endophytic fungi (Strobel and Daisy 2003; Strobel et al. 2004; Verma et al. 2009; Deshmukh and Verekar 2012). As the anti-infective branch is experiencing an inadequacy of lead compounds progressing into clinical trials, pristine antibacterial templates with novel mechanisms of action have several benifits over known antibiotics, especially in the fight against multi-drug resistant bacteria and emerging pathogens. Guanacastepene (Figure 2.4), a novel diterpenoid produced by a fungus isolated from the branch of *Daphnopsis americana* growing in Guanacaste, Costa Rica, has been reported to be evocative of a potentially new class of antibacterial agents presenting activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* (Singh et al. 2000).

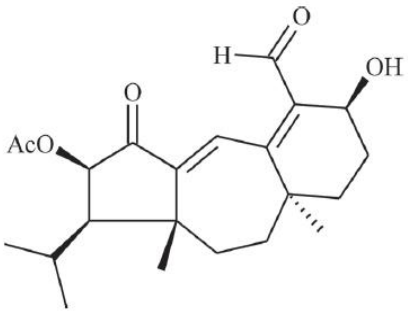


Fig. 2.4 Structure of Guanacastepene

The endophytic fungus *Chloridium* sp. produces Javanicin (Verma et al. 2007). This highly functionalized napthaquinone displays strong antibacterial activity against *Pseudomonas* sp. Three metabolites Phomoenamide, Phomonitroester and Deacetylphomoxanthone B have been reported from endophytic fungus *Phomopsis* sp. PSU-D15 (Ally et al. 2008; Sappapan et al. 2008). Phomoenamide was found to manifest moderate antimycobacterial activity against *Mycobacterium tuberculosis* H37Ra (Rukachaisirikul et al. 2008). Two polyketides, Clavatol and Patulin (a mycotoxin, commonly found in rotting apples and produced by variety of molds like *Aspergillus* and *Penicillium*) (Figure 2.5) produced by an endophytic fungus *Aspergillus clavatonanicus* isolated from *Taxus mairei* may be involved in the protection of *T. mairei* against attack by plant pathogens (Leuchtman 2003; Zhang et al. 2008).

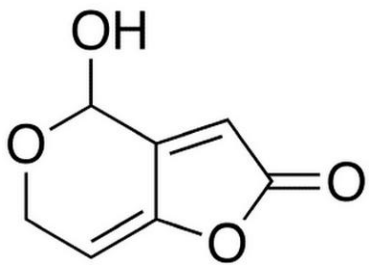


Fig. 2.5 Structure of Patulin

Endophytic fungus *Phomopsis* sp. YM 311483 produced four new ten membered lactones which showed antifungal activity against *Aspergillus niger, Botrytis cinere* and *Fusarium* sp. (Huang et al. 2008). These lactones were isolated from the extract of endophytic fungus *Xylaria* sp. PSU-D14 and exhibited two compounds: Sordaricin (Figure 2.6) and 2, 3-dihydro-5 hydroxy-2 methyl-4H-1-banzopyron-4-1, venaceum (Poncharoen et al. 2008). Endophytic *Fusarium* sp. from plant *Selaginella pollescens* collected in the Guanacaste conservation area of Costa Rica was screened for antifungal activity and showed potent activity against *Candida albicans* in agar diffusion assay (Strobel and Daisy 2003). Jesterone is another antifungal compound which was isolated from fungus *Pestalotiopsis jesteri.* It is the only compound from the endophytes in which total synthesis has been reported (Li and Strobel 2001).

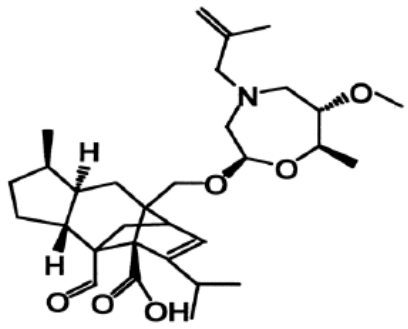


Fig. 2.6 Structure of Sordarcin derivative

(b) Endophytes and there antiviral compounds

Although apparently the probability for the discovery of endophytic compounds having antiviral activity is in its infancy, probably due to absence of suitable antiviral screening systems in most compound discovery programmes, only some propitious fungal metabolites have been found till now. Initially, two novel tridepside human cytomegalovirus (hCMV) protease inhibitors, cytonic acids A and B were isolated from *Cytonaema* sp. inhabiting the internal tissues of *Quercus* sp. (Guo et al. 2000). Further, research of the microbial flora

characteristic of oak trees resulted in the isolation of a prospectively valuable fungal specimen from the leaves of *Quercus coccifera*. This endophyte turned out to be a synthesizer of hinnuliquinone (Figure 2.7) - a potent inhibitor of the HIV-1 protease (Singh et al. 2004).

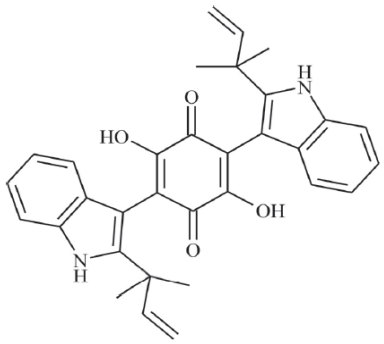


Fig. 2.7 Structure of Hinnuliquinone

(c) Antioxidant compounds produced by endophytes

Two isobenzofuranones, pestacin and isopestacin (Figure 2.8), with structural resemblance to the flavonoids - a well-established group of free-radical-scavengers, demonstrated to exceed the anti-oxidant activity of trolox (a vitamin E derivative) by at least one order of magnitude, as measured by the total oxyradical scavenging capacity (TOSC) assay. These new potent antioxidants were acquired from *Pestalotiopsis microspora*, an endophyte of *Terminalia morobensis* inhabiting the Sepik River drainage of Papua New Guinea (Strobel et al. 2002; Harper et al. 2003).

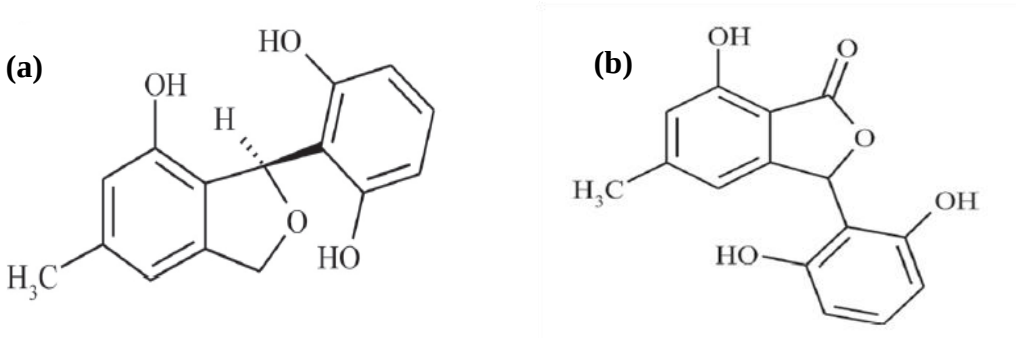


Fig. 2.8 Structure of **(a)** Pestacin **(b)** Isopestacin

(d) Other natural products by endophytes

(1) Antidiabetic agents

Pseudomassaria sp., a fungal endophyte convalescented from leaves of an undetermined plant collected near Kinshasa (Democratic Republic of Congo) was revealed to be a biofactory of nonpeptidal insulin mimetic L-783,281 (Figure 2.9). Its discovery encouraged a revolutionary notion in the therapy of diabetes, namely: an orally administered activator of the human insulin receptor (Zhang et al. 1999). Moreover, this fascinating endophytic metabolite was reported capable of triggering the *Trk* family of tyrosine kinase receptors, leading to the activation of multiple signalling cascades, culminating in neuroregenerative effects, involving neuronal survival and neurite outgrowth (Wilkie et al. 2001). Several other researchers have also reported anti-diabetic and anti-inflammatory activities from different endophytic fungi (Mishra et al. 2013).

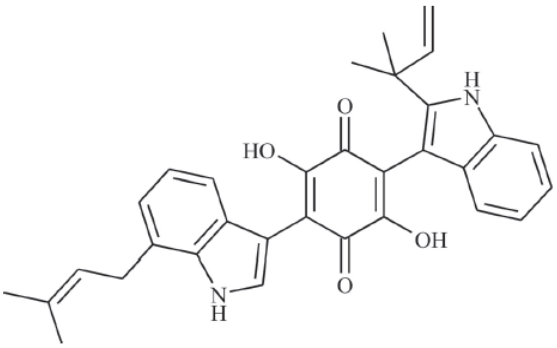


Fig. 2.9 Structure of L-783,281

(2) Immunosuppressive compounds

The quest for new immunosuppressants from endophytes resulted in the isolation of subglutinol A (Figure 2.10) and subglutinol B. These diterpene pyrones from *Fusarium subglutinans,* harboured by *Tripterygium wilfordii* (perennial twining vine), displayed substantial immunosuppressive activity while inducing none of the detrimental cytotoxic effects characteristic of cyclosporine A (Lee et al. 1995; US Patent 5648376, 1997).

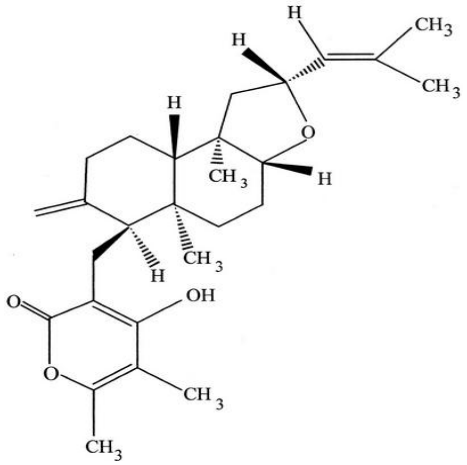


Fig. 2.10 Structure of Subglutinol A

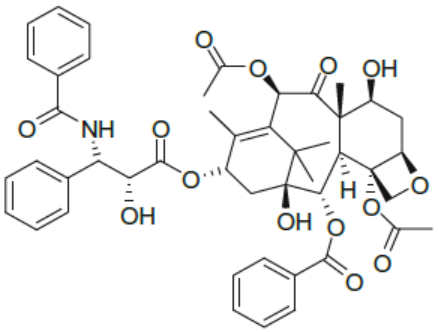
(3) Insecticidal activities

Several endophytic fungi are known to synthesize secondary metabolites having insecticidal properties. A novel compound with insecticidal activity was isolated and identified as phomosine G, obtained from *Phomopsis* sp. an endophyte of *Adenocarpus foliolosus* (Daija et al. 2005). Nodulisporic acid, a novel diterpene that displayed potent insecticidal properties against the larvae of the blowfly, is the introductory nodulisporium compound isolated from endophytes *Nodulispotium* sp. and *Bontia daphnoides* (Verma et al. 2007). This novel indole diterpene exhibits the insecticidal properties by activating insect glutamategated chloride channels (Strobel and Daisy 2003). Ergot alkaloids consist of distinct kinds of alkaloids and comprise of ergovaline, ergotamine, ergosine, ergostine and ergonine. Metabolites from endophytic fungi *Geotrichum* sp. AL4, isolated from leaves of *Azadirachta indica* were found to have nematicidal/insecticidal activities. The fungal metabolites were found to be chlorinated, empimeric compound 1, 3 oxazinane derivatives and were assessed for their nematicidal activity against nematodes. Another endophytic fungus, *Muscodor vitigenus*, isolated from *Paullina paullinioides*, yields naphthalene which is a widely harnessed insect repellent (Daisy et al. 2002). In recent years, a number of alkaloids have been discovered from endophytic fungi in plants, which showcased excellent insecticidal activities (Zhang et

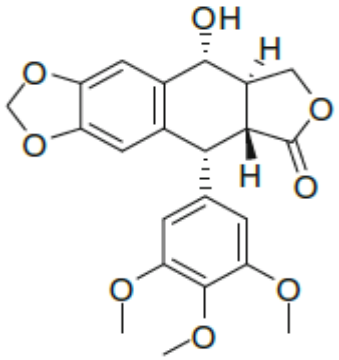
al. 2012). Recently, Biils et al. (2012) isolated *Hypoxylon pulicicidum*, a novel fungal species capable of producing a pantropical insecticide.

(e) Endophytic fungal products as anticancer agents

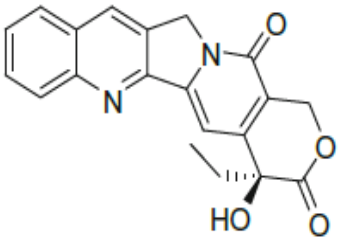
The hunt for natural products as potential anticancer agents precedes to 1550 BC, but the scientific era of this search is much more recent, emerging in the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine, and the isolation of the cytotoxic podophyllotoxins (Strobel et al. 1996; Strobel and Daisy 2003; Cragg and Newman 2004; Srivastava et al. 2005; Deshmukh and Verekar 2008; Deshmukh et al. 2009; Kharwar et al. 2011; Giridharan et al. 2012; Verekar et al. 2014). Plant-derived compounds have played an important part in the development of several clinically useful anticancer drugs e.g. vinblastine, vincristine, selerotiorin, the camptothecin (CPT) derivatives, topotecan and irinotecan, etoposide, derived from epipodophyllotoxin, with paclitaxel (Taxol) as the most impactful example (Figure 2.11).



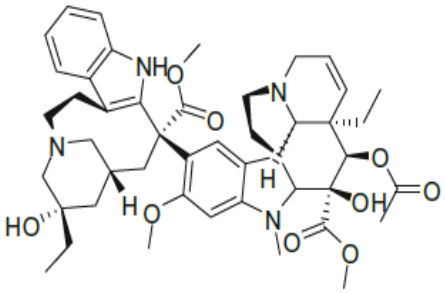
Taxol



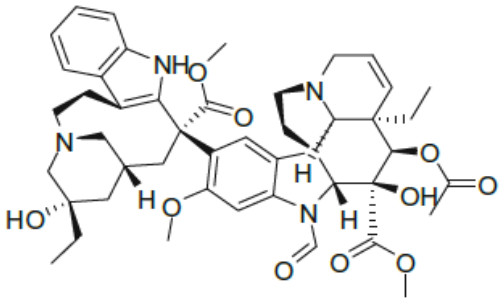
Podophyllotoxin



Camptothecin



Vinblastine



Vincristine

Fig. 2.11 Structures of some industrially relevant secondary metabolites used as anticancer compounds

(i) Camptothecin

CPT is a pentacyclic quinoline alkaloid, a potent antineoplastic agent. It has a unique mechanism of action involving interference with eukaryotic DNA. It primarily targets the intranuclear enzyme DNA topoisomerase I required for the swiveling and relaxation of DNA during DNA replication and transcription. It was first isolated by Wall et al. (1966) from the wood of *C. acuminata* Decaisne (Nyssaceae), a plant native to mainland China. This alkaloid has been proclaimed from several plant species e.g. *Ervatamia heyneana*, *Merrilliodendron megacarpum* and *Ophiorrhiza* species, with the extortionate yield found in *Nothapodytes nimmoniana* (Govindachari and Viswanathan 1972). CPT inhibits the replication of human

immunodeficiency virus *in vitro* and is also known to be effective in the complete abeyance of breast, cervical, lung and uterine cancer (Kusari et al. 2009). Puri et al. (2005) first reported an endophytic fungus *Entrophospora infrequens* obtained from *Nothapodytes foetida* that had the potential to produce CPT. Amna et al. (2006) conducted the kinetic studies on the growth and CPT accumulation of the endophyte *E. infrequens* in suspension culture and established that this endophytic fungus would be a potential alternate microbial source to produce CPT.

(ii) Vinca alkaloids

Vinblastine and vincristine are two natural bisindole alkaloids from *Catharanthus roseus* or *Vinca rosea* used as major drugs in the treatment of lymphoma and leukemia, respectively (Barnett et al. 1978). The two anticancerous drugs (vinblastine and vincristine) prevent mitosis in metaphase and bind to tubulin, thus preventing the cell from making the spindles it needs to divide.*C. roseus* L. (Apocynaceae) Madagascar Periwinkle is found to contain a very large number of alkaloids, about 100 of which have been isolated so far (Verpoorte et al. 1997; Samuelsson 1999; Hughes and Shanks 2002). Studies by Kharwar et al. (2008) on *Catharanthus roseus* endophytes (from two different ecosystems in North India) revealed that, *Alternaria* sp. and *Fusarium oxysporum* isolated from phloem of the plant material were capable for production of vinca alkaloids.

(iii) Podophyllotoxin

Podophyllotoxin is a pharmaceutically active natural drug belonging to the chemical group of lignans. It functions as a mitotic inhibitor by binding reversibly to tubulin and inhibiting microtubule assembly (Cragg and Suffness 1988). Etoposide and teniposide (its thiophene analog) are structurally affiliated to podophyllotoxin (Patel et al. 2010). So, podophyllotoxin is used as a precursor for the synthesis of these important antitumour drugs like etoposide

(VP-16-213) and teniposide (VM-26) which are used in the treatment of lung cancer, testicular cancer, a variety of leukemias and other solid tumors (Majumder and Jha 2009). Several reports are available showing production of podophyllotoxin from endophytes of *Podophyllum hexandrm*, *P. peltatum*, *Juniperus recurva* and *J. communis* L. Horstmann (Puri and Chawla 2006; Eyberger et al. 2006; Cao et al. 2007; Kour et al. 2008; Kusari et al. 2009; Nadeem et al. 2012).

(iv) Taxol

In comparison with other antineoplastic agents such as camptothecin, vinca alkaloids, podophyllotoxin and colchicine, the binding dynamics of taxol with tubulin are peculiar. The former enhance microtubule disassembly where as taxol promotes assembly and stabilization (Roberts and Hyams 1979). Because of its unique ability to bind specifically to β-tubulin and its cytotoxicity at lower concentrations, taxol is being used for the treatment of several classical tumors. Taxol has been successfully employed in clinical treatments of advanced, progressive and drug refractory ovarian cancer (McGuire et al. 1989; Einzig et al. 1991; Markman 1991) and breast cancer (Holmes et al. 1991). Presently, it is also used for the treatment of lung (Ettinger 1992), head and neck (Forastiere et al. 1993), renal, prostrate, colon, cervix, gastric and pancreatic cancers (Einzig et al. 1991; Arbuck et al. 1993; Roth et al. 1993; Brown et al. 1993).

Production of a plant-based natural drug is incessantly not up to the desired level. Moreover, it is generated at a specific developmental stage or under specific environmental condition, stress or nutrient availability. Also the plants may be very slow growing drawing several years to acquire a suitable growth phase for product accumulation and extraction. Same is the case with taxol, whose supply from the bark is limited (0.01-0.05 %) (Wheeler et al. 1992) because the plant is not abundantly found in nature (Cragg et al. 1993), grows slowly taking several decades to increase a few inches in diameter (Flores and Sgrignoli

1991), and contains trace amounts of Paclitaxel (0.01 % of dry weight o the bark) (Banerjee et al. 1996). Thus the supply of this potent antineoplastic agent was soon to become the matter of scarcity, as even early estimations indicated that the demand of paclitaxel for clinical treatment of cancer(s) might exceed 300 Kg, which would amount to 750000 yew trees per year (Stierle et al. 1995; Sohn and Okos 1998) i.e. 1 Kg of paclitaxel is produced after extraction from 10000 Kg of *Taxus* bark (Schippmann 2001) and a cancer patient needs approximately 2.5-3.0 gm of paclitaxel for the treatment (Bedi et al. 1996). Additionally, extraction of taxol from yew trees requires a complex system and specific purification techniques using advanced and expensive technology. The supply catastrophe, as well as the ecological implications resulting in the plant-endangered distinction, prompted the search for the development of alternative resource and potential strategy to fulfill the growing demand of the market and make the drug more widely available.

In the last 40 years, many effective approaches such as field cultivation, plant cell and tissue culture, chemical synthesize for paclitaxel production have been developed and much progression has been achieved (Table 2.1) (Fett-Neto et al. 1992; Holton and Nicolau 1994; Wang and Zhong 2002; Croteau et al. 2006; Zhou and Wu 2006). However, these approaches for the production of taxol are non realistic as these come with a number of problems like; are time consuming, product yield is lower and even the processes are non-economical.

Table 2.1 Various biotechnological approaches for the production of taxol other than obtaining it from yew bark and other plant parts.

Plant species	Culture type	Taxol yield	References
Taxus cuspidata	Callus	0.020 % DW	Fett-Neto et al. (1992)
Taxus brevifolia	*In vivo*	0.01 % of dry weight of the bark	Banerjee et al. (1996)
Taxus media	Cell suspension	115.2 mg/L	Yukimune et al. (1996)
Taxus chinensis	Elicitors *(Aspergillus niger)*	2-fold increase to control	Wang et al. (2001)
Taxus x media	Hairy root culture	Twice the amount of taxol than that in the bark of *T. brevifolia*	Furmanowa and Syklowska-Baranek (2000)
Taxus chinensis	Cell cultures in bioreactors	612 mg/L	Wang and Zhong (2002)

After several years of continuous efforts, a novel paclitaxel-producing endophytic fungus *Taxomyces andreanae* was successfully discovered from the Pacific yew in 1993 (Stierle et al.). This colossal finding firstly revealed that the plant endophytic fungi also had the potential to produce paclitaxel, giving us a novel and promising approach to produce this valuable compound. Since then many scientists throughout the globe have been expanding their interests in exploring fungal endophytes as potential candidates for producing paclitaxel. Comprehensive research for Paclitaxel-producing endophytic fungi from *Taxus* species as well as from other related plant species, microbial fermentation processes and genetic engineering for improving paclitaxel production has been developed and much advancement

has been achieved during the past two decades. Table 2.2 shows varying yields of taxol production by various endophytic fungi. In order to deal with low taxol yield, current studies need to be focused on isolation and identification of high taxol-producing cell lines, stable yield of taxol and optimization of fermenting conditions. Strain improvement and optimization of the media of taxol-producing fungus *Fusarium maire* has been discussed by Xu et al. (2006). The establishment of efficient transformation system of taxol producing endophytic fungus EFY-21 (*Ozonium* sp.) from *T. chinensis* var. *mairei* led to improved taxol production. Table 2.2 summarizes a list of taxol-producing endophytic fungi isolated from year 1993 till 2013.

Table 2.2 A list of taxol producing endophytic fungi isolated from yew associated and non-associated plants (1993-2013)

Endophytic fungus	Host	Taxol yield	References
Taxomyces andreanae	*Taxus brevifolia*	24-50 ng/L	Stierle et al. (1993)
Unidentified	*Taxus yunnanensis*	-	Qiu et al. (1994)
Pestalotiopsis microspora	*Taxus wallichiana*	60-70 µg/L	Strobel et al. (1996)
Pestalotiopsis microspora	*Taxodium distichum*	0.05-1.49 µg/L	Li et al. (1996)
Monochaetia* sp. & *Fusarium lateritium	*Taxus baccata*	102 & 130 ng/L	Strobel et al. (1996)
Alternaria* sp. & *P. microspora	*Taxus cuspidata*	157 & 268 ng/L	
Pestalotiopsis microspora	*Taxus wallichiana*	500 ng/L	
Pithomyces	*Taxus sumatrana*	95 ng/L	
Pestalotia bicilia	*Taxus baccata*	1081 ng/L	
Pestalotiopsis guepinii	*Wollemia nobilis*	0.17 µg/L	Strobel et al. (1997)
***Periconia* sp.**	*Torreya grandifolia*	30-830 ng/L	Li et al. (1998)
Seimatoantlerium tepuiense	Venezuelan Guyana	250-350 ng/L	Strobel et al. 1999
***Alternaria* sp.**	*Ginkgo biloba*	0.12-0.26 µg/L	Kim et al. (1999)

***Tubercularia* sp.**	*Taxus chinensis* var. *mairei*	185 µg/L	Wang et al. (2000)
***Taxomyces* sp.**	-	2.3 µg/L	Wan et al. (2001)
***Rhizoctonia* sp.**	-	1.43 µg/L	
Phoma	-	32.93 µg/L	
Botrytig	-	4.092 µg/L	Chen et al. (2003)
Penicillium	-	8.24 µg/L	
Trichoderma	-	19.586 µg/L	
Mucor	-	1.08 µg/L	
Chaetomium	-	21.1 µg/L	
Alternaria alternate	-	84.5 µg/L	Tian (2006)
***Ozonium* sp.**	*T. chinensis* var. *mairei*	4-18 µg/L	Guo et al. (2006)
Pestalotiopsis	-	8.5 µg/L	Li (2006)
Fusarium	-	2.7 µg/L	Cheng et al. (2007)
Fusarium mairei	-	20 µg/L	Dai and Tao (2008)
***Botrytis* sp.**	-	206.34 µg/L	Zhao et al. (2008a, b)
***Bartalinia robillardoides* Tassi**	*Aegle marmelos* Correa ex Roxb.	187.6 µg/L	Gangadevi et al. (2008)
Colletotrichum gloeosporioides	*Justicia gendarussa*	163.4 µg/L	Gangadevi et al. (2008)
Pestalotiopsis pauciseta	-	113.3 µg/L	Gangadevi et al. (2008)
Fusarium solani	*Taxus celebica*	1.6 µg/L	Chakravarthi et al. (2008)
Botryodiplodia theobromae	-	280.50 µg/L	Venkatachalam et al. (2008)
Cladosporium cladosporioides	*Taxus media*	800 µg/L	Zhang et al. (2009)
Phyllosticta dioscoreae	*Hibiscus rosa-senensis*	298 µg/L	Kumaran et al. (2009)
***Fusarium solani*, Tax-3**	*Taxus chinensis*	163.35 µg/L	Deng et al. (2009)
Phyllosticta tabernaemontanae	*Wrightia tinctoria*	461 µg/L	Kumaran et al. (2009)

Aspergillus niger* var. *taxi	*Taxus cuspidata*	273.46 µg/L	Zhao et al. (2009)
Metarhizium anisopliae	*Taxus chinensis*	846 µg/L	Liu et al. (2009)
***Gliocladium* sp.**	*Taxus baccata*	10 µg/L	Sreekanth et al. (2009)
Pestalotiopsis versicolor	*Taxus cuspidata*	478 µg/L	Kumaran et al. (2010)
Ozonium sp. (EFY-21)	*T. chinensis* var. *mairei*	-	Wei et al. (2010)
***Nigrospora* sp.**	*Taxus globosa*	0.142-0.221 µg/L	Ruiz-Sanchez et al. (2010)
***Botryodiplodia theobromae* Pat.**	*Morinda citrifolia* Linn.	-	Pandi et al. (2010)
Pestalotiopsis breviseta	-	0.064 mg/L	Kathiravan and Sri Raman (2010)
Lasiodiplodia theobromae	*M. citrifolia*	245 µg/L	Pandi et al. (2011)
***Phoma* species**	*Aloe vera*	73.66 µg/L	Rebecca et al. (2011)
Pestalotiopsis malicola	Plant debris (Yunnan, China)	186 µg/L	Bi et al. (2011)
Colletotrichum capsici	*Capsicum annuum*	687 µg/L	Kumaran et al. (2011)
***Didymostilbe* sp.**	*T. chinensis* var. *mairei*	8-15 µg/L	Wang and Tang (2011)
Stemphylium sedicola	*Taxus baccata*	6.9 µg/L	Mirjalili et al. (2012)
Fusarium oxysporum	*Rhizophora annamalayana*	172.3 µg/L	Elavarasi et al. (2012)
Guignardia mangiferae*, *Fusarium proliferatum* & *Colletotrichum gloeosporioides	*Taxus x media* (Anglojap yew)	720, 240 & 120 ng/L	Xiong et al. (2013)

2.6 Biosynthesis of taxol

As a natural diterpenoid, taxol is formed exclusively from geranylgeranyl diphosphate (GGPP), which is synthesized from three isopentenyl diphosphate (IPP) molecules and the isomer dimethyl diphsophate (DMAPP) by the enzyme geranylgeranyl diphosphate synthase (Fig. 2.12). This enzyme is of special interest as it leads to the formation of a branched point progenitor of a variety of diterpenoids and tetraterpenoids. According to Eisenreich et al. (1996), the IPP involved in the biosynthesis of the taxane ring is formed by the plastidic route. However, other studies (Lansing et al. 1991; Zamir et al 1992; Cusido et al. 2007; Exposito et al. 2009) have shown the involvement of the cytosolic pathway. Srinivasan et al. (1996) suggested that cytosolic IPP could play a role in taxol production in the initial growth phase of *Taxus* cells. Additionally, Wang et al. (2003) after supplementing *T. chinensis* cell suspensions with two inhibitors of metabolite translocation, suggested that translocation of IPP through the plastidic membrane only occurs during late growth phase of the culture. A recent study in *T. baccata* cell cultures showed that while taxol biosynthesis was blocked by the addition of fosmidomycin (an inhibitor of the plastidic pathway), it was also reduced by mevinolin (an inhibitor of the cytosolic pathway), indicating that both pathways could be involved in the biosynthesis of taxol (Cusido et al. 2007).

The first committed step of taxol biosynthesis is the cyclization of geranylgeranyl diphosphate (GGPP) to the taxa-(4,5),(11,12)-diene, a reaction catalyzed by taxadiene synthase (TS), amonomeric protein of 79 kDa (Fig. 2.13). The enzyme was purified and characterized by Hezari et al. (1995) and the gene that codifies for TS has been cloned and functionally expressed in *E. coli* by Wildung and Croteau (1996). Afterwards, oxygen and acyl groups are added to the taxane core by oxygenation at multiple positions mediated by cytochrome P450 mono-oxygenases. The hydroxylation at the C5 position of the taxane ring by the enzyme cytochrome P450 taxadiene-5α-hydroxylase (T5αH) results in the formation

of taxa-4(20),11(12)-dien-5α-ol, which is the second step in taxol biosynthesis (Jennewein et al. 2004). T5αH is a protein of 56 kDa with an N-terminal sequence of insertion in the membrane of endoplasmic reticulum. This enzyme, apart from its hydroxylating activity, also conditions the migration of the double bond from 4(5) to 4(20). Although these two metabolic steps, cyclization and hydroxylation; are slow, they do not seem to be rate-limiting in taxol biosynthesis (Hefner et al. 1996).

The next step in the pathway is catalyzed by a specific taxadiene-5α-ol-O-acetyl transferase (TDAT) that acylates taxa-4(20),11(12)-dien-5α-ol at the C5 position to form taxa- 4(20),11(12)-dien-5α-yl-acetate. This enzyme is a protein of 50 kDa that bears no N-terminal organellar targeting information (Walker et al. 2000). The product of this reaction is then hydroxylated by taxoid 10β-hydroxylase (T10βH) at C10. T10βH is a P450-dependent monooxygenase cloned and functionally characterized in yeast (Schoendorf et al. 2001). Another Cyt P450-dependent hydroxylase leading to the formation of taxa-4(20), 11(12)-dien-5α-13α-diol has been found (Jennewein et al. 2001). The fact that this enzyme uses the same substrate as TDAT, the taxa-4(20), 11(12)-dien-5α-ol suggests that taxol biosynthesis is not a linear pathway and that there are branch points that can lead to other related taxoids. It has been observed that this alternative step is especially frequent in cell cultures elicited with methyl jasmonate (Wheeler et al. 2001). The taxoid 14β-hydroxylase (T14βH) is responsible for the formation of taxa-4(20), 11(12)-dien-5α-acetoxy-10β-14β-diol (Jennewein et al. 2003). This enzyme does not use substrates already hydroxylated at the C13 position but only those hydroxylated at the C10 position, suggesting that T14βH cannot be involved in the production of taxol, which does not present any hydroxylation at the C14 position.

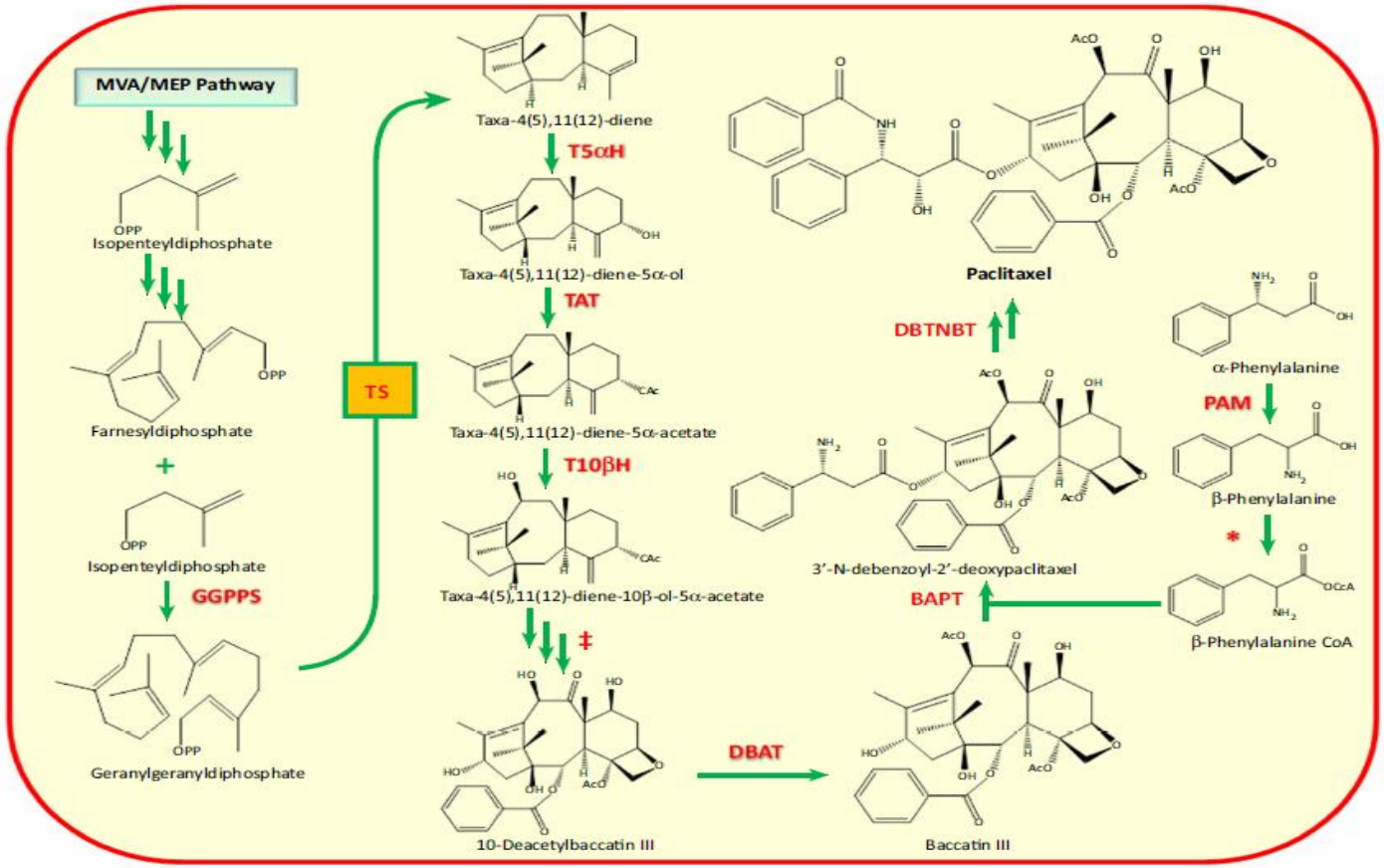


Fig. 2.12 Prevalent consensus biosynthetic route for Taxol in *Taxus* species. Abbreviations: MVA: mevalonic acid; MEP: 2-C-methyl-D-erythritol-4-phosphate; GGPPS: geranylgeranyldiphosphate synthase; TS: taxa-4(5),11(12)-diene synthase that catalyzes the committed step of this pathway; T5αH: taxa-4(5),11(12)-diene-5α-hydroxylase; TAT: taxa-4(5),11(12)-diene-5α-ol-O-acetyltranseferase; T10βH: taxane-10β-hydroxylase; ‡: 'oxetane ring' formation and branch migration enzymes including taxane 2α-O-benzoyltransferase (T2BT or DBBT = debenzoyltaxane-2´-α-O-benzoyltransferase) as well as C-13 hydroxylation and steps taking pathway flux towards non-Taxol-type molecules; DBAT: 10-deacetylbaccatin III-O-acetyltransferase; BAPT: baccatin III 13-O-(3-amino-3-phenylpropanoyl) transferase; DBTNBT: 3´-N-debenzoyl-2´-deoxytaxol-N-benzoyltransferase which follows hydroxylation in the side chain by an unknown enzyme; PAM: phenylalanineaminomutase; *: β-phenylalanine coenzyme A ligase. Multiple arrows imply more than one biosynthetic step. The Taxol biosynthetic pathway is proposed to have about 20 different enzymatic steps in *Taxus* plants (Kusari et al. 2014)

The last steps in the taxol biosynthetic pathway, after the formation of taxa-4(20),11(12)-dien-5α,10β-diol 5-acetate, include several hydroxylations at the C1, C2, C4 and C7 positions, oxidation of C9 and epoxidation at the C4C5 double bond. It is known that the hydroxylations are mediated by Cyt P450 enzymes but not exactly in which order. Taking into account the oxidation frequency of the taxoids found in cell cultures, a probable sequence validated by phylogenetic analyses of previously cloned taxoid P450 oxigenases could be: C5, C10, C2, C9, C13, C7 and finally C1 (Vongpaseuth et al. 2007). However, rather than intermediates in taxol biosynthesis, some of these taxoids might be commodities of the *in vitro* cultures.

Although different mechanisms for the oxetane ring formation have been proposed (Floss et al. 1995; Walker et al. 1999; Jennewein et al. 2001), it is currently accepted that the process involves epoxidation of the 4(20) double bond followed by migration of the α-acetoxy group from the C5 to the C4 position together with the expansion of the oxirane to the oxetane group. It is possible that this step precedes hydroxylation at C1 in taxol biosynthesis, and in this case the hypothetical polyhydroxylated intermediate would be a taxadien-hexaol rather than a heptaol hydroxylated at C1 (Croteau et al. 2006). The enzyme that epoxidates the C4-C20 double bond has not yet been functionally characterized and the expansion of the oxirane-to-oxetane ring is also an incompletely known step. After the formation of the hypothetical polyhydroxylated precursor by the activity of the enzyme 2α-O-benzoyl transferase (DBT), a protein of 50 kDa, the next compound obtained is 10-deacetylbaccatin III. Another identified transacetylation reaction in the taxol biosynthetic pathway involves hydroxylation at the C10 position of the 10-DAB (10-deacetylbaccatin III), which is catalyzed by the enzyme 10-deacetyl-baccatin III-10-O-acetyl transferase (DBAT). It leads to the formation of a diterpene intermediate, baccatin III, using 10-DAB and acetyl CoA as substrates (Fig. 2.13).

An essential step in the taxol biosynthesis is the esterification of the C13 hydroxyl group of baccatin III with the β-phenylalanoyl-CoA side chain. The side chain is obtained from the amino acid β-phenyalanine by the action of phenylalanine aminomutase (PAM) (Walker et al. 2004). An unknown ester CoA ligase probably activates the compound so it can bind to baccatin III. The enzyme that catalyzes the conjugation of the β-phenylalanoyl-CoA side chain to baccatin III is C-13-phenylpropanoyl-CoA transferase (BAPT), yielding the compound 3´-N-debenzoyl-2´-deoxytaxol (Fig. 2.13). This compound, by the action of an unknown Cyt P450-dependent hydroxylase that hydroxylates the C2´ position and the enzyme 3´-N-debenzoyl-2´-deoxytaxol N-benzoyl transferase (DBTNBT) that conjugates benzoyl-CoA to 3´-N-debenzoyl-2´-deoxytaxol, yields taxol as the final compound. This enzyme can be exploited to improve the production of taxol in genetically engineered systems (Walker et al. 2002).

2.7 Extraction and determination of fungal taxol

***Fermentation of endophytic fungi*:** Endophytic fungi fermentation is always carried out by a two-stage culture process. Fungi are first inoculated into basal liquid medium for several days to ensure sufficient mycelium growth and then are transferred to fermentation medium to harvest good yields of secondary metabolites. For example, Xiang et al. (2002) inoculated several fungal mycelia, preserved in PDA solid media, into 250 mL flasks containing 50 mL basal liquid medium. The mycelium was cultured at 28°C for 3 days and then transferred to 500 mL fermentation liquid medium and cultured at 28°C for another 8-10 days in a rotary shaker. The conditions of the fermentation greatly affected the yield. Different factors include temperature, pH value, amounts of inoculation, volumes of fermentation liquid medium, and fermentation time affect the final yield (Dai and Tao 2008). When different taxol precursors such as; sodium benzoate, special fungal regulators, extracts of *Taxus* needles, and different saccharines were added to fungal fermentation broth at different phases of the fermentation

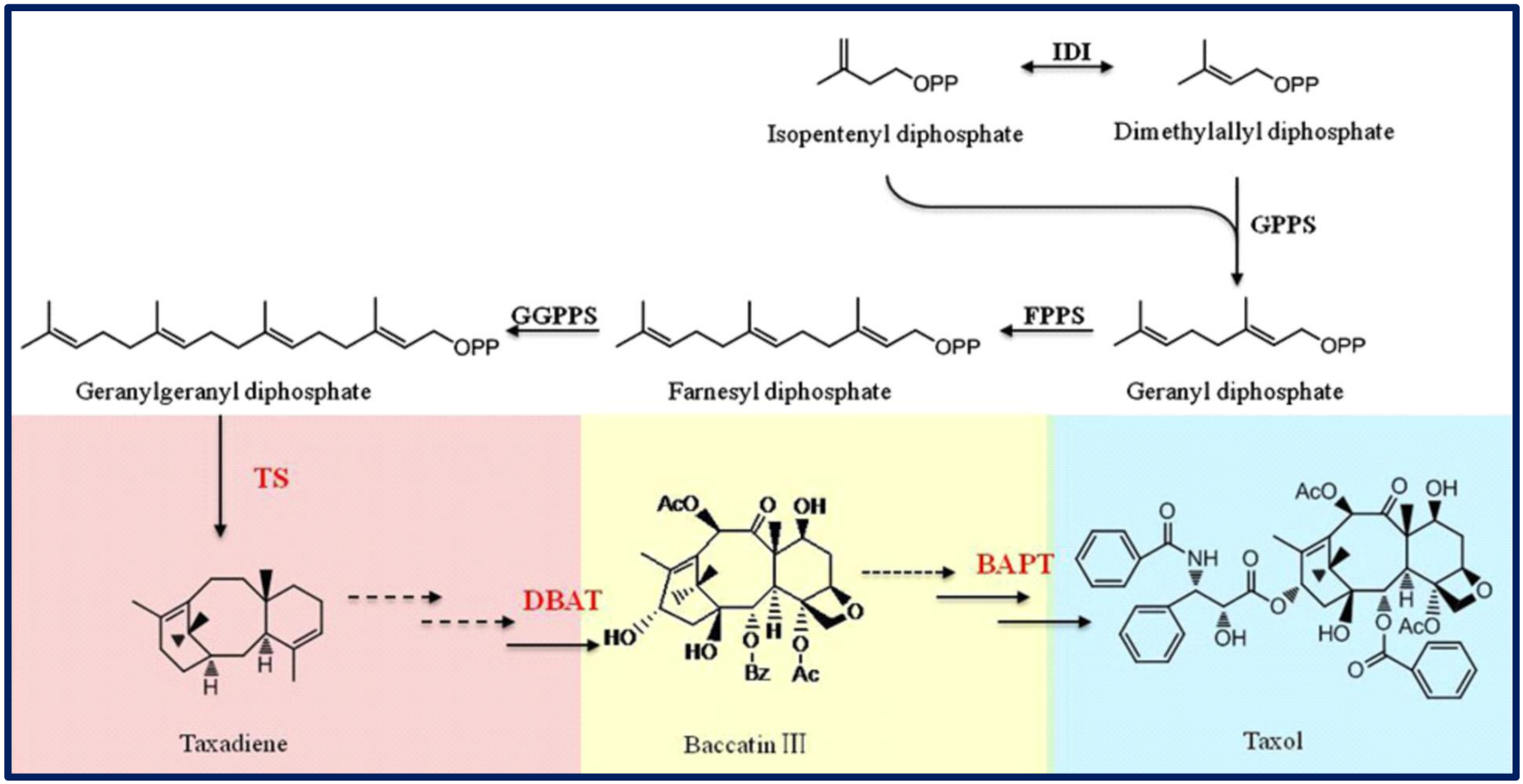


Fig. 2.13 Key genes involved in biosynthesis for Taxol in *Taxus* species. Abbreviations: GGPPS: geranylgeranyldiphosphate synthase; TS: taxa-4(5),11(12)-diene synthase that catalyzes the committed step of this pathway; DBAT: 10-deacetylbaccatin III-O-acetyltransferase; BAPT: baccatin III 13-O-(3-amino-3-phenylpropanoyl) transferase. Multiple arrows imply more than one biosynthetic step (Xiong et al. 2013)

process, taxol yields were increased (Stierle et al. 1995). Studying the growth conditions of different fungi is very important because it could help to increase the taxol yield.

***Choice of different solvents and extraction methods*:** Until now, different organic solvents were used to extract taxol, such as ethyl acetoacetate, dichloromethane (DCM), chloroform, and methyl alcohol (Dai and Tao 2008; Stierle et al. 1995; Chen et al. 2004). At the same time, different extraction methods were adopted by various study groups for several fungal species. After the specified fermentation period, some researchers centrifuged the culture and collected the supernatant. The mycelium were homogenized for 15-20 min to release the target material and centrifuged again. The two supernatant were collected together, filtered, evaporated at 50°C to one third of its original volume and then extracted with two equal volumes of chloroform chloride. All the organic extracts were evaporated at 35°C and the residue is resuspended in a certain amount of methanol and subjected for taxol detection (Dai and Tao 2008). Others use an easier method that extracts the mycelium directly. Mycelia are dried overnight (35-40°C), crushed and extracted thrice in methanol. The methanolic fraction is centrifuged and supernatant is reconstituted with an equal volume of distilled water and then portioned with DCM. The culture broth is also extracted with three equal volumes of DCM. The DCM fractions are collected, combined and evaporated under pressure at 35°C. Then, the obtained residue is resuspended with a small quantity of chloroform and purified with silica gel column filled with silica particles (Zhou et al. 2001; Chen et al. 2004). The advantage of this method is that it can efficiently extract all the taxol in the fungal cultures. In summary, to extract taxol from fungal culture, pre-treatment, solvent selection and evaporation temperature must be considered critically.

***Determination of taxol in fungal fermentation products*:** The methods that can detect taxol in fungal fermentation products include thin-layer chromatography (TLC) (Chen et al. 2004), high performance liquid chromatography (HPLC) (Huang et al. 2001; Chen et al. 2004),

liquid chromatography-mass spectroscopy (LC-MS) (Zhou et al. 2007; Zhu et al. 2008), ultraviolet (UV) immunity analysis and many others (Oehme et al. 2002). In addition to the above-mentioned techniques, competitive inhibition enzyme immunoassay (CIEIA) is a method that uses mAb3C6 (McAb possesses specificity to taxol) and mAb8A10 (McAb possesses specificity to taxol analogues) to bioassay taxol; CIEIA is easy to use and sensitive (Grothaus et al. 1993). However, up to now HPLC remains the most popular method to be used for determining concentration and also for tracing and isolating taxol (Nadeem et al. 2002; Zhou et al. 2004; Wang et al. 2007; Gangadevi et al. 2008; Zhao et al. 2009).

2.8 Antimitotic activity of fungal taxol

Traditional medicine based on legends, beliefs, customs, and casual observations or indications has produced leads for new antitumor and antibiotic drugs (Wedge and Camper 1999), *Vinca rosea* was traditionally used for diabetes and was found to decrease white blood cell counts (Lippincott and Lippincott 1975). *Vinca* alkaloids are used to treat pediatric leukemia and other neoplasms today. *Podophyllum peltatum* has been reported to be used by the Penobscot Indians to treat cancer. Others used the resin to remove venereal warts and it is still the treatment of choice (Gordaliza et al. 1994). Derivatives of *Podophyllum* are used today to treat small lung and testicular cancer. Native American Indians used *Taxus* spp. as an abortifacient as well as for skin cancers. Taxol from *Taxus brevifolia* is used for ovarian and breast cancer. The development of a pharmaceutical of ethnobotanical, anecdotal or folkloric origin can be expensive and require years of experimentation. Verification of claimed biological activity requires testing in bioassays and ultimately clinical testing. Bioassays can provide information about the biological activity of a plant and fungal extracts and can also be used to direct fractionation of the extract to identify active components.

Bioassay methods used in assessing the antitumor activity of plant and fungal extracts have varied over the years. These methods have yielded important discoveries including

vincristine, vinblastine, the podophyllotoxins derivatives, 10-hydroxy-camptothecin and Taxol (Suffness and Douors 1979; Wani et al. 1980). The 3PS (P388) (methylcholanthrene-induced) leukemic mouse assay and the *in vitro* screening for 9KB (human nasopharyngeal carcinoma) cytotoxicity (Jackson et al. 1984; Wall and Wani 1977) came to be the assays of choice. New primary *in vitro* screening assays were developed by developmental therapeutics program of National Cancer Institute (NCI) and consist of a panel of over 50 human cancer cell lines (McLaughlin and Rogers 1998). These assays identify agents having cell-type selective toxicity (Paclitaxel). However, they are costly, require specialized laboratories and equipment, and may present problems in interpreting data obtained with plant extracts.

The inhibition of *Agrobacterium tumefaciens*-induced tumors (or Crown Gall) in potato disc tissue is an assay based on antimitotic activity and can detect a broad range of known and novel antitumor effects (McLaughlin and Rogers 1998). Crown Gall is a neoplastic plant disease caused by *A. tumefaciens* (Kahl and Schell 1982; Lippincott and Lippincott 1975). The validity of this bioassay is predicted on the observation that certain tumorigenic mechanisms are similar in plants and animals (Becker 1975; Braun 1972; Karpas 1982). It has been shown that the inhibition of Crown Gall tumor initiation on potato discs and subsequent growth showed good correlation with compounds and extracts active in the 3PS leukemic mouse assay (Galsky et al. 1980; Galsky et al. 1981). Ferrigni et al. (1982) showed that the potato disc tumor assay was statistically more predictive of 3PS activity than either the 9KB or the 9PS cytotoxicity assays. Podophyllin, Taxol, Camptothecin, Vincristine and Vinblastine has all shown significant tumor inhibition in the 3PS (also referred to as P-388) leukemic mouse assay (Gordaliza et al. 1994; Kahl and Schell 1982; Lewis and Elvin- Lewis 1977; Riley 1999). The bacterium, *A. tumefaciens,* is a gram-negative rod that is the causative agent of Crown Gall Disease. It is a disease in which a mass of tissue bulging from stems and roots of woody and herbaceous plants is produced. These masses (tumors) may be spongy or

hard, and may or may not have a deleterious effect on the plant. The tumors produced are histologically similar to those tumors found in humans and animals (Agrios 1997). During infection of plant material with *A. tumefaciens*, a tumor-producing plasmid (Ti-plasmid), found in the bacterial DNA, is incorporated into the plant's chromosomal DNA. When plant tissue is wounded it releases phenols, etc., which will activate the Ti plasmid in *A. tumefaciens*. The Ti-plasmid causes the plant's cells to multiply rapidly without going through apoptosis, resulting in tumor formation similar in nucleic acid content and histology to human and animal cancers (Agrios 1997). McLaughlin (1991) concluded that the Crown Gall tumor (potato disc) assay could be used as a fairly rapid, inexpensive and reliable pre-screen for antitumor activity.

Mechanism-based assays detect potential anticancer agents that interfere with neoplastic growth, or focus on target receptors that were discovered as the mechanism of drug action (McLaughlin, 1991). Crown Gall is a neoplastic disease of plants induced by specific strains of *A. tumefaciens*. Galsky et al. (1980) first demonstrated that inhibition of Crown Gall tumor distribution on potato discs correlated with compounds and plant extracts known to be active in the 3PS leukemic mouse tumor assay. The antitumor effect is independent of antibiosis (Galsky et al. 1980). Thus, as a pre-screen, antibiosis is not a problem.

The Potato Disc Tumor Assay is a simple, inexpensive and fast screen for antitumor agents. It was used to identify the antitumor activity of ellagic acid and an extract of *Meliva volkensii* fruit (Wedge and Camper 1999). Also, Coker et al. (2003) conducted this assay using camptothecin, podophyllin, taxol, vinblastine and vincristine as the test drugs. Camptothecin served as a positive control and inhibited tumor production at all concentrations tested. There was a significant difference in activity between camptothecin, podophyllin and vincristine at 0.001 ppm; but camptothecin was not significantly different

from paclitaxel at the same concentration. Camptothecin was significantly more active at 0.01 ppm than podophyllin, vinblastine or vincristine at the same concentration. No significant difference was observed between camptothecin at 0.1 ppm and podophyllin, vinblastine, or taxol at the same rate. Camptothecin at 0.1 ppm was significantly more inhibiting than vincristine at 0.1 ppm. All compounds tested (Podophyllin, taxol, vinblastine and vincristine), inhibited tumor production in potato disc tumor assay at all tested concentrations. The study reported herein affirms the use of this assay as a first general screen in the search for new antitumor agents, whether their mode of action is inhibition of topoisomerase, interference with tubulin function, or prevention of microtubule reorganization.

2.9 Optimization of conditions for maximum taxol production by statistical tool Response Surface Methodology (RSM)

Production of various biotechnological products required at large scale often needs to be optimized for conditions which provide their maximum level. It is prerequisite to find all the factors and conditions involved in a process and optimize the parameters so that their response reaches the optimum level. This operation relates to several methods of statistical experimental design. Several conventional methods for optimization of a process exist e.g. 'one variable at time' (OVAT) approach. This strategy has the advantage that the individual effects of medium components can be seen on a graph, without the need to revert to statistical analysis.

But there are several drawbacks with OVAT, as:

1. They are time consuming

2. Require huge sets of experiments to be conducted

3. Are uneconomical

4. Lack mutual interactions among variables (Rathi et al. 2002; Kunamneni 2005)

Orthogonal and Plackett-Burman (PB) designs are often applied to reduce the number of trials and determine the critical variables. PB design (Plackett and Burman 1946) is a well-established and widely used statistical technique for selecting the most effective component with high significance levels for further optimization, while ignoring interactions among variables (Chattopadhyay et al. 2002; Naveena et al. 2005; Prakesh and Srivastava 2005; Srivastava and Srivastava, 2005; Chauhan et al. 2006; Sayyad et al. 2007; Jin et al. 2008; Rajendran et al. 2008; Lu et al. 2009; Kiran et al. 2010). PB design is a class of fractional two-level factorial designs, which does not describe interaction among factors (nutrient components) and is used only to screen and evaluate important factors influencing the response (Hymavathi et al. 2010). It is a resolution III design which confounds main effects with two-factor interactions. However, despite the compounded alias structure, in contrast to fractional factorial designs, PB design is advantageous as the effects are not fully confounded and in certain circumstances even allow interactions to be estimated simultaneously (Tyssedal and Samset 1997). Factor sparsity can be an appropriate assumption at an early stage of an investigation; this assumption states that only a few factors are responsible for variation in the data (Box and Meyer 1985). Hence, based on the assumption of factor sparsity and the principle of effect heredity, which says that for an interaction to be significant, at least one of the corresponding factors main effects also needs to be significant (Tyssedal and Samset 1997), a 12-run (non-geometric) PB design was used for screening of significant nutrients affecting growth and taxol production (Luo and He 2004; Xu et al. 2006).

Based on the results of the PB design, a process optimization tool, response surface methodology (RSM), was then used to determine the optimum concentrations of selected significant factors (independent variables, i.e. medium nutrients) affecting the desired response (dependent variable, i.e. biomass or taxol production) (Theodore and Panda 1995;

Luo et al. 2004; Xu et al. 2006). RSM may be summarized as a collection of experimental strategies, mathematical methods, and statistical inference methods for exploring the functional relationship between a response value and a set of design variables (Han et al. 2008; Kumar and Gupta 2008). In this way, the interactive effects of possible influencing parameters can be evaluated and taken into consideration within a limited number of planned experiments, thus enhancing productivity. A Box-Behnken design (BB design) or Central Composite Design (CCD), using the Design-Expert software, is usually used to acquire data that are then fitted to an empirical, polynomial model. The experimental design coupled with a polynomial model is a powerful combination that usually provides an adequate representation of most continuous-response surfaces over a relatively broad factor domain (Srivastava and Srivastava 2005).

A lot of studies have proved the successful application of RSM as a powerful statistical tool for rapid search of key factors from a multivariable system, minimizing the error in determining the effect of various parameters and the results are achieved in an economical manner. Over the years RSM has been widely used for enhanced production of various enzymes, fermentation products, biomolecules and secondary metabolites in different organisms and systems using computer software such as Design-Expert (Vohra and Satyanarayana 2002; Xiong et al. 2004; Lv et al. 2008; Singh and Bishnoi 2012). Luo and He (2004) employed RSM for optimizing elicitors and precursors for paclitaxel production in cell suspension culture of *Taxus chinensis* which resulted in 2 times higher paclitaxel production (54 mg/L) in comparison to unoptimized medium. Combination of orthogonal design and RSM was used for optimization of medium for the production of secondary metabolite squalestatin S1 by *Phoma* sp. (Parra et al. 2005). Confirmatory experiments of optimal composition produced 434 mg/L (60% improvement in maximum litre production) of the metabolite. Chang et al. (2006) worked on optimization of medium components for

submerged culture of *Ganoderma lucidum* using statistical tool RSM. When the strain was grown in optimized medium under optimal operating conditions, improvement in mycelium formation from 1.70 to 18.70 g/L was recorded along with the increase in polysaccharide production from 0.140 to 0.420 g/L.

Xu et al. (2006) employed RSM for enhancement of taxol production by endophytic fungus *Fusarium maire*. After optimization of media components the yield of taxol increased from 20 to 225 µg/L. Similarly, enhancement in cordycepin production by *Cordyceps militaris* CCRC 32219 was attained by use of RSM strategy (Shih et al. 2007). Promising results were obtained by Banik and Pandey (2008) while optimizing medium components for enhancing oleanolic acid production from *Lantana camara* (ornamental plant). Yin and Dang (2008) employed RSM for enhancing the yield of polysaccharides from *Lycium barbarum* and found that predicted values coincided with that of the experimental results. Niladevi et al. (2009) applied RSM for optimization of different nutritional and physical parameters for production of laccase by a novel filamentous bacterial strain *Streptomyces psammoticus* MTCC 7334 in submerged fermentation and reported 3 fold increases in optimized medium. Enhanced production of butyric acid (maximum yield of 12.05 g/L) by *Clostridium thermobutyricum* in shake flask culture using RSM was reported by Zhang et al. (2009). Similarly, Majumder et al. (2009) applied the statistical tool RSM for enhancing glucan production by *Leuconostoc dextranium* and found that gulcan produced experimentally (1015 ± 4.5 mg/L) was in aggrement with the predicted amount. Production of aspergiolide A by the marine-derived fungus *Aspergillus glaucus* HB1-19 and in (+)-terrein by *Aspergillus terreus* PF26 has been reported via medium optimization using statistical methodology (Cai et al. 2009; Yin et al. 2012). Guo et al. (2010) reported optimization of β-galactan, polysaccharide of *Phellinus igniarius* by RSM and maxium yield (50.39 mg/g) of crude intracellular polysaccharide (IPS) from mycelia was obtained in optimized medium. Wu et al.

(2011) also implied RSM for enhancing the production of fumigaclavine C (FC) and helvolic acid (HA) by *Aspergillus fumigatus* CY018 and found higher yields of 17.20 mg/L for FC and 16.88 mg/L for HA under optimized medium conditions. Srivastava and Srivastava (2012) employed statistical tool RSM for media optimization for enhanced azadirachtin production in hairy root culture of *Azadirachta indica*. The optimized medium composition yielded a root biomass production of 14.2 gm/L and azadirachtin accumulation of 5.2 mg/g (68% more than unoptimized medium). RSM was also used for media optimization by Bajaj et al. (2012) for enhancing the production of sophorolipids (SLs) by *Starmerella bombicola* NRRL Y-17069 and maximum yield of SLs was found to be 54.16 g/L which was in aggrement with the predicted value. Varshney et al. (2013) performed statistical optimization of medium components to increase the manganese peroxide productivity (MnP) by *Phanerochaete chrysosporium* NCIM 1197 and reported 70.20 U/ml enhancements in MnP production. Hedge et al. (2013) reported 6 fold increase (BC concentration 1.72% w/v) in bacterial cellulase (BC) production by *Gluconacetobacter persimmonis* with the implications of RSM. Enhancement of palmarumycin C_{13} production in liquid culture of endophytic fungi *Berkleasmium* sp. Dzf12 after treatment with metal ions was reported by Mou et al. (2013). A 6 fold increase (203.85 mg/L) was obtained when the endophytic fungus was grown in liquid medium with optimized nutritional parameters. Similarly, Baadhe et al. (2013) used RSM for enhancing production of amorphadiene (amorpha-4,11-diene; precursor of antimalarial drug) by an engineered yeast strain *Saccharomyces cerevisiae* (YCF-AD1) and the experimental yield was found to be in agreement with the predicted values. Wang et al. (2013) employed RSM for optimizing media for 2´,4´-dihydroxy-6´-methoxy-3´,5´-dimethylchalcone production by *Ceriporia lacerata*. Compared to unoptimized medium, the production of DCM was increased 6 folds in optimum medium. Madhusudhan et al. (2014) used the statistical tool RSM for enhanced production of insoluble melanin by *Streptomyces lusitanus*

DMZ-3. Vijayaraghavan and Vincent (2014) reported statistical optimization of fibrolytic enzyme produced by *Pseudoalteromonas* sp. IND11 and an enzyme activity of 1573 U/ml was obtained under optimized medium parameters which was three times higher than unoptimized medium.

CHAPTER 3

Material and Methods

3.1 Sample collection areas and geographic features

Bark samples of *Taxus baccata* L. subsp. *wallichiana* (Zucc.) Pilger (Himalayan yew) were collected from different locations of Northern Indian Himalayan Region (IHR)**.** It extends from Kashmir to Uttrakhand with latitudes 30º-38º N and longitudes 78º-89º E and includes parts of Jammu and Kashmir, Himachal Pradesh and Uttrakhand. In the present study, samples were collected from Bhaderwah (district Doda, Jammu and Kashmir) which is situated 32.98º N 75.72º E, Shimla (Himachal Pradesh) situated 31.61º N 77.10º E, and Almora (Uttrakhand) situated 29.62º N 79.67º E. Bark samples were collected from Bhadrewah in the month of October 2009, from Shimla in the month of November 2009 and Almora in the month April 2010.

3.2 Isolation and cultivation of endophytic fungi from *Taxus baccata* bark

Bark samples (1 × 3 cm) were harvested from the stem of relatively young *T. baccata* subsp. *wallichiana* growing at different locations of the northern Himalayan region. Collected bark pieces were placed in a sealed plastic bag, transported to the laboratory, and stored at 4°C. For isolation of endophytic fungi, bark pieces were washed meticulously under running tap water, followed by final washing with sterile distilled water under aseptic conditions. The bark samples were surface sterilized by treating with 70% aqueous ethyl alcohol (v/v) for 60 s to kill epiphytic micro-organisms, followed by washing in 4 % sodium hypochlorite for 60-90 sec and rinsing twice in sterilized distilled water. The excess moisture on the bark surface was blotted using sterile filter paper. The outer bark was teased apart with the help of flame sterilized sharp blade in order to procure inner bark. Surface disinfected small pieces (~ 0.5 ×

0.5 × 0.5 cm) of inner bark were excised and placed on the surface of potato dextrose agar (PDA) medium supplemented with ampicillin (50 μg/ml) in Petri plates. The plates were incubated at 28ºC for 5-10 days to permit the growth of endophytic fungi. The plates were periodically checked for the growth of endophytic fungal colonies and culture purity. Pure fungal cultures of endophytic isolates were acquired by the hyphal tip method (Strobel et al. 1996). All the fungal isolates were coded and stock cultures were preserved by subculturing at monthly intervals. For long term preservation, isolates were stored in sterile distilled water as agar plugs. From an actively growing stock culture, sub-cultures were made on fresh plates and after 7 days of incubation at 25ºC, these were used as the commencing material for molecular screening and biochemical detection of taxol.

3.3 Screening of taxol producing fungi based on PCR amplification

3.3.1 Isolation of fungal genomic DNA

The fungal isolates were inoculated aseptically and individually in 20 ml of potato dextrose broth in 150 ml Erlenmeyer flasks. Cultures were incubated at 25-28°C at 120 rpm for 3-5 days and the mycelium of each fungus was harvested by centrifugation at 12,000 rpm for 10 min. Genomic DNA was extracted from the mycelia using the CTAB method (Zhang et al. 1996).

3.3.1.1 CTAB method for DNA extraction

1. Taken 3 gm of mycelium in an autoclaved mortar, ground in liquid nitrogen to fine powder. Immediately transferred (do not allow to thaw) to 50 ml oak ridge tube and added preheated CTAB buffer to make slurry along with 200 μL β-mercaptoethanol. Incubated at 60ºC for 1h in water bath with mixing at regular intervals.
2. Added equal volume of chloroform:iso-amyl alcohol (24:1), mixed for about 5 min. and centrifuged for 10 min at 5000 rpm.

3. Removed aqueous phase with wide-bore pipette (cut off tip from mouth) to clean oak ridge tube. Repeated chloroform extraction if extract was still colored.
4. Precipitated DNA with 0.66 volumes of cold isopropanol and incubated for 1 h at -20ºC.
5. Centrifuged at 10000x g for 15 min.
6. Supernatant was discarded and dissolved the pellet in 1 mL TE buffer (Appendix I) and taken solution in microfuge tube.
7. 2 µL RNase solution (10 mg/mL stock) was added and incubated at 37ºC for 1 h. RNase stock solution was preheated for 5 min at 60ºC before its use.
8. Added equal volume of phenol:chloroform (1:1 v/v) and shaken slowly. Centrifuged (1000 x g, 10 min) and retained aqueous phase.
9. Added 0.3 volume of 3M sodium acetate and 0.6 volume of chilled iso-propanol. Incubated for 1 h at -20ºC.
10. Centrifuged (10000 x g, 8 min) and retained pellet. Washed pellet with 30 µL of 70% EtOH and air-dried pellet.
11. Dissolved pellet in TE buffer and stored at -20ºC.

3.3.1.2 DNA Purification

The DNA was purified by elution through the Wizard DNA Clean up system (Promega, USA) according to manufacturer's instructions in order to remove contaminants, which can hamper in manipulation of DNA.

3.3.1.3 Checking of DNA (Agarose Gel Electrophoresis)

1. Made 0.8 % (w/v) gel in TBE buffer (Appendix I) by boiling in microwave. Added ethidium bromide (1µL/30mL) after cooling down and poured the gel in mini gel tray with comb. Allowed the gel to solidify.

2. Placed gel tray in electrophoresis unit, filled the unit with 0.5X TBE buffer (Appendix I) and removed the comb carefully.
3. Prepared samples by adding tracking dye to DNA samples and mixed.
4. After loading the samples in wells, electrophoresis was carried out at 60 V for 30-45 minutes and visualized on a U.V. transilluminator.

3.3.1.4 Quantification of DNA using nanodrop

The concentration of extracted DNA in suspension was estimated by using a nanodrop spectrophotometer (Thermo Fisher Scientific Inc. USA). For DNA quantification 1 µl of sample was loaded onto the lower optical surface and then the lever arm was lowered. The upper optical surface engages with the sample, establishing a liquid column with the path length determined by the gap between the two optical surfaces. The sample is analysed at both 1 mm and 0.2 mm path, allocating a large dynamic range of nucleic acid detection. The quality of the DNA was evaluated by measurement of the A_{260}/A_{280} and the A_{230}/A_{260} ratios. Ideally, the A_{260}/A_{280} ratio should be 1.8-2.0 while the A_{230}/A_{260} ratio should be 0.3-0.9. Ratios (A_{260}/A_{280}) less than 1.8 indicate protein or phenol contamination, while ratios greater than 2.0 indicate the presence of RNA.

3.3.1.5 Ethidium bromide fluorescent DNA quantification

DNA was migrated electrophoretically in an agarose gel (0.8% w/v) containing ethidium bromide (1µL/30mL). The quantity of DNA was visually determined with reference to a known DNA concentration of lambda phage (Fermentas, USA) by comparing the intensity of fluorescence.

3.3.2 Ribonucleic acid (RNA) isolation and complementary DNA (cDNA) synthesis

3.3.2.1 RNA isolation (Chuang et al. 1993)

1. One gm of mycelium was taken in an autoclaved mortar, ground in liquid nitrogen to fine powder. Immediately transferred (do not allow to thaw) to sterile eppendorf and added preheated extraction buffer (CTAB buffer) to make slurry along with 40 µL β-mercaptoethanol. Incubated at 65ºC for 20 min in water bath with mixing at regular intervals.
2. Added equal volume of chloroform:iso-amyl alcohol (24:1), mixed for about 5 min and centrifuged at room temperature for 10 min at 12000 g. Transferred aqueous phase to a clean eppendorf. This step was repeated twice.
3. Added ¼ volumes of 10M Lithium chloride to the supernatant and mixed well.
4. RNA was allowed to precipitate overnight at 4ºC and harvested by centrifugation at 12000 g for 30 min.
5. Washed pellet with 200 µL of 75% EtOH (12000 g at 4ºC for 10 min) and air-dried pellet.
6. Dissolved pellet into 25µl of DEPC water and stored at -20ºC

3.3.2.2 cDNA synthesis

Extracted RNA was quantified using a nanodrop spectrophotometer (Thermo Fisher Scientific Inc. USA). cDNA synthesis was performed using a RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific Inc. USA) as per the manufacturer's instructions.

3.3.3 Molecular screening of endophytes using gene specific primers

The conserved sequences of two key genes involved in the taxol biosynthetic pathway, *DBAT* (10-deacetylbaccatin III-10-O-acetyl transferase) and *BAPT* (C-13 phenylpropanoid side chain-CoA acyltransferase), were used as molecular markers for the primary screening of taxol producing fungi. The following specific primers (Table 3.1) as described by Li et al.

(2006) were used for PCR amplification, which was accomplished in the GeneAmp® PCR system 2700 (Applied Biosystems, USA).

Table 3.1 Specific primer sequences for *DBAT* and *BAPT* genes

Gene	Primer sequences
DBAT	*dbat*-F 5′-GGGAGGGTGCTCTGTTTG-3′
	dbat-R 5′-GTTACCTGAACCACCAGAGG-3′
BAPT	*bapt*-F 5′-CCTCTCTCCGCCATTGACAA-3′
	bapt-R 5′-TCGCCATCTCTGCCATACTT-3′

First, the fungal isolates were screened for the existence of the *DBAT* gene. Amplification was done using *dbat*-F and *dbat*-R primers in a 20 µl PCR mixture. The PCR reaction mixture consisted of 1X PCR buffer (Fermentas, USA), each dNTPs at a concentration of 200 µM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.1 µM, 2.5 units of Taq DNA polymerase (Fermentas, USA) and 50 ng DNA in a final volume of 20 µl. The PCR programme consisted of the following primer extension conditions (Table 3.2) as mentioned in Zhang et al. (2008).

Table 3.2 Primer extension conditions for amplification with *dbat* primers

Cycle No.	Reaction step	Time	Temperature
1	Denaturation of template DNA	6 min	95ºC
35	Denaturation	50 sec	94ºC
	Annealing of primers	30 sec	50ºC
	Elongation	50 sec	68ºC
1	Final elongation	10 min	68ºC

The amplified DNA fragments were examined by agarose gel electrophoresis, and fungi showing amplification for *DBAT* gene were further subjected to *BAPT* gene amplification. PCR amplification was done using *bapt*-F and *bapt*- R primers in a 20 µl reaction mixture, with the following primer extension conditions (Table 3.3) as mentioned in Zhang et al. (2008). Reaction mixture for the PCR contained 1X PCR buffer (Fermentas, USA), each dNTPs at a concentration of 200 µM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.1 µM, 2.5 units of Taq DNA polymerase (Fermentas, USA) and 50 ng DNA in a final volume of 20 µl.

Table 3.3 Primer extension conditions for amplification with *bapt* primers

Cycle No.	Reaction step	Time	Temperature
1	Denaturation of template DNA	6 min	95ºC
35	Denaturation	50 sec	94ºC
	Annealing of primers	50 sec	55ºC
	Elongation	50 sec	68ºC
1	Final elongation	10 min	68ºC

The amplified DNA fragments were examined by agarose gel electrophoresis. Those fungal isolates which gave PCR positive results for both the molecular markers (*DBAT* and *BAPT*) were first identified and then subjected to biochemical screening.

3.3.3.1 Cloning and characterization of *BAPT* gene

The PCR amplified product of *BAPT* gene of positive fungal isolates were purified using a QIAquick® PCR purification kit (Qiagen Inc., USA). The purified PCR products were ligated to pTZ 57R/T vector (Thermo scientific) and transformed into *E. coli* DH5α (Invitrogen). Transformed colonies were carefully picked and the inserts were sequenced. The *BAPT* gene sequences of endophytic isolates were compared by using BLASTx and

aligned with the protein sequences of the *BAPT* gene of various *Taxus* species using Clustal W software (Larkin et al. 2007).

3.4 Identification of endophytic fungi

3.4.1 Morphological characterization

The fungal isolates which showed positive results in molecular screening were characterized based on its morphological characters. For examining the cultural and morphological characters, endophytic isolates were subcultured onto fresh media (PDA) and incubated at 28ºC for 2 weeks. Cultural characters such as colour and nature of the growth of the colony were intented by visual observation. Morphological characteristics of the fungal endophytes like mycelia, conidiophores and conidia were microscopically (Nikon Eclipse E200) examined (Barnett et al. 1998; Wei 1979).

3.4.2 Molecular phylogenetic analysis [Amplification of Internal Transcribed Spacer (ITS) region]

ITS regions of the rDNA from genomic DNA of selected endophytic fungi together with the 5.8S rRNA gene were amplified using the universal primers, ITS1 and ITS4 (Table 3.4) as described by White et al. (1990).

Table 3.4 Primer sequences for ITS 1 and ITS 4 genes

	Universal primers
ITS1	5′-TCCGTAGGTGAAC CTGCGG-3′
ITS4	5′-TCCTCCGCTTATTGATATGC-3′

Reaction mixture for the PCR contained 1X PCR buffer (Fermentas, USA), each dNTPs at a concentration of 200 µM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.1 µM, 2.5 units of Taq DNA polymerase (Fermentas, USA) and 50 ng DNA in a final volume of 50 µl. The

amplification of ITS region from isolates was carried out with a GenAmp thermocycler (Applied Biosystem, USA). The PCR programme consisted of primer extension conditions as described in table 3.5.

Table 3.5 Primer extension conditions for amplification with ITS universal primers

Cycle No.	Reaction step	Time	Temperature
1	Denaturation of template DNA	5 min	94ºC
35	Denaturation	1 min	94ºC
	Annealing of primers	1 min	54ºC
	Elongation	1 min	72ºC
1	Final elongation	10 min	72ºC

Controls containing no DNA template were included for verifying that there is no contamination of reagents and reaction buffer. Successful amplifications were confirmed by agarose gel (1.5% w/v) electrophoresis and visualization on a U.V. transilluminator.

3.4.2.1 Purification of PCR products

Amplified ITS products were purified by agarose gel (0.8%) electrophoresis prior to cloning. The DNA fragment was excised from the gel, using the QIAquick gel extraction kit (Qiagen Inc., USA) following the instructions of the manufacturer. Purified PCR products were eluted with 30 µl TE buffer (pH 8.0). Purified PCR products were then straightway used for cloning in T-vector.

3.4.2.2 Ligation in pTZ57R/T vector

The ITS PCR products were cloned using the restriction independent InsTA Cloning Kit, following the manufacturer's protocol (Fermentas, USA). The fungal ITS amplicons were ligated into pTZ57R/T vector. The reaction mixture was prepared as described in table 3.6.

The ligation reaction mixture was incubated overnight at 4°C, analyzed on 0.8% agarose gel and was used for transformation.

Table 3.6 Ligation reaction mixture

Plasmid pTZ57R/T (55ng/µl)	3 µl
Insert (75ng/µl)	4 µl
Buffer (5X)	6 µl
T_4 Ligase	1 µl
H_2O	16 µl

3.4.2.3 Genetic Transformation of ITS products into *E. coli* DH5α cells

1. A single colony of *E. coli* DH5α from a freshly grown plate was inoculated into 25 ml of LB broth in a 250 ml flask and incubated for 16-20 hrs at 37°C under shaking condition (120 rpm). Aseptically 200 µl of the above-saturated culture was transferred into 25 ml of fresh LB broth in a 250 ml flask. The culture was further incubated with vigorous shaking at 37°C for 2-3 hrs. To monitor the growth of the culture, the OD_{590} was determined at every one-hour (OD_{590} should be ~ 0.5).
2. The above culture was transferred to sterile, disposable, ice-cold 50 ml polypropylene tubes. The culture was cooled to 0°C by storing the tubes on ice for 10 min. The cells were harvested by centrifugation at 8,000 rpm for 10 minutes at 4°C.
3. The pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and stored on ice for 15 min. Further, the cells were recovered by centrifugation at 8,000 rpm for 10 min at 4°C.
4. The cell pellet was resuspended in 1 ml of ice-cold 0.1 M $CaCl_2$. $CaCl_2$ treatment for 2 hours induces considerably a transient state of "competence" in the *E. coli* cells.

5. One hundred micro litre of the suspension of competent cells were transferred to a sterile and prechilled microfuge tube (1.5 ml capacity).
6. The plasmid DNA sample (~100 ng in a volume of 5 μl or less) was added to each tube. The content of the tubes were mixed gently and stored the tubes on ice for 30 min.
7. The tubes were incubated in a circulating water bath that has been preheated to 42°C for exactly 2 min without shaking.
8. The tubes were rapidly transferred to an ice bath and chilled the cells for 1-2 min. One ml of LB broth was added to each tube and incubated the cultures for 45-60 min at 37°C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid.
9. One hundred micro liter of transformed cells were spread on Luria agar-Ampicillin-X-Gal-IPTG plates and incubated at 37°C. Transformed colonies appeared in 12-16 hrs.

3.4.2.4 Blue/white screening for recombinant plasmids

For the selection of transformants, the *E. coli* DH5 α (LacZ-) bacterial host cells were plated on Luria Agar medium containing 50 μg/ml ampicillin. X-Gal and IPTG were used to screen colonies containing a recombinant plasmid. The cloning site in the pTZ57R/T vector is located in the multiple cloning site (MCS) of the plasmid's *lacZα* gene; if no insert is present, functional β-galactosidase is produced, and the transformed bacterial colony is blue. These few blue colonies occur due to the presence of supercoiled vector molecules, which have escaped linearization. However, if the host cell receives a recombinant plasmid containing an ITS insert in the *lacZα* gene, the resulting transformant colony is white (Lac Z-).

3.4.2.5 Isolation and purification of plasmid DNA from recombinant bacteria by alkaline lysis method

1. A single transformed *E. coli* white colony was transferred into 2 ml of Luria broth containing appropriate antibiotic (ampicillin, used in a final concentration of 50 μg/ml) in a loosely capped 15 ml tube and incubated the culture overnight at 37°C with vigorous shaking.
2. 1.5-2.0 ml of the above-saturated culture was poured into a microfuge tube and cells were harvested by centrifugation at 8,000 rpm for 1 min.
3. The bacterial pellet was resuspended in 200 μl of ice-cold Solution I (Appendix I) by vigorous vortexing to ensure that the bacterial pellet is completely dispersed in this solution.
4. Further, 200 μl of freshly prepared Solution II (Appendix I) was added and the contents were mixed by gentle inversion of the tubes, five to ten times. Vortexing is avoided here. The tubes were stored on ice for 5 min.
5. Finally, 300 μl of ice-cold Solution III (Appendix I) was added and mixed by inversion to disperse Solution III (Appendix I) through the viscous bacterial lysate. The tubes were stored on ice for 10 min.
6. The tubes were centrifuged at 12,000 rpm for 10 min in a microfuge.
7. The upper aqueous phase was then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1).
8. To precipitate extracted plasmid DNA, 0.7 volumes isopropanol were added to the aqueous phase, followed by 10 min centrifugation at 12,000 rpm.
9. The DNA pellets were washed with 750 μl ethyl alcohol (70%) and microfuged for another 10 min.

10. Finally, the pellets were resuspended in 40 µl TE buffer/milliQ water and stored at -20°C for further use.

3.4.2.6 Size screening for recombinant plasmids

Clones containing ITS inserts of approximately 500-750 bp were identified by PCR screening; using the rapid protocol for preparation of template DNA from single bacterial colony, using M13-forward (5'-GTAAAACGACGGCCAGT-3') M13-reverse (5'-CAGGAA ACAGCTATGAC-3') plasmid primers. The amplification products were checked by agarose gel (1.0% w/v) electrophoresis.

3.4.2.7 Sequencing

The ITS inserts were sequenced for both strands using M13 forward and reverse primers, used for pTZ57R/T vectors. The sequence was generated by chain termination method (Sanger et al. 1977) using an Applied Biosystems automatic sequencer (DNA Sequencing Facility, Department of Biochemistry, South Campus, Delhi University, New Delhi, India).

3.4.2.8 Analysis of sequence data

The ITS nrDNA sequences of all the isolates were compared with those available in GenBank databases using BLAST search program (Altschul et al. 1997) to find the possible homologous sequences of the newly sequenced taxa for each fungus. The sequences of closely related strains and uncultured fungi retrieved from GenBank were aligned to minimize the number of inferred gaps. The sequences were edited with BioEdit 5.0.6 (Hall 1999) and aligned using multiple alignments ClustalW software (Larkin et al. 2007). The phylogenetic analysis was carried out by the maximum parsimony method and the Kimura two-parameter distance calculation by MEGA5.1 software. In computational phylogenetics, maximum parsimony delineates an appropriate non-parametric statistical method for the construction of phylogenies. The favoured phylogenetic tree, in this application is the tree

that assumes the least evolutionary change to explain observed data i.e. is maximally parsimonious. On the other hand, Kimura's two parameter model corrects for multiple hits, taking into account transitional and transversional substitution rates, while presuming that the four nucleotide frequencies are the same and the rates of substitution do not vary among sites. The bootstrap was 1,000 replications to assess the reliable level for the nodes of the tree (Tamura et al. 2011). All sequences were annotated using Sequin software and submitted to the NCBI GenBank database. Cultures of selected fungal endophytes were deposited in the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India.

3.5 Biochemical screening of fungal taxol

3.5.1 Fungal cultivation

Production of taxol by the five fungal endophytes with positive results of primary screening was studied by a two stage fermentation procedure. In the first phase, these fungi were grown in submerged culture and in the second phase they were grown as stationary culture. Flasks with 100 ml of sterilized S7 medium (Appendix I) were inoculated with agar plugs containing mycelium from 7 days old cultures of the five selected endophytes, incubated at 28ºC on a rotary shaker (250 rpm) for 5 days (Stierle et al. 1993). These cultures were used as seed cultures (First stage). For taxol production, 10-20 ml seed cultures were transferred to 2 litre Erlenmeyer flasks containing 500 ml of modified S7 medium as described by Stierle et al. (1993). The flasks were incubated at 28ºC for three weeks (21 days) as stationary culture (Second stage).

3.5.2 Extract preparation

After 3 weeks of incubation the cultures were harvested and passed through four layers of muslin cloth to part the mycelial mat from the culture filtrate. Then, 0.2 mg Na_2CO_3 was

added to the culture filtrates with frequent shaking to minimize the amount of fatty acids that might contaminate the taxol in the broths. The harvested mycelia were frozen by liquid nitrogen, crushed rigorously in a mortar and extracted 3 times in 10 ml of methanol. Then, the fermentation broths were extracted with three equal volumes of dichloromethane (DCM), and the methanol fractions were reconstituted with an equal volume of distilled water and portioned with DCM. The DCM fractions were combined and the solvents were removed from the organic extracts by rotary evaporation under reduced pressure at ~ 35ºC (Strobel et al. 1994). The residues were re-dissolved in chloroform for column chromatography.

3.5.3 Column chromatography

Column chromatography was carried out on a glass column packed with silica gel to a 12 cm bed volume (Silica gel 60-120 mesh; Merck). The column was equilibrated in chloroform ($CHCl_3$) and rinsed several times before loading the sample. Sample previously dissolved and stored in $CHCl_3$ was loaded on the column and again rinsed 2-3 times with chloroform. Taxanes were eluted in 20 ml of acetonitrile and the elute was dried *in vacuo* and finally dissolved in 1 ml of HPLC grade methanol (MeOH) for TLC, HPLC, and LC-MS analyses.

3.5.4 Thin layer chromatography (TLC)

Thin layer chromatography (TLC) analysis was carried out on a 0.5 mm (20 × 20 cm) silica gel (lab prepared) preparative TLC plate (Silica gel GF 254; Merck). The methanolic extracts from column chromatography as well as the methanolic solution of Paclitaxel standard (Paclitaxel Taxus brevifolia; Sigma Chemicals Co., St. Louis, USA) were chromatographed on the TLC plate, and developed in chloroform:methanol 7:1 (v/v). A spray reagent that consisted of 1% vanillin (w/v) in sulfuric acid was used to detect the compound of interest on the TLC plate (Cardellina 1991; Wang et al. 2000). Another TLC plate loaded with the same fungal sample and authentic taxol was also exposed to iodine-potassium iodide fumes in a

glass chamber for confirming the presence of the compound. The fungal taxol was identified by comparison with authentic taxol, on the basis of its chromatographic mobility and Rf values. The TLC plates were also visualized under short-wave UV light at 254 nm to visualize the bands of desired compound. After TLC, the area of plate containing crude compound was rigorously removed by scrapping off silica at the appropriate Rf and eluting it with pure methanol.

3.5.5 High performance liquid chromatography (HPLC)

Taxol identification was carried out by injecting 20 µl of putative samples to HPLC (Acquity HPLC; Waters, USA) equipped with a reverse phase column (RP-C_{18} pre-packed column; Waters company) and detected with online DAD (diode array detector) set at a wavelength of 232 nm. Elution was carried out in an isocratic mode with mobile phase methanol:acetonitrile:water (20:40:40 v/v) at a flow rate of 1 ml/min and run time of 30 min, at room temperature.

3.5.6 High performance liquid chromatography-Mass spectroscopy (HPLC-MS)

A Waters Acquity HPLC-MS apparatus with atmospheric pressure electrospray ionisation and triple-quadrupole tandem mass spectrometer operating in the positive mode was used for LC-MS analysis. The instrument was equipped with a HPLC micrograd ient pump (Waters, USA) and an autosampler. Elution was in an isocratic mode with acetonitrile:water (49:51) as mobile phase. The samples in 100 % methanol were infused into the mass spectrometer through a reverse phase C_{18} column and separated at a flow rate of 0.3 ml/min with column temperature of 25ºC and spray voltage of 2.2 kV by the loop injection method. The MS scanning ranged from 100 to 1,000 m/z and the mass spectral fragment ions of taxol were observed. Nitrogen was used both as the nebulizer and curtain gas at a pressure of 0.7 Torr and a flow rate of 13 mL/min.

3.6 Antitumorigenic Activity Assay

The antimitiotic activity of fungal taxol was assayed by potato disc tumor induction as described in Coker et al. (2003) using *Agrobacterium tumefaciens* as the tumor causing agent. In this assay:

1. Healthy potatoes were surface sterilized, and using an autoclaved and flame-sterilized cork borer (10 mm), cylinders were cut from the potato. The cylinders were given a final wash in sterile distilled water, and 0.5 cm thick discs were cut from the cylinders using a flame-sterilized blade.
2. The discs were placed aseptically in Petri plates containing 15% water agar.
3. *Agrobacterium tumefaciens* (MTCC No. 431) grown on yeast extract medium (YEM) for 48 h at 28ºC was used for inoculation. The cell suspension was centrifuged and suspended in phosphate buffer saline (PBS: 0.043% KH2PO4, 0.148% Na2PO4, and 0.72% NaCl) to attain the absorbance of 0.96 ± 0.02 at 600 nm.
4. Paclitaxel (Sigma Chemicals) was dissolved in dimethylsulfoxide (DMSO) at a concentration of 1 mg/ml and then further diluted to 0.1, 0.01, and 0.001 µg/µl, respectively. Standard taxol served as positive inhibitory control. Other controls included DMSO with PBS, DMSO without bacterium, and DMSO with the bacterium.
5. The test solutions consisted of 400 µl of the drug (Paclitaxel) or fungal extract + 100 µl of sterile water + 400 µl of standardized bacterium suspension. Each disc in the petriplate was overlaid with 50 µl of the appropriate extract/water/bacterial mix, incubated at room temperature for 15-20 days, and observed regularly.
6. After the incubation time, the discs were stained with Lugol's reagent (I_2KI: 5 % I_2 + 10% KI in distilled water). Stained potato discs were viewed under a dissecting microscope and tumors were counted. The experiment with fungal extracts of selected endophytic fungi was repeated thrice at all dilutions and the results were analyzed.

7. Bacterial viability was determined by incubating the drug (Paclitaxel: 0.001 mg/ml) and extracting with bacterial suspension (in PBS solution) in YEM broth. After 3, 6, 9, and 12 h of inoculation, the growth was monitored by taking absorbance at 600 nm. All the experiments were performed in triplicate.

3.7 Optimization of process parameters and media components for taxol production by Response surface methodology

Fusarium redolens (TBPJ-B), which showed maximum taxol production, was selected for process optimization of medium components and growth conditions.

3.7.1 Optimization of basic parameters

3.7.1.1 Estimation of radial growth

Mycelial disc of 5 mm cut from the edge of actively growing culture was inoculated at the centre of potato dextrose agar (PDA) in Petri plates. The plates were incubated at 28ºC for 10 days and the growth [diameter (in cm)] was measured at daily intervals.

3.7.1.2 Estimation of fungal biomass (Growth kinetics)

To determine the biomass produced by *F. redolens*, it was grown in S7 liquid medium in 250 mL Erlenmeyer flasks and incubated at 28ºC. After 5, 10, 15, 20, 25 and 30 days of incubation, the mycelium was harvested and the fresh weight (FW) was recorded.

3.7.1.3 Optimization of medium-to-flask volume ratio (V_m/V_f ratio)

The impact of medium-to-flask volume ratio in the range of 0.01 to 0.03 was tested on mycelial growth and taxol production. The fungus was grown in 250 mL Erlenmeyer flasks with different volumes of S7 medium analogous to each volumetric ratio. Mycelium was harvested from the fermentation broth after 20 days. The incubation was carried out under dark conditions, temperature maintained at 28ºC and medium pH of 6.8. The fresh weight of

the mycelium was recorded and taxol production was determined from culture filtrate and the mycelium as described in Nadeem et al. (2002).

3.7.1.4 Estimation of optimal pH and temperature

The culture was grown in 50 ml of S7 liquid medium in 250 mL Erlenmeyer flasks at different pH (3.5, 4.5, 5.5, 6.5, 7.5, 8.5 and 9.5) and temperature (5, 15, 25 and 35 ºC). The pH of medium was adjusted with 0.5 M NaOH and 0.5 M HCl. After 20 days of incubation, the fresh weights of mycelium and taxol production were determined.

3.7.2 Experimental design

Statistical design strategies were implemented to optimize the fermentation medium. The one-factor-at-a-time strategy was first used to ascertain the basic medium composition. Then, a two-level Plackett-Burman design was selected to analyse the significance of each factor. Finally, a Box-Behnken design was used to locate the optimum values for the screened factors given by response prediction.

3.7.2.1 One-factor-at-a-time strategy (OFAT)

3.7.2.1.1 Effect of carbon and nitrogen sources on biomass and taxol production

The culture was grown in 250 mL Erlenmeyer flasks containing 50 ml of S7 medium supplemented with different concentrations of carbon sources (4%, 6%, 8%, 10% and 12%) such as sucrose, glucose and fructose. Similarly, liquid medium was supplemented with varying concentrations of nitrogen sources (0.4%, 0.6%, 0.8% and 1%) such as ammonium nitrate (NH_4NO_3), peptone and calcium nitrate [$Ca(NO_3)_2 \cdot 4H_2O$]. The flasks were incubated at 25ºC with an initial medium pH of 6.5 for 20 days. After 20 days the fresh weight of the mycelium was recorded and taxol production was determined as described in Nadeem et al. (2002).

3.7.2.2 Statistical methods

3.7.2.2.1 Plackett-Burman design (PB design)

The Plackett-Burman design was the statistical approach chosen for this optimization study to determine the effect of trace elements. PB design permits the investigation of up to N-1 variables with N experiments. This design eases to identify the most significant nutrients and their concentrations from a group for further optimization. This design is effective, especially when the investigator is encountered with a number of factors and is unassertive which settings are likely to produce optimal or near-optimal response. In this investigation, eight micronutrients [ferric chloride ($FeCl_3 \cdot 6H_2O$), potassium dihydrogen phosphate (KH_2PO_4), manganese chloride ($MnCl_2 \cdot 4H_2O$), magnesium sulphate ($MgSO_4 \cdot 7H_2O$), zinc sulphate ($ZnSO_4 \cdot 7H_2O$), sodium acetate (NaOAc), thiamine and phenylalanine] were evaluated. Trial experimental protocols were framed using Design-Expert version 8.0.7.1 software (Stat-Ease Corporation, Minneapolis, MN). Each variable was analysed at two levels, a high (+) and a low (-) level of concentration i.e. the higher level of the components was chosen to equal four times their lower levels, as shown in table 3.7. Twelve experiments were formulated using PB design and the response was measured in terms of taxol production. Experiments were performed in 250 mL Erlenmeyer flasks containing 50 mL of S7 medium (optimized V_m/V_f ratio at 0.20) at 25ºC with medium pH of 6.5 as still cultures in dark. After 20 days of incubation, the mycelium was harvested from the fermentation broth for biomass analysis (FW) and taxol production. The scrutiny of the experimental results was performed based on the first-order model assumption to compute the coefficient value of each selected constituent. A high positive coefficient value (*t* value) implies that the analysed factor has a major influence on response and a low *p* value of variables ($p<0.05$) implies a significant effect. The components giving high positive *t* value and *p* value less than 0.05 were chosen for subsequent concentration optimization.

Table 3.7 The two levels of medium components used in the Plackett-Burman design

Components	**Symbol**	**Low level (-1) (g/l)**	**High level (+1) (g/l)**
$FeCl_3$	**A**	0.001	0.004
KH_2PO_4	**B**	0.2	0.8
$MnCl_2$	**C**	0.001	0.004
$MgSO_4$	**D**	0.25	1
NaOAc	**E**	0.001	0.004
$ZnSO_4$	**F**	0.5	2
Thiamine (Vit. B1)	**G**	0.05	0.2
Phenylalanine	**H**	0.002	0.008

3.7.2.2.2 Response surface methodology (RSM)

Selected pertinent factors i.e. those having high *t* value and low *p* value, were subjected to RSM for determining their optimum concentrations while the rest of the medium components were retained at constant level. A three coded level Box-Behnken design (BB design) using the Design-Expert software was employed for determining the optimal concentrations of the screened factors in PB design. Seventeen experiments were formulated using BB design (Table 3.8) for the three independent variables [NH_4NO_3 (conc. range 2.5 to 10 gm/l), $MgSO_4 \cdot 7H_2O$ (conc. range 0.25 to 1 gm/l) and NaOAc (conc. range 0.5 to 2 gm/l)] each at three levels of concentrations used to optimize the taxol production. The rest of the parameters and media components were kept at constant level. The experiments were carried out in 250 ml Erlenmeyer flasks containing 50 ml of S-7 medium at 25ºC with initial pH of 6.5 as still cultures in dark. After 20 days of incubation, the biomass (FW) was harvested and

Table 3.8 Experimental recipe in Box-Behnken experimental design protocol for medium optimization

Trials	Actual values (g/L)		
	NH_4NO_3	$MgSO_4$	NaOAc
1	2.50	0.63	0.50
2	2.50	0.63	2.00
3	10.00	0.63	0.50
4	10.00	0.63	2.00
5	6.25	0.25	0.50
6	6.25	0.25	2.00
7	6.25	1.00	0.50
8	6.25	1.00	2.00
9	2.50	0.25	1.25
10	10.00	0.25	1.25
11	2.50	1.00	1.25
12	10.00	1.00	1.25
13	6.25	0.63	1.25
14	6.25	0.63	1.25
15	6.25	0.63	1.25
16	6.25	0.63	1.25
17	6.25	0.63	1.25

taxol production was determined. The data on taxol production obtained from RSM was subjected to analysis of variance (ANOVA). The behaviour of the system was explained by the following quadratic equation:

$$Y = \beta_o + \Sigma \beta_i \chi_i + \Sigma \beta_{ij} \chi_i \chi_j + \Sigma \beta_{ii} \chi_i^2$$

Y is the predicted response, β_o the offset term, β_i the linear offset, β_{ii} the squared offset, β_{ij} the interaction effect and χ_i is the dimensionless coded value of X_i.

The statistical significance of the above model equation was determined by Fisher's *F*-test value and the proportion of variance explained by the model was given by the multiple coefficient of determination, R^2 value. Contour plots (2D) were generated by the statistical Design-Expert software on the premise of the response (taxol production) analysis to visualize the interactive effect of the significant factors on taxol production. Unless otherwise mentioned all experiments were performed in triplicates.

3.7.3 Analytical methods

After 20 days of incubation, the fermentation broth was filtered through 4 layers of cheese cloth to remove the mycelia. The mycelium was blotted on a filter paper to depose excess of medium and weighed for fresh weight (FW) estimation. For the extraction of taxol, the mycelium was dried at 30-35ºC (until a constant weight was obtained), crushed in liquid nitrogen and extracted thrice with methanol. The methanolic fraction was reconstituted with equal volume of distilled water and portioned with dichloromethane (DCM). The fermentation broth was also extracted with three equal volumes of DCM. The DCM fractions were carefully pooled and evaporated under vacuum at 35ºC. The residue was taken up in HPLC grade methanol. The amount of taxol present in each sample was estimated by using HPLC (Waters, USA) equipped with a reverse-phase C_{18} column (column temperature maintained at 35ºC) as stationary phase, while methanol:acetonitrile:water (20:40:40 v/v) was

employed as the mobile phase with a flow rate of 1 ml/min and run time of 30 min. The absorbance of taxol was measured at 232 nm by a diode array detector (DAD).

3.8 Statistical Analysis

The data were expressed as mean values and standard deviation (SD). The data were analyzed by analysis of variance (ANOVA) and the means were compared with Tukey's test at $P < 0.05$. All the analyses were performed by using Graph Pad Prism 5.1 software (GraphPad software, Inc. USA).

Chapter 4

Results

4.1 Isolation and characterization of taxol producing endophytic fungi

4.1.1 Isolation of endophytic fungi

Bark samples of *Taxus baccata* L. subsp. *wallichiana* (Zucc.) collected from various sites of Indian Himalayan region (IHR) (Fig. 4.1a, b) were carefully packed and transported to laboratory. The bark pieces before inoculating on PDA were surface disinfected and the plates were sealed and incubated at 28ºC (Fig. 4.2). Pure fungal cultures obtained after several sub-cultures were coded, maintained by sub-culturing at regular intervals and stored in sterile dd H_2O as agar plugs for long term preservation (Fig. 4.3). In total, 60 endophytic fungi with different morphotypes harbouring in the bark of *Taxus baccata* subsp. *wallichiana* (Himalayan yew) were isolated.

4.1.2 Molecular screening (Primary screening of taxol-producing endophytic fungi)

4.1.2.1 Screening for taxol-producing endophytic fungi using *dbat* and *bapt* specific primers

Genomic DNA of 60 isolated endophytic fungi was extracted from fungal mycelium using the CTAB method. Purified fungal DNA of all endophytes were quantified using a nanodrop and were accordingly diluted to get a final template concentration of 50-100 ng/µl. All the fungal morphotypes were screened for the presence of *dbat* and *bapt* genes. Firstly endophytes were screened for the presence of *dbat* gene. Eight out of the sixty fungi showed positive for *dbat* gene amplification with approximately 200 bp amplified fragments of this gene (Fig. 4.4 a). These eight endophytes were further screened for the presence of *bapt* gene.

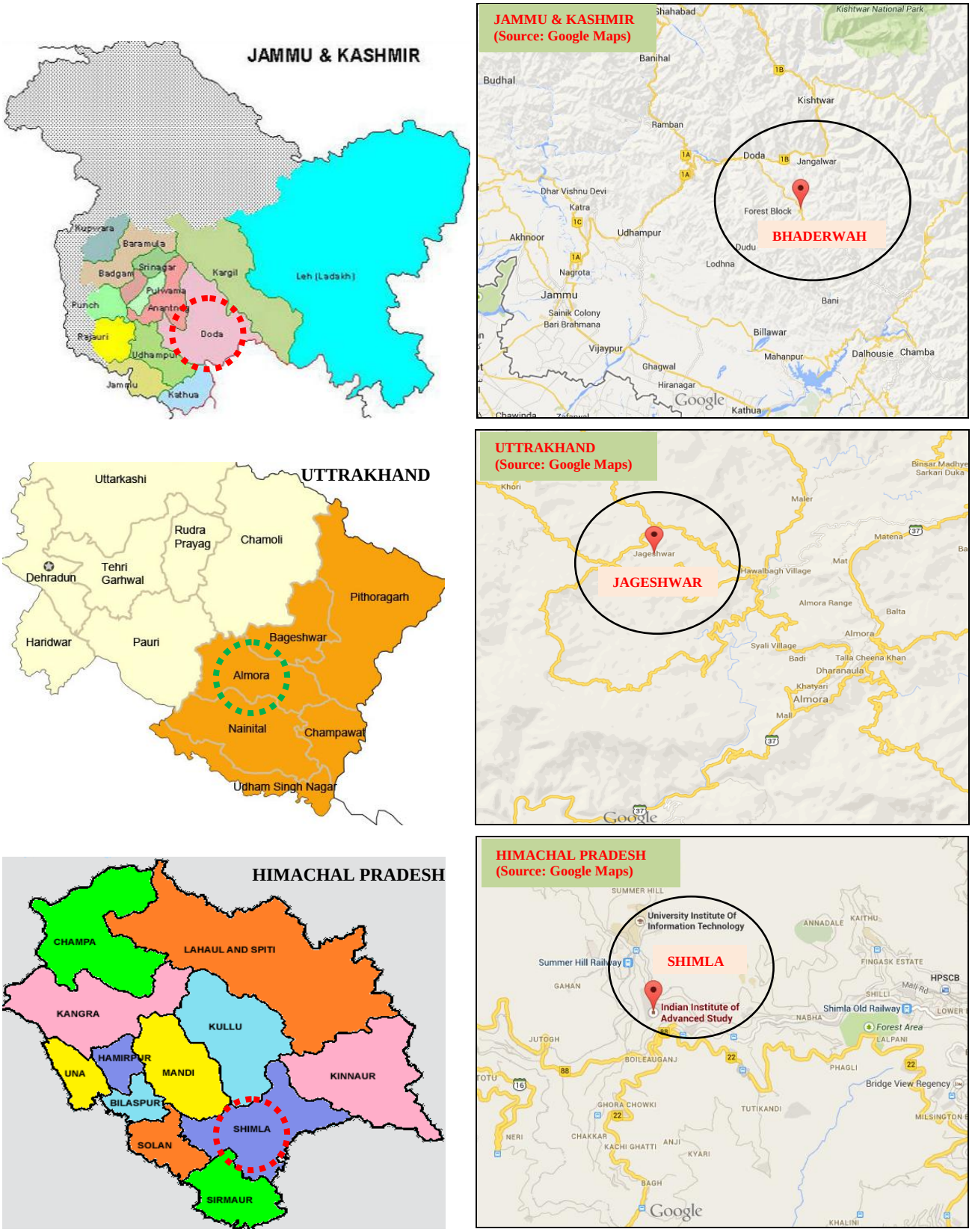


Fig. 4.1a *Taxus baccata* bark sample collection sites (Source: Google images and Google maps)

Fig. 4.1b *Taxus baccata* bark samples collected from various sites of northern Himalayan region

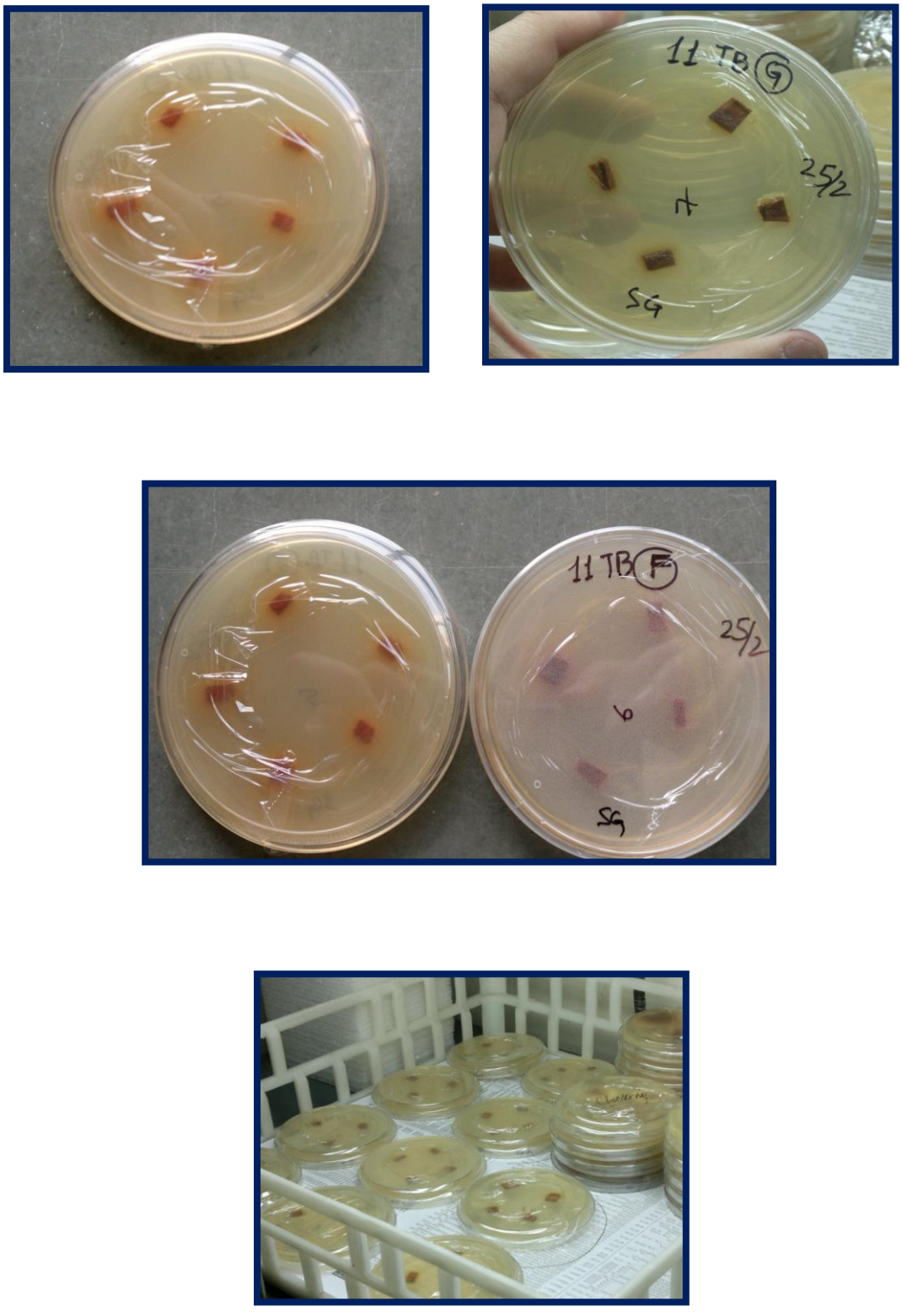


Fig. 4.2 Inoculation of surface sterilized bark pieces on potato dextrose agar (PDA)

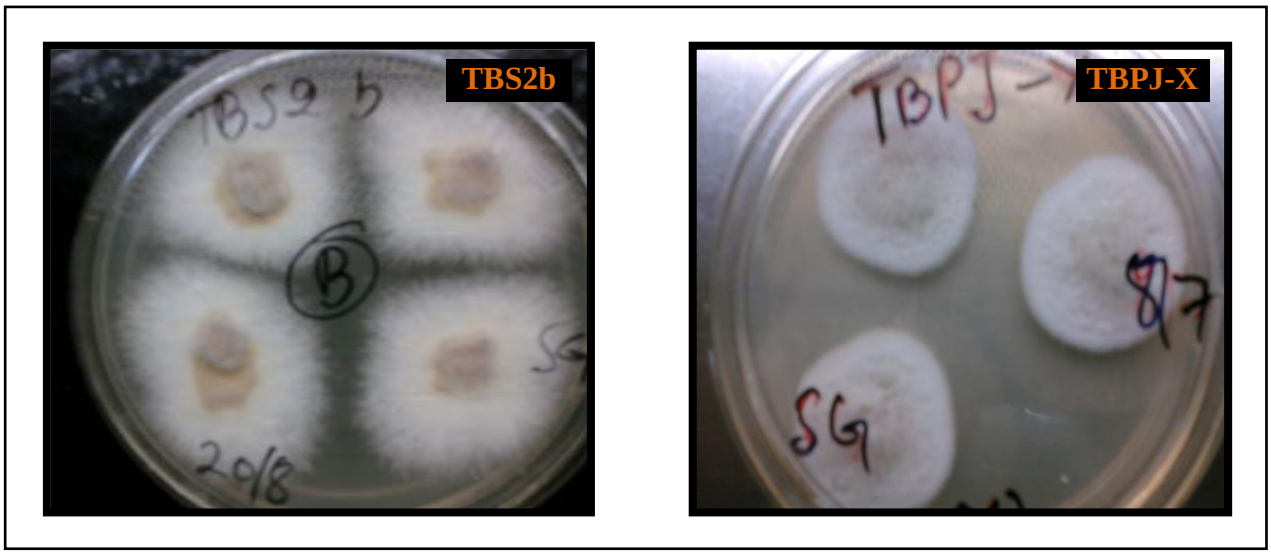
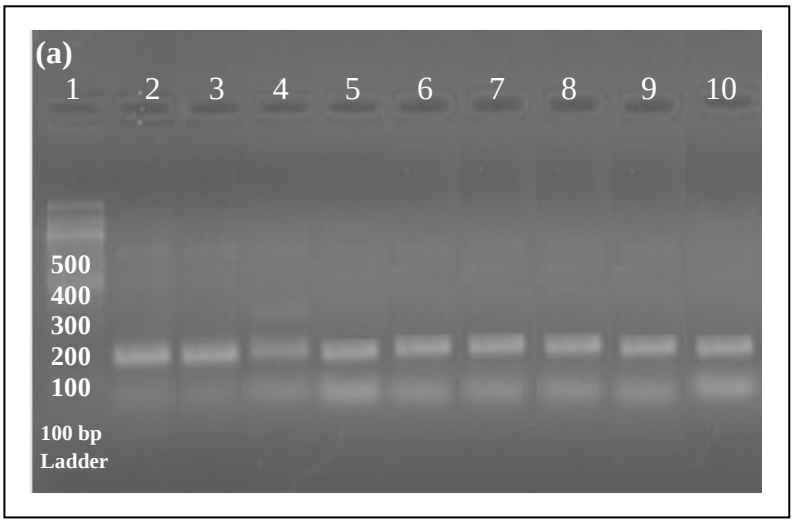
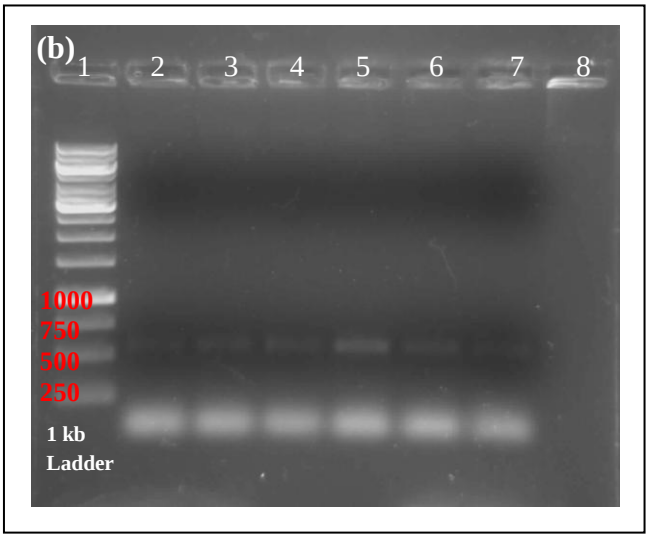


Fig. 4.3 Isolated pure cultures of endophytic fungi

Out of 8 endophytes screened, five gave positive result for *bapt* gene amplification of about 500 bp and these isolates were designated as TBPJ-B, TBPJ-A, TBPJ-13, B-7 and C-1 (Fig. 4.4 b). For the above mentioned molecular screening, genomic DNA was used as template and out of 60 endophytic fungi 8 isolates were harbouring *dbat* gene and 5 isolates harboured *bapt* gene. But, it is a well known fact that introns (intervening sequences) are present in genomic DNA of eukaryotes. So to clarify that the amplification results obtained with genomic DNA is reproducible and also those fungal isolates which gave negative results for *dbat* and *bapt* gene presence is not because of the interference of introns, complimentary DNA (cDNA) for all 60 endophytes was synthesized using ribonucleic acid (RNA) isolated from the same. cDNA of all endophytes was subjected for *dbat* and *bapt* gene screening. The results obtained from screening of cDNA coincided with that of genomic DNA. So, in primary screening of isolated endophytes for the selection of promising isolates harbouring genes involved in taxol biosynthetic pathway, five fungi gave positive hits for both *dbat* and *bapt* genes.



Lane: (1) Ladder 100 bp; (2) *Taxus baccata* DNA; (3) TBPJ-X; (4) TBPJ-6; (5) TT-21a; (6) TBPJ-B; (7) TBPJ-A; (8) TBPJ-13; (9) B-7; (10) C-1

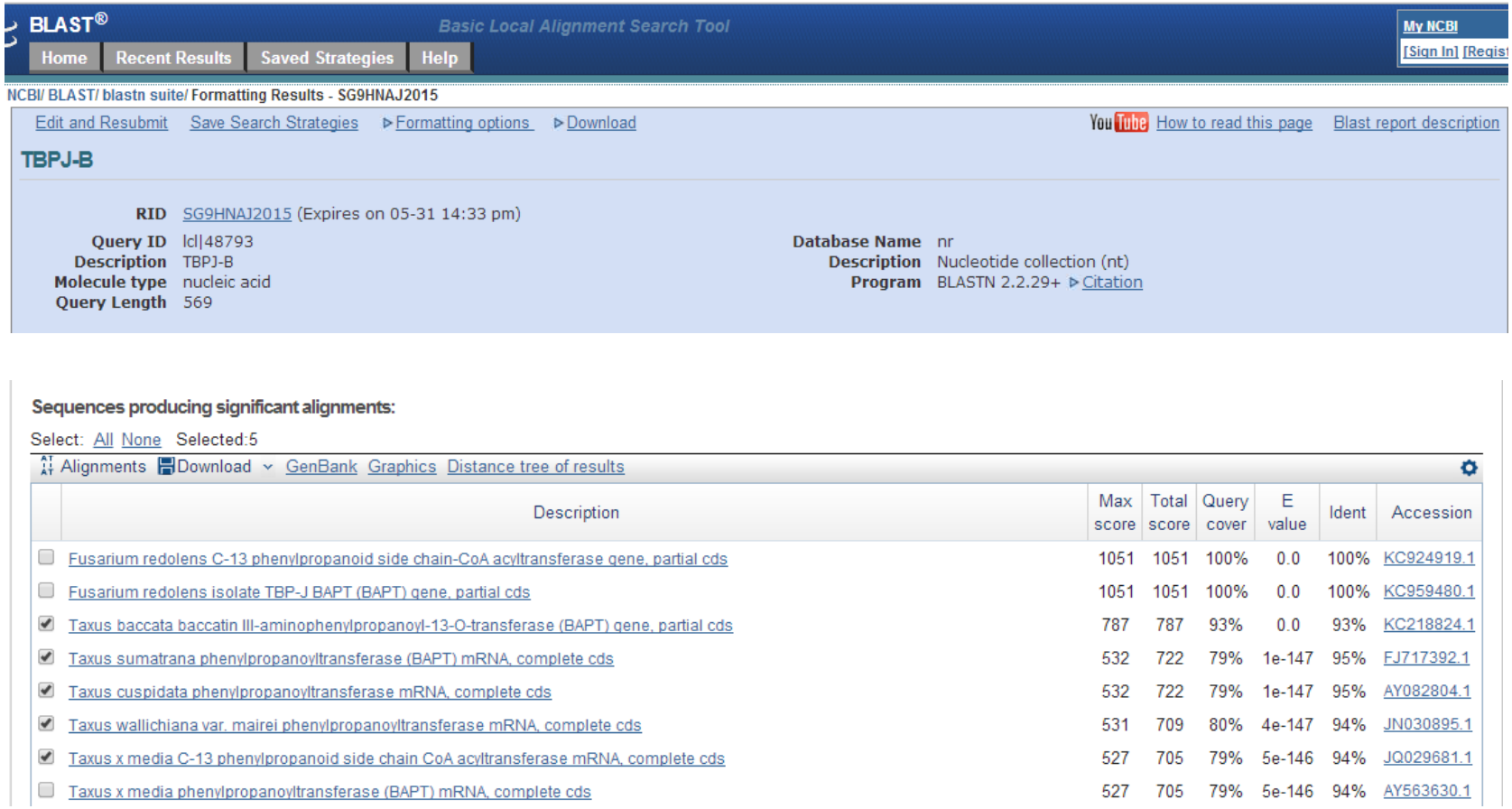


Lane: (1) Ladder 1kb; (2) *Taxus baccata* DNA; (3) B-7; (4) TBPJ-A; (5) TBPJ-B; (6) TBPJ-13; (7) C-1; (8) TT-21a

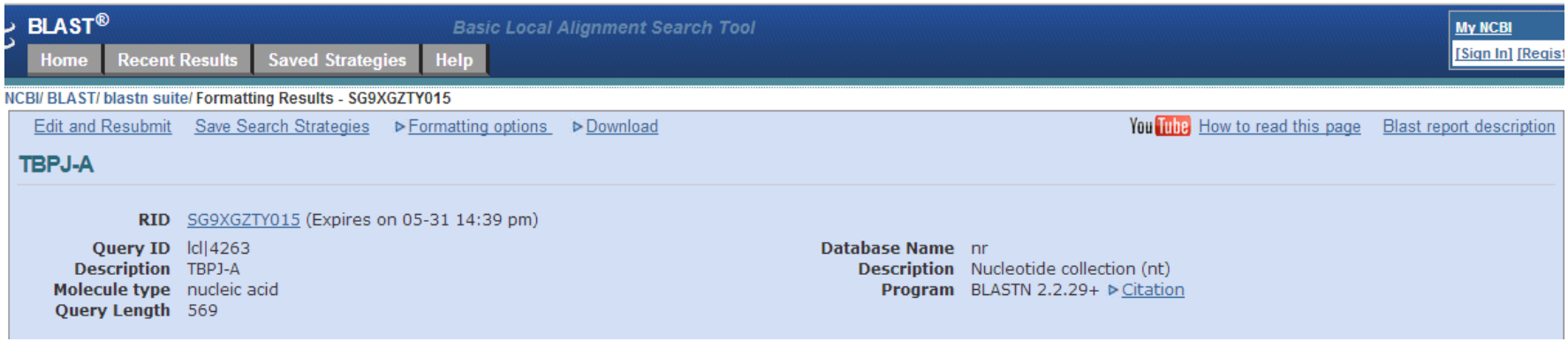
Fig. 4.4 Representative PCR analysis for the presence of **(a)** *DBAT* gene amplification and **(b)** *BAPT* gene amplification in fungal isolates

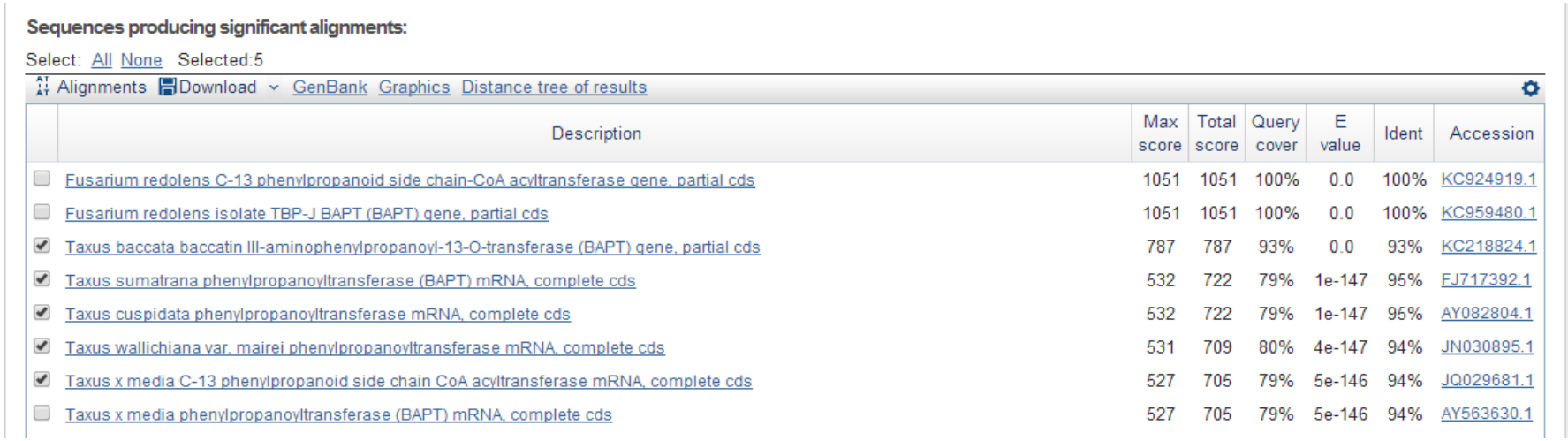
4.1.2.2 Cloning and characterization of *BAPT* gene

After pre-screening for the presence of *BAPT* gene, those endophytes which showed positive PCR amplification results were subjected for *BAPT* gene characterization. The amplified DNA fragments of *bapt* gene from all 5 endophytic isolates were bulk amplified and purified (PCR purification kit, Qiagen). The purified products were ligated into pTZ 57R/T vector (Fermentas, Thermo Fisher Scientific Inc. USA) as per the manufacturer's instructions and transformed into *E. coli* DH5α cells and sequenced. The received sequences were screened for the presence of vector sequences using VecScreen (NCBI) online software and were deleted. *BAPT* gene nucleotide sequence for each fungus sized approximately 500 bp and each sequence was individually analysed for sequence identity in GenBank database using blastx (NCBI) search program. Each fungal gene sequence showed similarity with existing *bapt* gene sequences of various *Taxus* species in NCBI database (Fig. 4.5 A-E). The partial protein sequences of *bapt* gene of all five endophytes along with the protein sequences of *bapt* gene of various related *Taxus* species were edited (using BioEdit) and aligned using Clustal W software (Larkin et al. 2007). The Clustal W alignment results depicted in figure 4.6, clearly showed high homology between protein sequences of fungal and plant (*Taxus* species) *bapt* gene. These results confirmed the presence of taxol biosynthetic machinery in all the five endophytic fungal strains. The partial protein sequences of *bapt* gene of all 5 endophytic strains were deposited in the GenBank of NCBI data library under the accession numbers: KC924919 for TBPJ-B, KF010845 for TBPJ-A, KF010844 for TBPJ-13, KF010842 for B-7 and KF010843 for C-1 (Table 4.6).



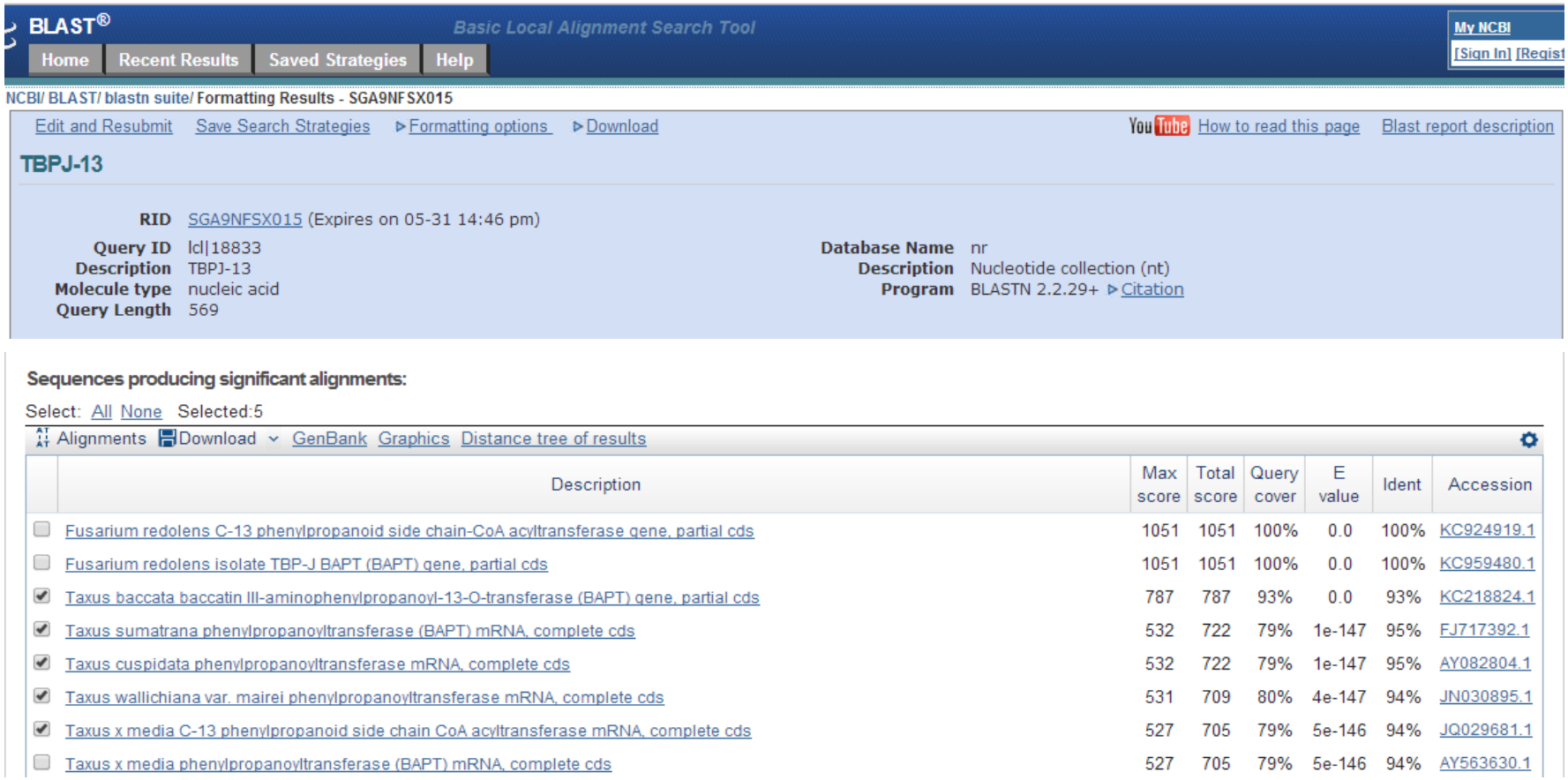
(A) Blastx result for TBPJ-B



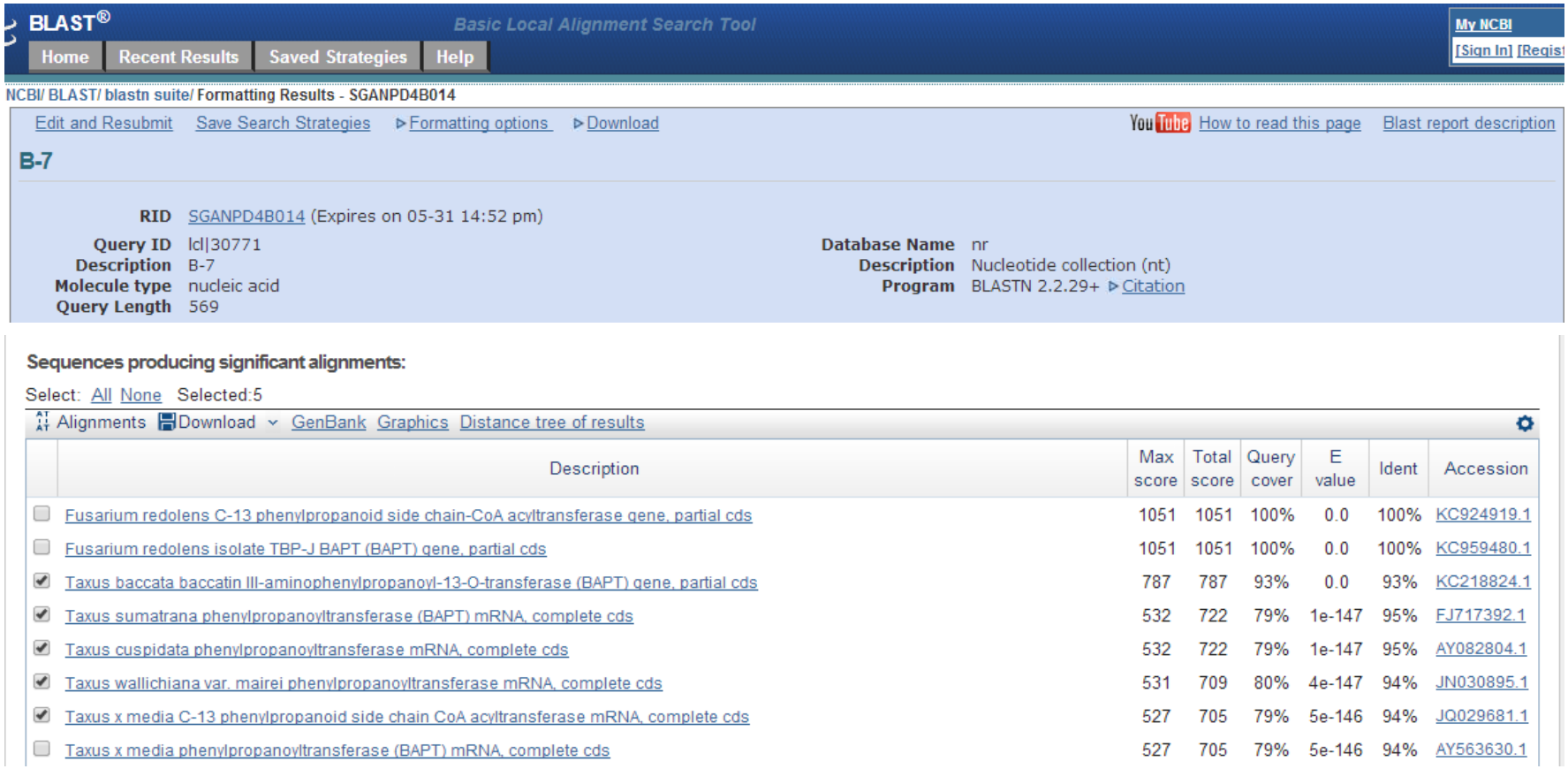


Description	Max score	Total score	Query cover	E value	Ident	Accession
Fusarium redolens C-13 phenylpropanoid side chain-CoA acyltransferase gene, partial cds	1051	1051	100%	0.0	100%	KC924919.1
Fusarium redolens isolate TBP-J BAPT (BAPT) gene, partial cds	1051	1051	100%	0.0	100%	KC959480.1
Taxus baccata baccatin III-aminophenylpropanoyl-13-O-transferase (BAPT) gene, partial cds	787	787	93%	0.0	93%	KC218824.1
Taxus sumatrana phenylpropanoyltransferase (BAPT) mRNA, complete cds	532	722	79%	1e-147	95%	FJ717392.1
Taxus cuspidata phenylpropanoyltransferase mRNA, complete cds	532	722	79%	1e-147	95%	AY082804.1
Taxus wallichiana var. mairei phenylpropanoyltransferase mRNA, complete cds	531	709	80%	4e-147	94%	JN030895.1
Taxus x media C-13 phenylpropanoid side chain CoA acyltransferase mRNA, complete cds	527	705	79%	5e-146	94%	JQ029681.1
Taxus x media phenylpropanoyltransferase (BAPT) mRNA, complete cds	527	705	79%	5e-146	94%	AY563630.1

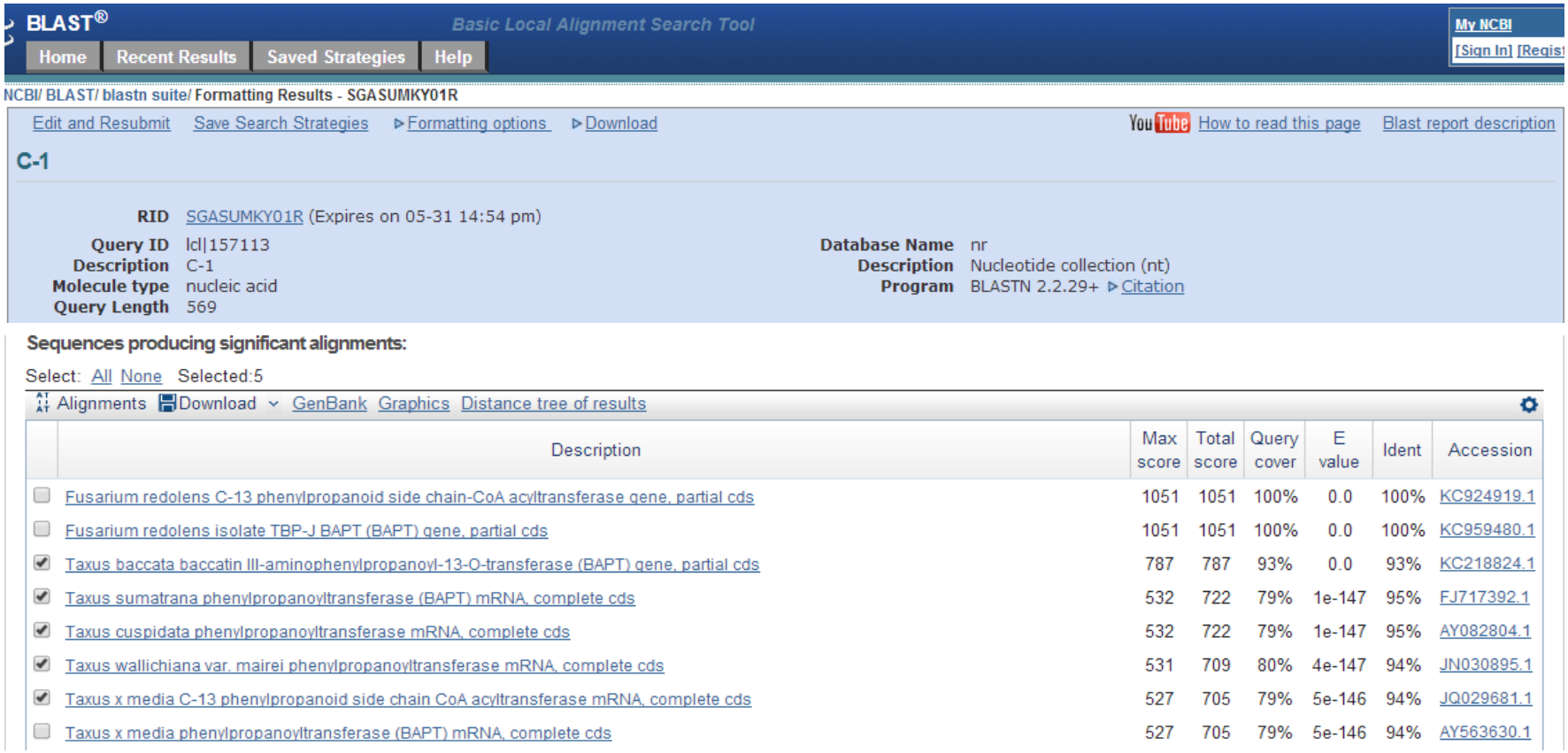
(B) Blastx result for TBPJ-A



(C) Blastx result for TBPJ-13



(D) Blastx result for B-7



Description	Max score	Total score	Query cover	E value	Ident	Accession
Fusarium redolens C-13 phenylpropanoid side chain-CoA acyltransferase gene, partial cds	1051	1051	100%	0.0	100%	KC924919.1
Fusarium redolens isolate TBP-J BAPT (BAPT) gene, partial cds	1051	1051	100%	0.0	100%	KC959480.1
Taxus baccata baccatin III-aminophenylpropanoyl-13-O-transferase (BAPT) gene, partial cds	787	787	93%	0.0	93%	KC218824.1
Taxus sumatrana phenylpropanoyltransferase (BAPT) mRNA, complete cds	532	722	79%	1e-147	95%	FJ717392.1
Taxus cuspidata phenylpropanoyltransferase mRNA, complete cds	532	722	79%	1e-147	95%	AY082804.1
Taxus wallichiana var. mairei phenylpropanoyltransferase mRNA, complete cds	531	709	80%	4e-147	94%	JN030895.1
Taxus x media C-13 phenylpropanoid side chain CoA acyltransferase mRNA, complete cds	527	705	79%	5e-146	94%	JQ029681.1
Taxus x media phenylpropanoyltransferase (BAPT) mRNA, complete cds	527	705	79%	5e-146	94%	AY563630.1

(E) Blastx result for C-1

Fig. 4.5 A-E Screen shots: *BAPT* gene sequence identity with the existing sequences in GenBank DNA database for 5 endophytic fungi

Fig 4.6 Multiple sequence alignment of *bapt* protein of various *Taxus* species aligned with *bapt* protein of different endophytic fungi. NCBI accession numbers of *bapt* gene of fungal isolates are as follows: *Gibberella avenacea* (KF010843), *Paraconiothyrium brasiliense* (KF010844), *Fusarium tricinctum* (KF010842), *Microdiplodia* sp. (KF010845) and *Fusarium redolens* (KC924919)

4.1.3 Molecular phylogenetics

4.1.3.1 PCR amplification of ITS region

Genomic DNA of the 5 endophytic isolates was used for PCR amplification of ITS region of the nrDNA using universal primers (ITS1 and ITS4). After completion of PCR cycles the resultant PCR products were subjected to agarose gel electrophoresis and amplicons of size ranging from 500-750 bp (approx.) were observed under UV transilluminator (Fig. 4.7). ITS products of all 5 endophytes were bulk amplified and purified using a PCR purification kit (Qiagen, Inc. USA). The purified ITS amplicons were ligated into pTZ 57R/T vector, transformed into *E. coli* DH5α cells and were sequenced. ITS sequences of the five endophytic fungi were found to be of lengths: 567 bp for TBPJ-B, 752 bp for TBPJ-A, 643 bp for TBPJ-13, 517 bp for B-7 and 590 bp for C-1 (Appendix II). Individually ITS sequences were searched for sequence identity in GenBank DNA database using BlastN (NCBI) search program (Altschul et al. 1997). The Blast results revealed that the ITS sequences of all 5 endophytic fungi had 99-100% similarity with the existing sequences of NCBI database (Table 4.1). TBPJ-B showed similarity with *Fusarium redolens* strain Ppf2 (EF495234) with 99% percentage identity (Fig. 4.8). Similarly, TBPJ-A showed similarity with *Microdiplodia* sp. G16A (EF432267) with 100% percentage identity, TBPJ-13 had first hit with *Paraconiothyrium brasiliense* isolate F01 (JF439492) with 100% identity, B-7 had first hit with *Fusarium tricinctum* isolate 6477 (JQ846085) with 100% identity and C-1 with *Gibberella avenacea* isolate FA08 (EU255798) with 99% percentage identity (Fig. 4.8). The ITS sequences of all 5 endophytic strains were deposited in the GenBank of NCBI data library under the accession numbers: KC924920 for TBPJ-B, KF010841 for TBPJ-A, KF010840 for TBPJ-13, KF010839 for B-7 and KF010838 for C-1 (Table 4.2).

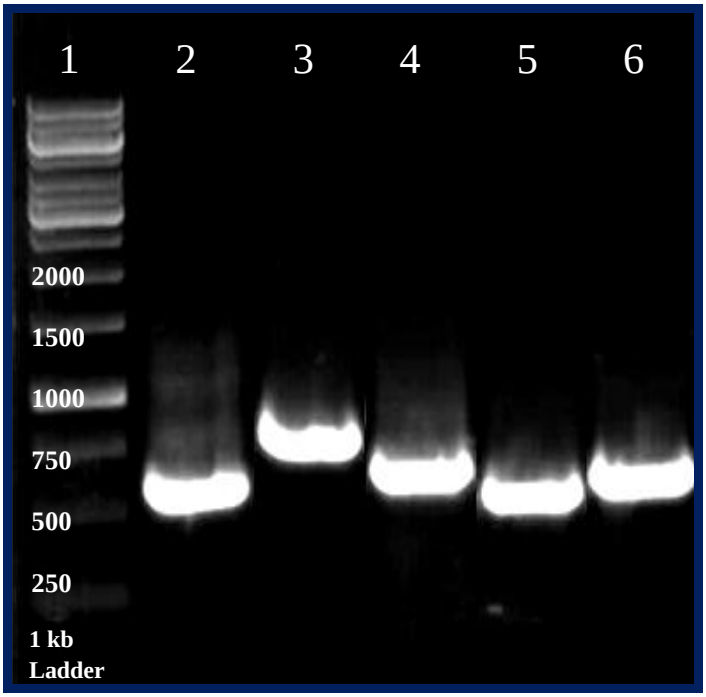
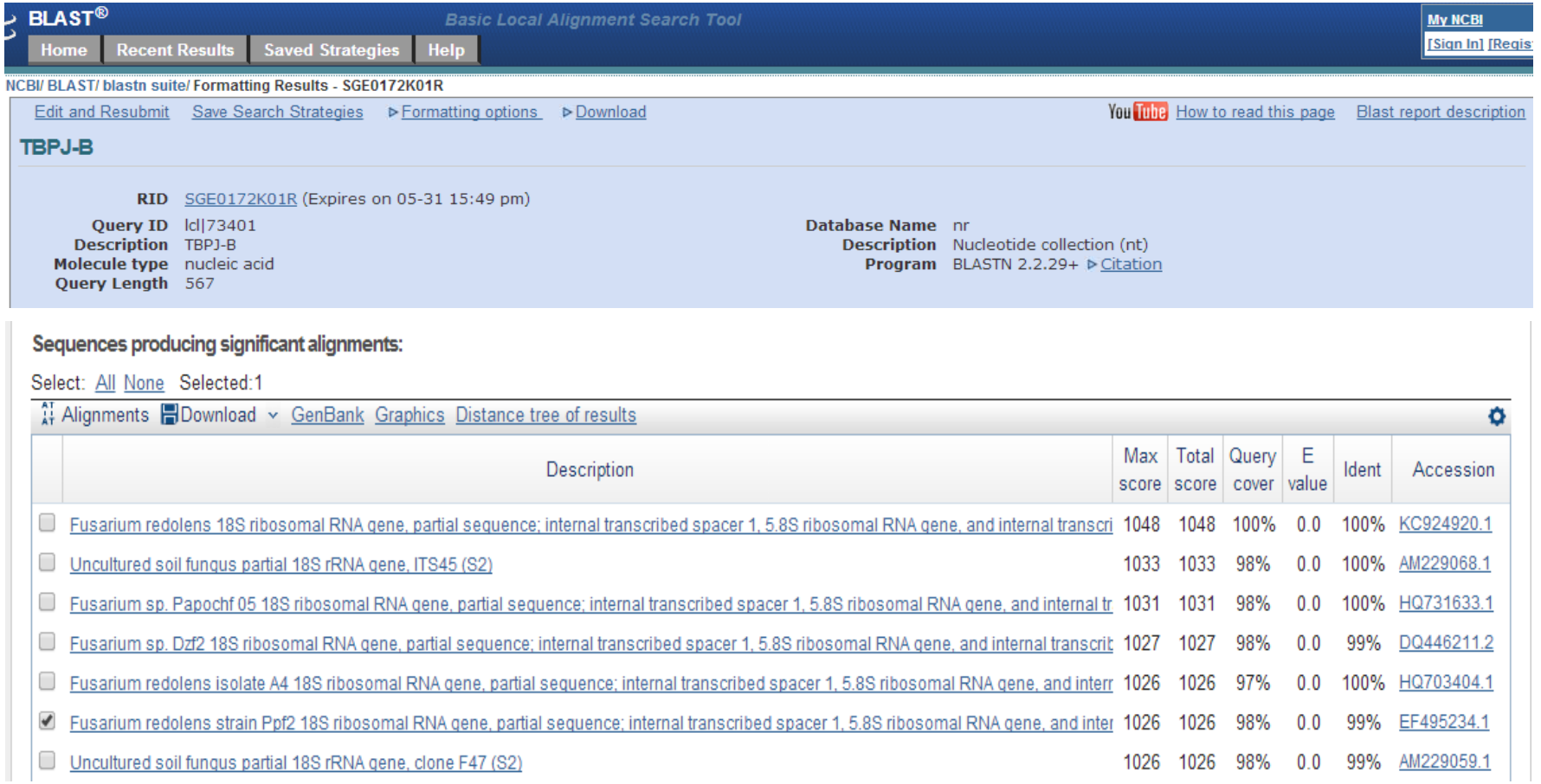


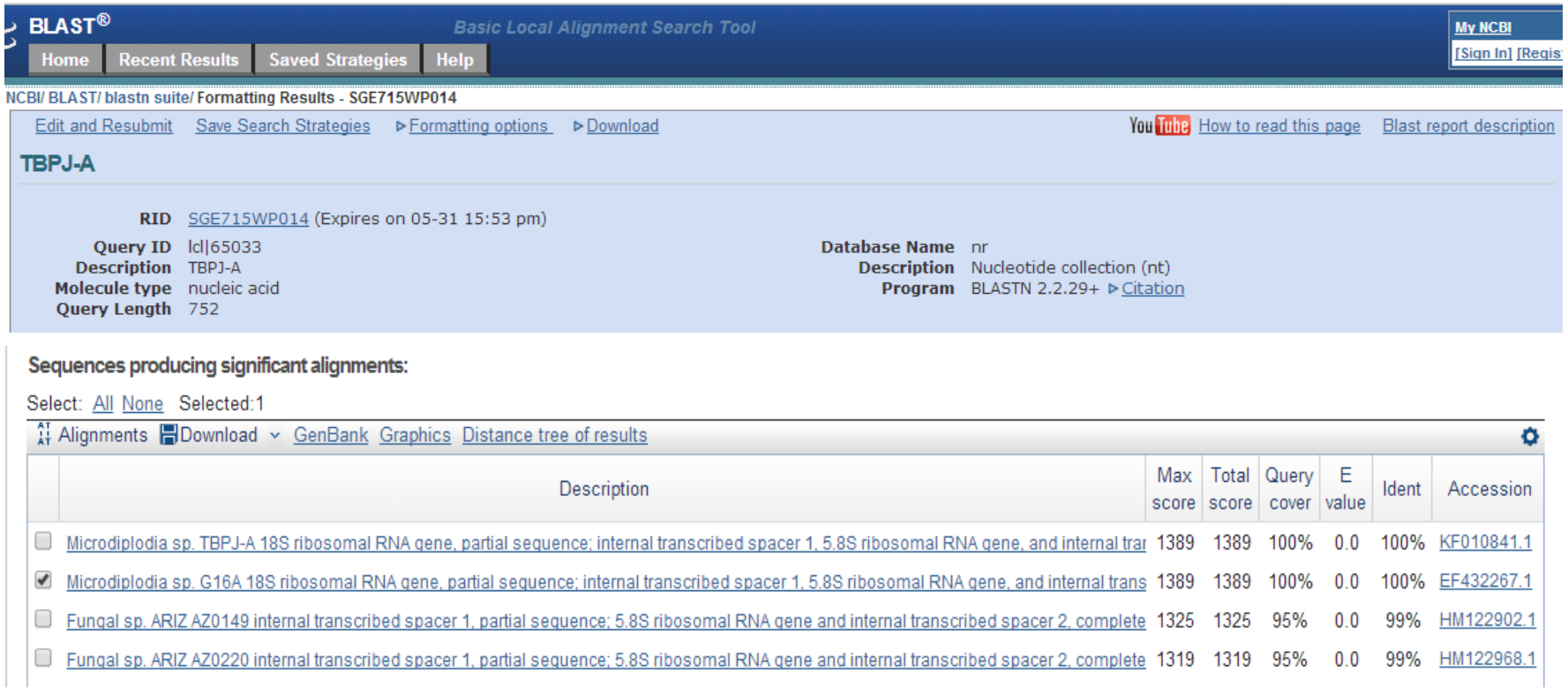
Fig. 4.7 ITS amplification of different fungal isolates of the present study. **Lane (1)** Ladder (1 kb); **(2)** TBPJ-B; **(3)** TBPJ-A; **(4)** TBPJ-13; **(5)** B-7; (6) C-1

Table 4.1 Analysis and putative taxonomic affinities of fungal endophytes isolated from *Taxus baccata* (Himalayan yew)

Strain and site of isolation	Length of sequenced ITS region (bp)	Molecular identification	
		Blast and first hit	% identity
TBPJ-B **(Bhaderwah, J & K)**	567	*Fusarium redolens* stain Ppf2 (EF495234)	99%
TBPJ-A **(Almora, Uttrakhand)**	752	*Microdiplodia* sp. G16A (EF432267)	100%
TBPJ-13 **(Shimla, H.P.)**	643	*Paraconiothyrium brasiliense* isolate F01 (JF439492)	100%
B-7 **(Shimla, H.P.)**	517	*Fusarium tricinctum* isolate 6477 (JQ846085)	100%
C-1 **(Almora, Uttrakhand)**	590	*Gibberella avenacea* isolate FA08 (EU255798)	99%



(A) BlastN result for TBPJ-B



BLAST® Basic Local Alignment Search Tool

Home | Recent Results | Saved Strategies | Help

My NCBI [Sign In] [Regis

NCBI/ BLAST/ blastn suite/ Formatting Results - SGE715WP014

Edit and Resubmit | Save Search Strategies | ▷Formatting options | ▷Download | YouTube How to read this page | Blast report description

TBPJ-A

RID SGE715WP014 (Expires on 05-31 15:53 pm)

Query ID lcl|65033
Description TBPJ-A
Molecule type nucleic acid
Query Length 752

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.2.29+ ▷Citation

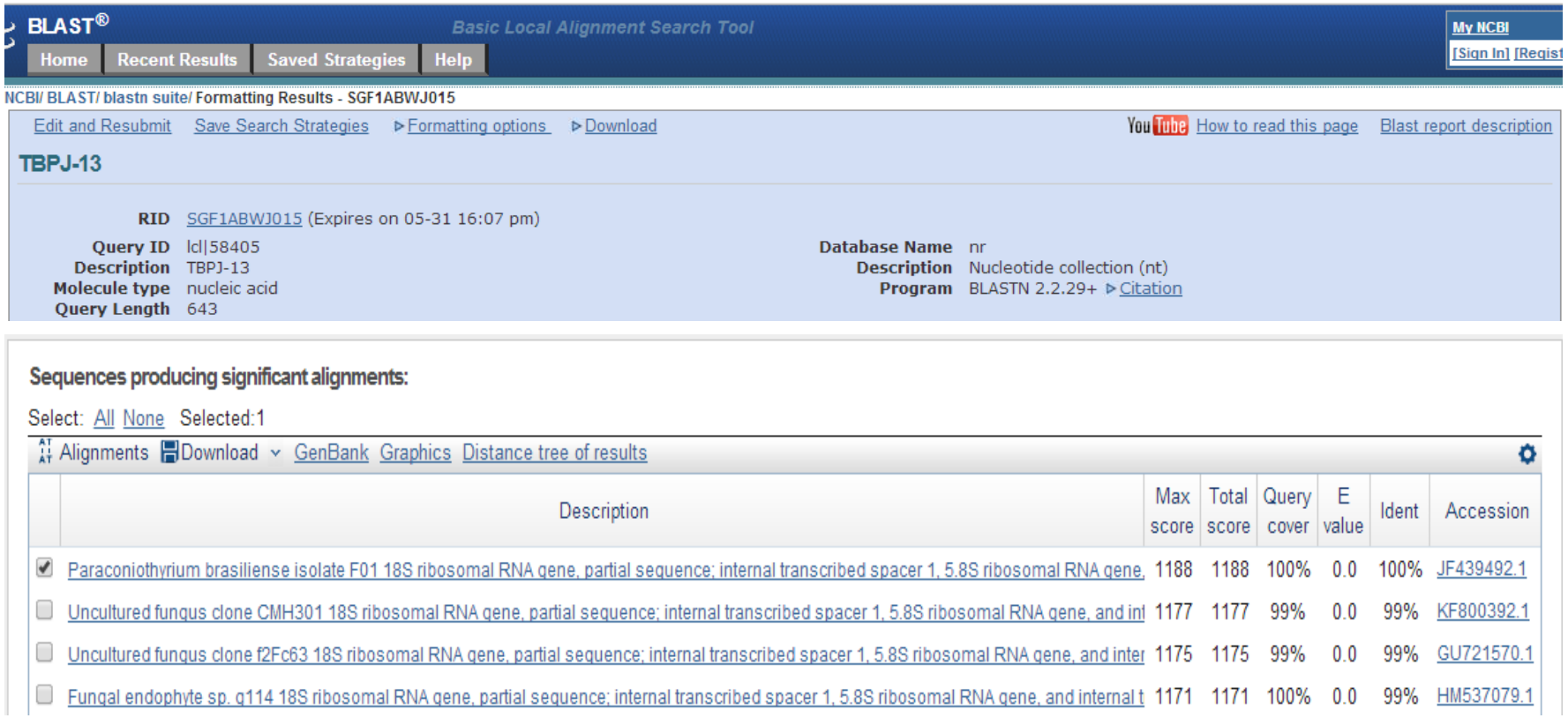
Sequences producing significant alignments:

Select: All None Selected:1

Alignments | Download | GenBank | Graphics | Distance tree of results

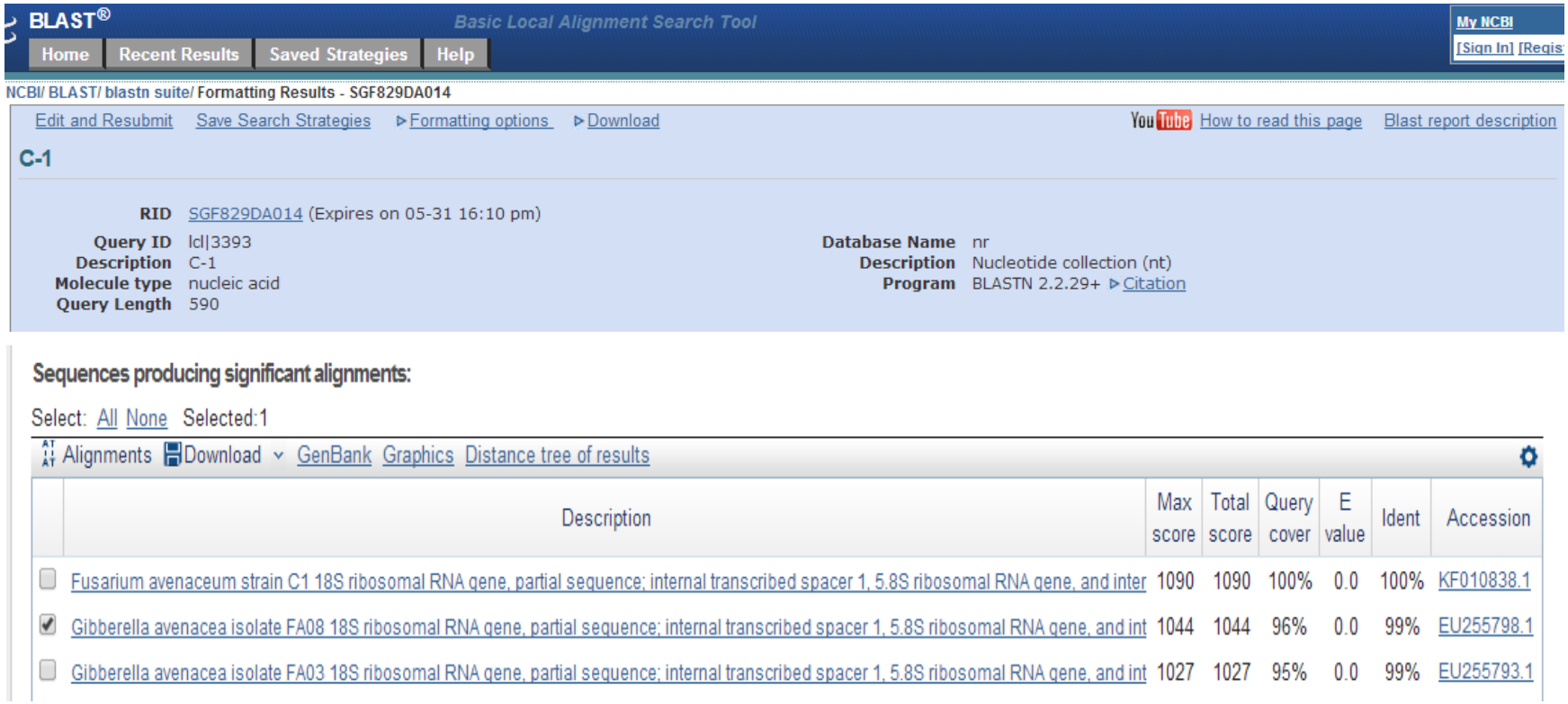
	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Microdiplodia sp. TBPJ-A 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal trar	1389	1389	100%	0.0	100%	KF010841.1
☑	Microdiplodia sp. G16A 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal trans	1389	1389	100%	0.0	100%	EF432267.1
☐	Fungal sp. ARIZ AZ0149 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete	1325	1325	95%	0.0	99%	HM122902.1
☐	Fungal sp. ARIZ AZ0220 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete	1319	1319	95%	0.0	99%	HM122968.1

(B) BlastN result for TBPJ-A



(C) BlastN result for TBPJ-13

(D) BlastN result for B-7



(E) BlastN result for C-1

Fig. 4.8 A-E Screen shots: ITS sequence identity with the existing sequences in GenBank DNA database for 5 endophytic fungi

4.1.3.2 Phylogenetic analyses

The ITS sequence for each of the 5 endophytic fungi was individually compared with those available in GenBank database using Blast search program and sequences of closely related fungal strains retrieved from the database were edited (BioEdit software) and aligned (Clustal W software) before the construction of phylogenetic tree using molecular evolutionary genetics analysis (MEGA 5) software. The phylogenetic tree reconstructed using ITS sequences of TBPJ-B, TBPJ-A, TBPJ-13, B-7 and C-1 is shown in Fig. 4.9.

Phylogenetic tree constituted of four clades. Strain TBPJ-B formed as clade 1 with a boot-strap value of 99% was clustered with *Fusarium redolens* (KC924920), B-7 was clustered with *Fusarium tricinctum* (KF010839) in clade 2 (boot-strap support 96%) and C-1 with *Gibberella avenacea* (KF010838) in clade 3 (boot-strap support 93%). Similarly, TBPJ-A clustered with *Microdiplodia* sp. G16A (KF010841) with a boot-strap value of 74% and TBPJ-13 with *Paraconiothyrium brasiliense* (KF010840), were clustered in clade 4. Strain TBPJ-13 with a low boot-strap value of 42% shared sequence similarity of 100% with *Paraconiothyrium brasiliense*.

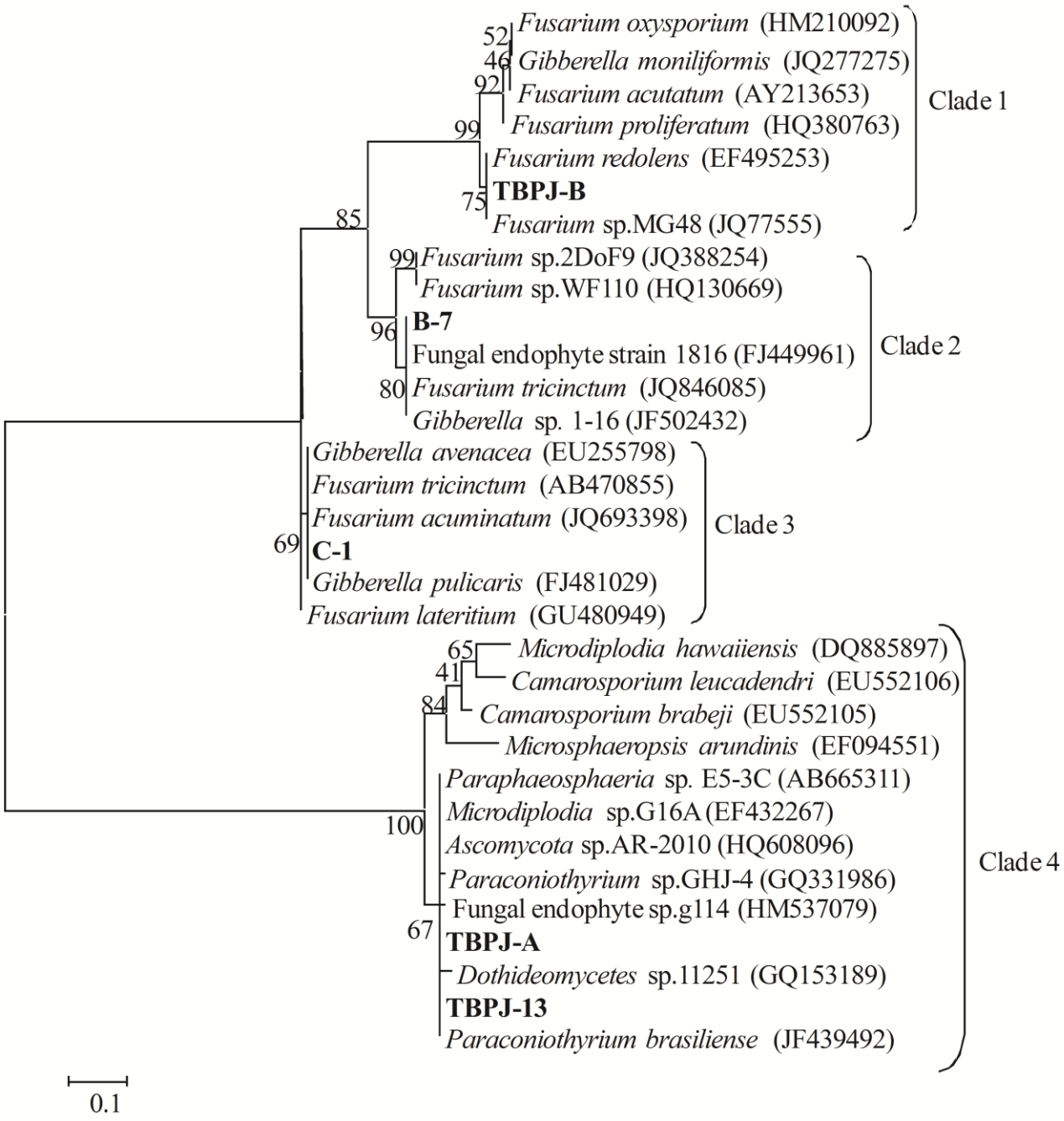


Fig. 4.9 Phylogenetic tree of different endophytic fungi based on ITS nrDNA sequence data using parsimony analysis. Number at nodes are bootstrap scores (above 50%) obtained from 1,000 replications. *Neurospora crassa* is used as an out-group. The scale bar indicates the number of nucleotide substitutions per site

Table 4.2 Accession Numbers of fungal cultures, ITS sequences and partial protein sequences of *bapt* gene

Endophytic Fungal Code	**Location of Bark Collection**	**MTCC Accession No. (Fungal Cultures)**	**NCBI GenBank Accession No. (ITS Sequences)**	**NCBI GenBank Accession No. (Partial protein sequence of *bapt* gene)**
TBPJ-B	Bhaderwah, Jammu & Kashmir	MTCC11742	KC924920	KC924919
TBPJ-A	Almora, Uttrakhand	MTCC11759	KF010841	KF010845
TBPJ-13	Shimla, Himachal Pradesh	MTCC11758	KF010840	KF010844
B-7	Shimla, Himachal Pradesh	MTCC11743	KF010839	KF010842
C-1	Almora, Uttrakhand	MTCC11754	KF010838	KF010843

4.1.4 Fungal morphology

4.1.4.1 Macroscopic characteristics

(a) TBPJ-13 colonies grew on PDA with a radial growth rate of 2.5 cm per week in dark conditions. The endophytic fungus grew giving a pure white, creamish centre from front while creamish dark brown color on the reverse of colony (Fig. 4.10). The fungus grew with raised elevation on media and had wooly appearance.

(b) TBPJ-A colony gave whitish color from front and creamish dark brown centered when viewed from reverse of culture plate (Fig. 4.11). Margins were regular, smooth and submerged, and the radial growth on PDA at 25°C under dark conditions was 3.5 cm per week.

(c) C-1 on PDA under dark conditions produced less abundant white mycelium. The fungus filled the Petri plate completely in 8 days. Colony front appeared dull beige in color while the reverse appeared black with light brownish edges (Fig. 4.12).

(d) Aerial mycelia of TBPJ-B on PDA under dark conditions appeared pink white pigmented were abundant and densely fluffy. Colony reverse appeared pink at centre with orangish towards edges (Fig. 4.13). The culture had some unique fragrance when culture plate was opened.

(e) On PDA media at 25°C under dark conditions, B-7 aerial mycelia produced violet pinkish pigments (Fig. 4.14). The fungus grew with a radial growth rate of 3.5 cm per week, appearing cottony with flat spreading.

The colonial morphology of five fungal endophytes is summarized in Table 4.3.

Table 4.3 Colonial morphology of five fungal endophytes

Sl. No.	Fungus code	Color				Appearance	Growth (on PDA at 25°C)	Elevation	Special characters
		Front	Centre	Edges	Back				
1	**TBPJ-13**	Pure white	Creamish	White	Creamish, dark brown	Round, wooly	5 cm/14 days; slow, compact but dense with broken edges	Raised	-
2	**TBPJ-A**	Whitish	Dull white	Rich white	Creamish, dark brown centered	Cottony, round	7 cm/14 days; moderate, non dense, compact, margins regular and smooth	Flat but slightly raised	Growth is circular like co centric rings with creamish towards centre and gets whitish as moving towards edges
3	**C-1**	Dull beige	Beige	Beige	Black with light brownish edges	Cottony, round	Filled Petri plate completely in 8 days; dense, broken edges	Raised	Fast growing fungi
4	**TBPJ-B**	Pink with whitish tinge	Pinkish	Dull white	Pink centre with orangish towards edges	Cottony, round	8 cm/week; moderate, broken edges	Flat spreading	Culture having some unique fragrance when culture plate is opened
5	**B-7**	Violet, pinkish	White-pinkish	Pinkish	Dark pinkish centre, creamish towards edges	Round, cottony	7 cm/14 days; moderate, non dense, broken edges	Complete flat spreading	Unique pinkish color

4.1.4.2 Microscopic characteristics

1. TBPJ-13

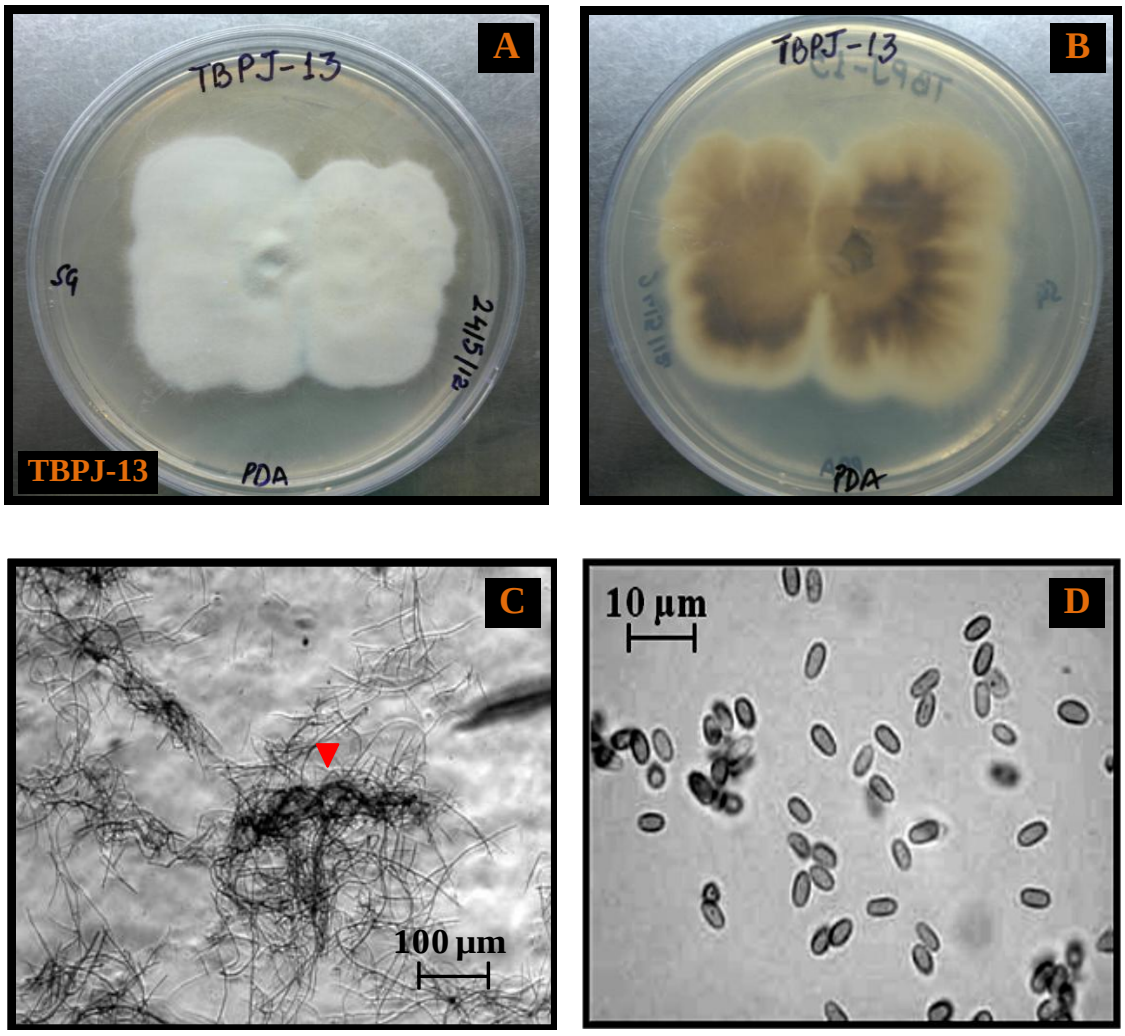


Fig. 4.10 Macroscopic and microscopic characteristics of *Paraconiothyrium brasiliense* (TBPJ-13). **A-B**: colony features on PDA upper and lower surface respectively; **C**: conidiomatal formation; **D**: conidia

Under microscope, conidiomatal initials were evident as small knots of fungal hyphae (Fig. 4.10). Also intertwining of several hyphae was observed which resulted in primodium formation. One-celled, ellipsoid to short-cylindrical conidia were observed. Conidia were rounded at both ends containing minutely granular conidial mass which appeared dark brown to black in color and measured (3.3-5.6) × (2.2-3.6) µm in size.

2. TBPJ-A

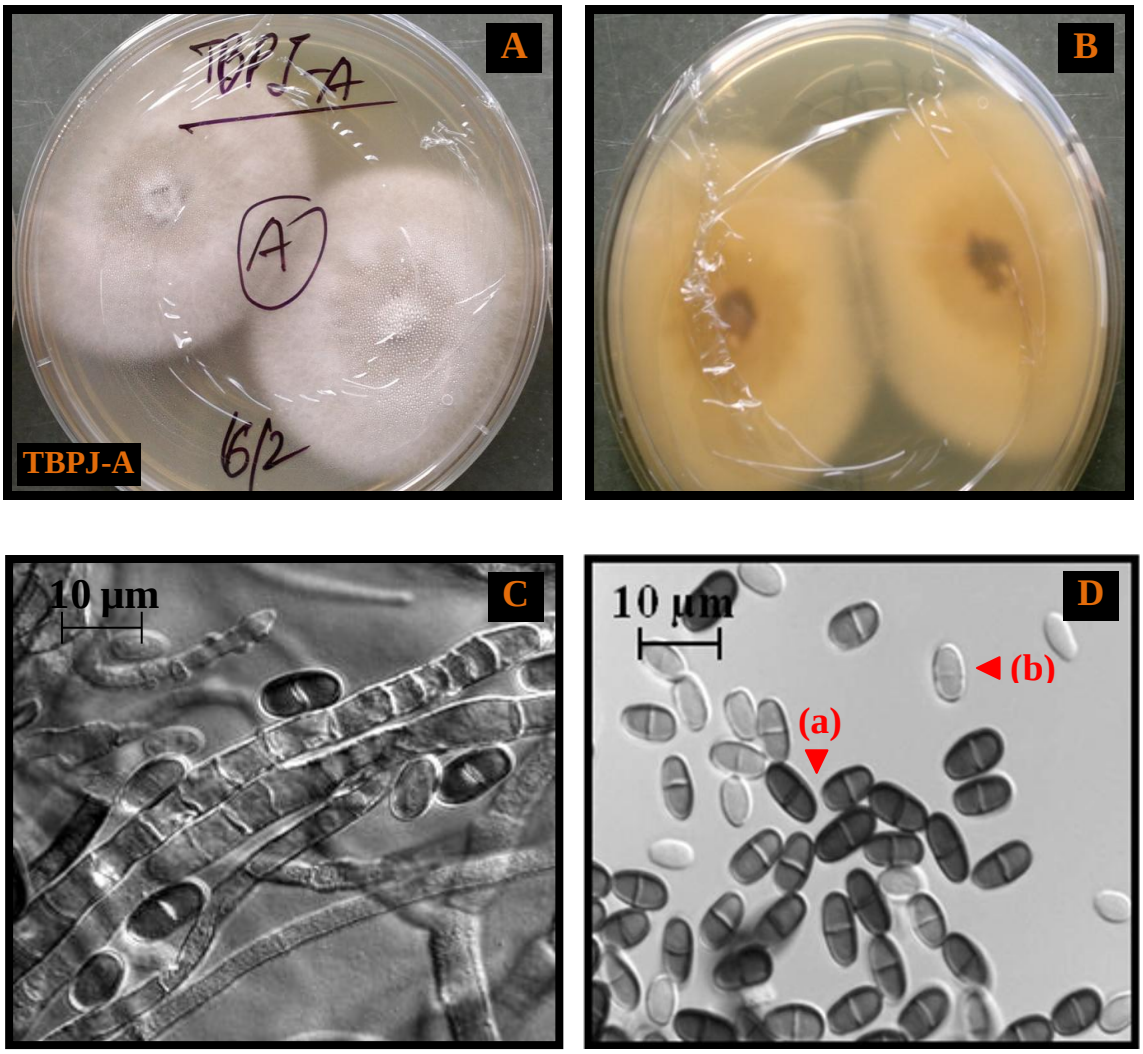


Fig. 4.11 Macroscopic and microscopic characteristics of *Microdiplodia* species (TBPJ-A). **A-B**: colony features on PDA upper and lower surface respectively; **C**: conidiophores; **D**: brown, one septate conidia **(a)** and hyaline, aseptate conidium **(b)**

When viewed under microscope the aerial mycelium was sparse and conidiogenous cells were found lining the inner conidiomatal cavity (Fig. 4.11). Conidiophores of aerial mycelium mostly monophialidic but occasionally polyphialidic in nature were observed. Ellipsoidal to subcylinderical conidia with obtuse apex and obtusely rounded base were present. 1/2 septate thick walled conidia, initially hyaline, smooth becoming golden-brown measured (10-12) × (4-5) µm in size.

3. C-1

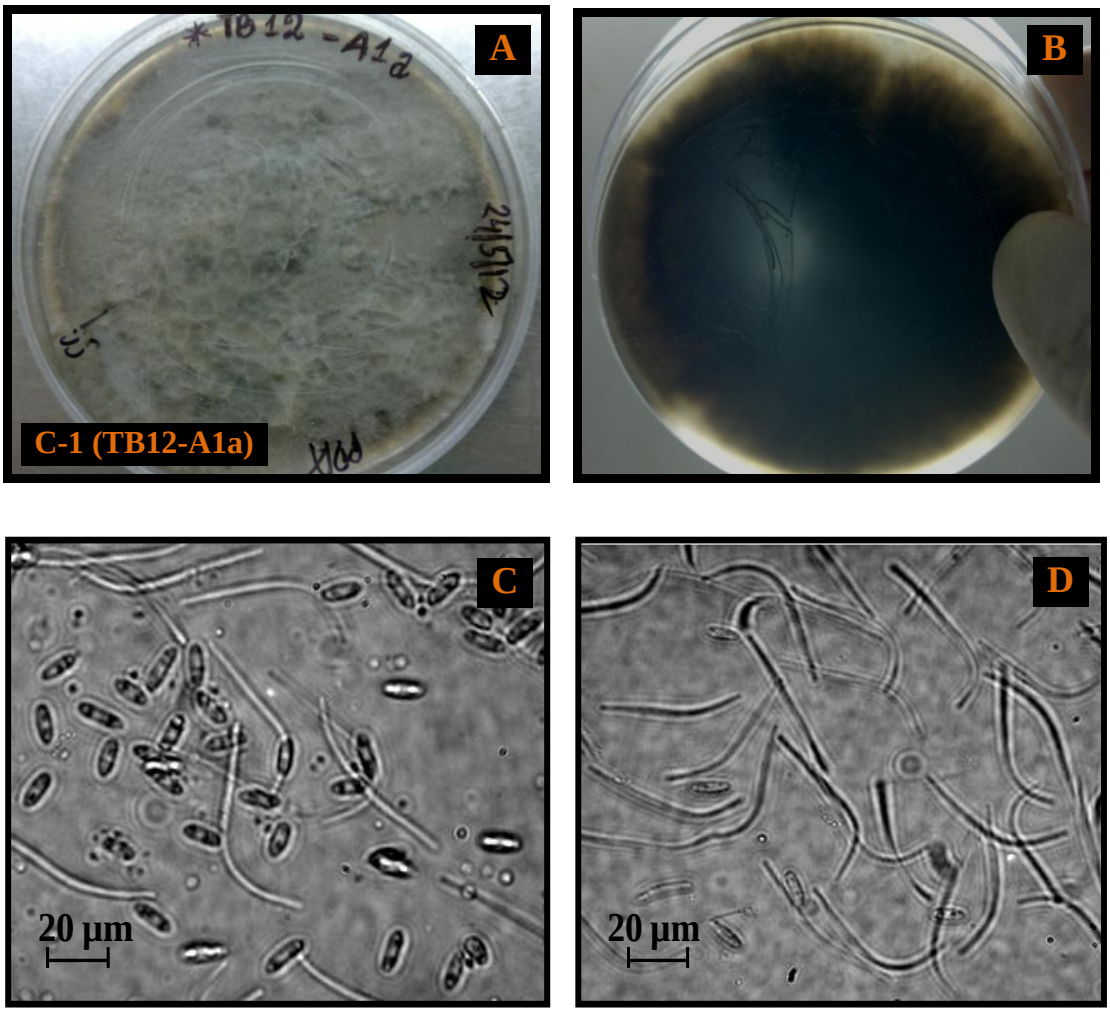


Fig. 4.12 Macroscopic and microscopic characteristics of *Phomopsis* species (C-1). **A-B**: colony features on PDA upper and lower surface respectively; asexual conidia of *Phomopsis* sp. **C**: *α*-conidial spores (elliptical); **D**: hair-like filamentous *β*-conidial spores (needlelike)

Microscopic morphologies indicated presence of colorless, elliptical, single α-spores with 2 oil drops measuring (4.5-7.0) × (2.0-2.8) µm and needle/hair-like, filamentous, colorless β-spores, curved at one end measuring (15-25) × (0.5-1.5) µm in size (Fig. 4.12).

4. TBPJ-B

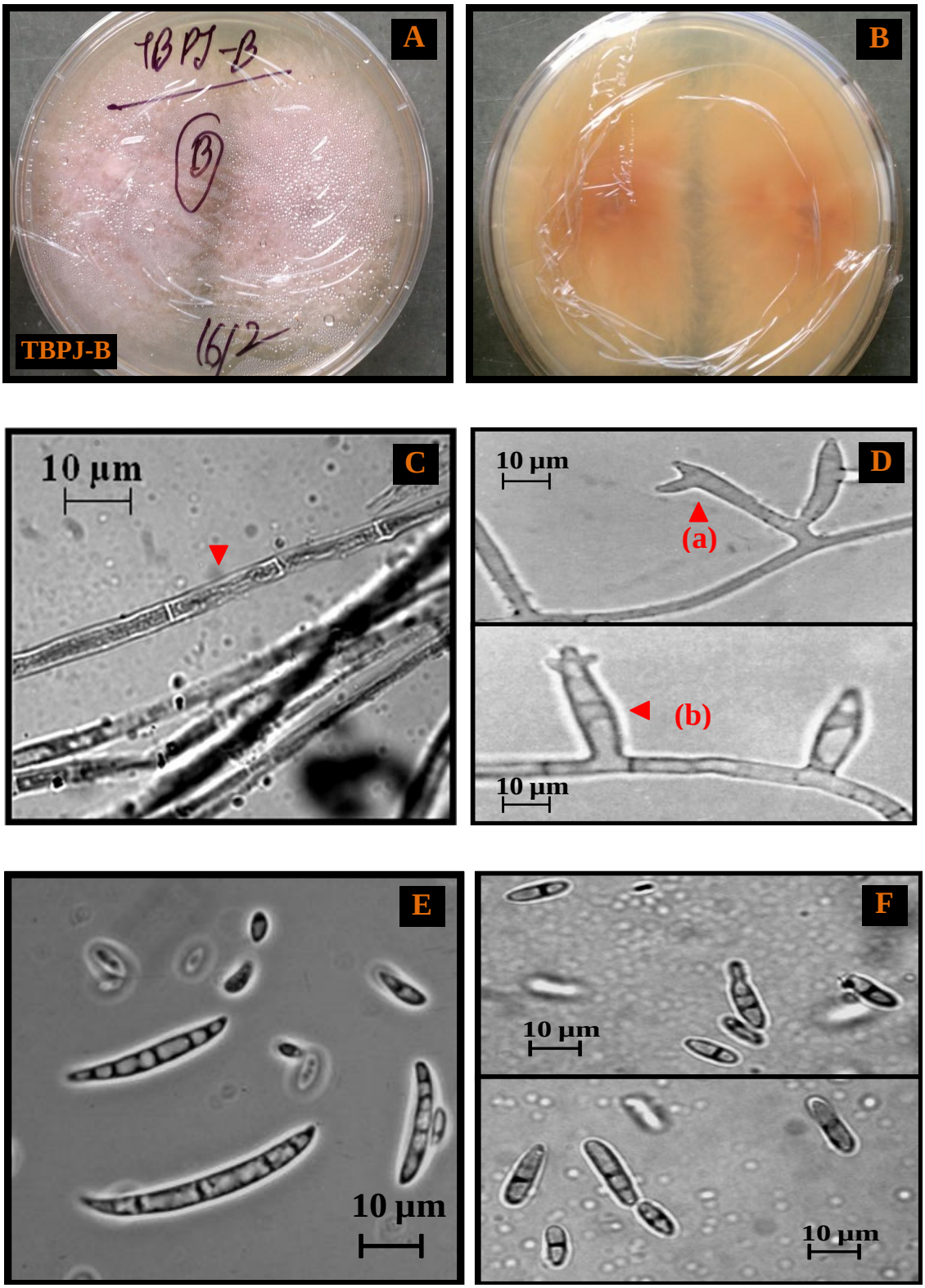


Fig. 4.13 Macroscopic and microscopic characteristics of *Fusarium redolens* (TBPJ-B). **A-B**: colony features on PDA upper and lower surface respectively; **C**: tubular, thick walled, septate hyphae; **D**: polyphialidic conidiophores **(a)** and monophialidic conidiophores **(b)** of the aerial mycelium; **E**: long sporodochial conidia; **F**: microconidia

Microscopic morphologies indicated presence of tubular, thick walled, septate hyphae, simple or branched (Fig. 4.13). Conidiophores consisting of short monophialides up to 15 µm long 4 µm wide and polyphialides up to 32 µm long and 4 µm wide, appeared in the culture incubated in dark. Microconidia were sparse, one celled, smooth, ovoid, septate, present solitary and measured (1.75-2.5) × (1.0-1.5) µm in size. Macroconidia borne on branched conidiophores were abundant, thin walled, typically fusiform with slightly curved apical cell and a foot-shaped basal cell, bending equally towards both ends. Two-septate conidia measured (17.0-29) × (3.5) µm, three-septate conidia measured (22-35) × (4) µm and five-septate ones measured (55-60) × (3.5-4.6) µm in size. Chlamydospores were not present.

5. B-7

Under microscope both macro and micro conidia were observed (Fig. 4.14). Macroconidia produced were long, slender, straight and thin walled measuring about (36.5-50.0) × (3.7-4.0) µm with three-septates. The apical cell of the macroconidia was curved and tapered, and the basal cell was notched. Microconidia were typically single celled, small, hyaline, oval with a flat base containing a single nucleus. Microconidia were present 0-2 septate measuring (3-4) × (2.5- 3.0) µm, and were found in long catenate chains formed from morphologically simple monophialides that occur in V-shape pairs. Phialides bearing the microconidia chains measured (15-29) × (3-4.5) mm in size. No chlamydospores were observed.

Table 4.4 summarizes the microscopic characteristics of fungal isolates of the present study.

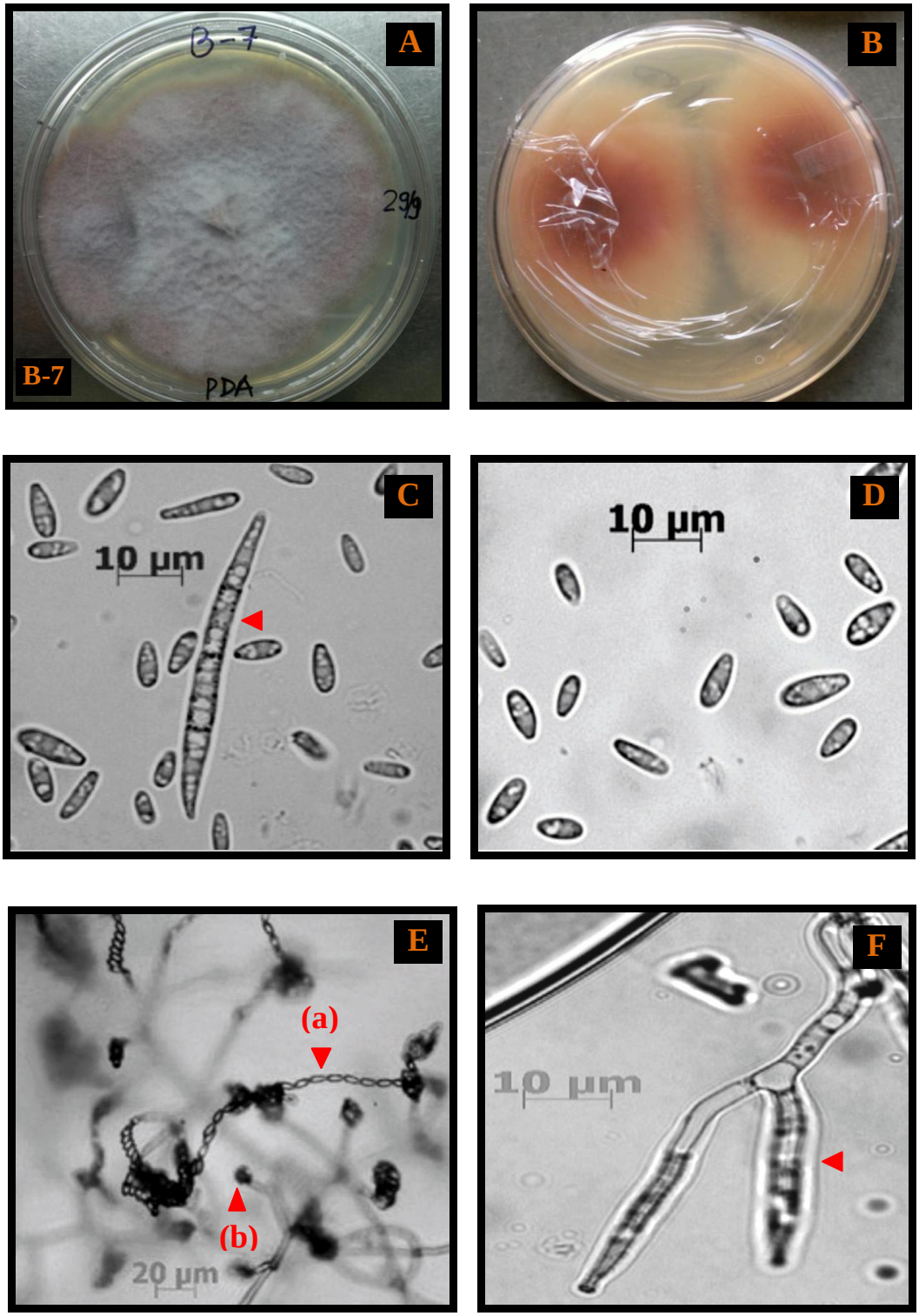


Fig. 4.14 Macroscopic and microscopic characteristics of *Fusarium verticillioides* (B-7). **A-B**: colony features on PDA upper and lower surface respectively; **C**: macroconidia; **D**: microconidia; **E**: microconidia forms long chains **(a)** and false head **(b)**; **F**: branched monophialides

Table 4.4 Microscopic characteristics of different fungal isolates of the present study

Sl. No.	Fungus code	Microscopic characteristics
1	**TBPJ-13**	Conidiomatal initials evident as small knots of fungal hyphae; intertwining of several hyphae resulted in primodium formation; one-celled, ellipsoid to short-cylindrical conidia were observed.
2	**TBPJ-A**	Aerial mycelium were sparse; conidiogenous cells lining the inner conidiomatal cavity; conidiophores mostly monophialidic but occasionally polyphialidic in nature; ellipsoidal to subcylinderical, golden brown conidia.
3	**C-1**	Colorless, elliptical, single spores of α-conidia; needle like, filamentous spores of β-conidia were observed.
4	**TBPJ-B**	Tubular, thick walled, septate hyphae; monophialidic and polyphialidic conidiophores present; one celled ovoid microconidia were sparse; thin walled fusiform macroconidia were abundant; chlamydospores absent.
5	**B-7**	Long 3-septate macroconidia; small single celled 0-2 septate microconidia; microconidia formed long chains originating from phialides.

Table 4.5 Identification of endophytic fungal isolates based on molecular analysis and morphological studies

Strain code	Molecular identification (ITS sequence analysis)	Morphological identification (Based on macroscopic and microscopic features)
TBPJ-13	*Paraconiothyrium brasiliense* isolate F01 (JF439492)	*Paraconiothyrium brasiliense*
TBPJ-A	*Microdiplodia* sp. G16A (EF432267)	*Microdiplodia* species
C-1	*Gibberella avenacea* isolate FA08 (EU255798)	*Phomopsis* species
TBPJ-B	*Fusarium redolens* stain Ppf2 (EF495234)	*Fusarium redolens*/ *verticillioides*
B-7	*Fusarium tricinctum* isolate 6477 (JQ846085)	*Fusarium verticillioides*

Table 4.6 A-E Scientific classification of 5 endophytic fungi

(A) Scientific classification TBPJ-13	
Kingdom	Fungi
Phylum	Ascomycota
Subphylum	Pezizomycotina
Class	Dothideomycetes
Subclass	Pleosporomycetidae
Order	Pleosporales
Family	Montagnulaceae
Genus	*Paraconiothyrium*
Paraconiothyrium brasiliense	

(B) Scientific classification TBPJ-A	
Kingdom	Fungi
Phylum	Ascomycota
Class	Dothideomycetes
Order	Botryosphaeriales
Family	Botryosphaeriaceae
Genus	*Microdiplodia*
***Microdiplodia* species**	

(C) Scientific classification C-1	
Kingdom	Fungi
Phylum	Ascomycota
Class	Sordariomycetes
Subclass	Sordariomycetidae
Order	Diaporthales
Family	Diaporthaceae
Genus	*Phomopsis*
***Phomopsis* species**	

(D) Scientific classification TBPJ-B	
Kingdom	Fungi
Subkingdom	Dikarya
Phylum	Ascomycota
Subphylum	Pezizomycotina
Class	Sordariomycetes
Order	Hypocreales
Family	Nectriaceae
Genus	*Fusarium*
Fusarium redolens	

(E) Scientific classification B-7	
Kingdom	Fungi
Subkingdom	Dikarya
Phylum	Ascomycota
Subphylum	Pezizomycotina
Class	Sordariomycetes
Order	Hypocreales
Family	Nectriaceae
Genus	*Fusarium*
Fusarium verticillioides	

Conclusions and salient findings

Sixty different endophytic fungi were isolated from the bark and samples of *T. baccata*. Out of these, 8 fungi showed positive amplification with *DBAT* gene. These were further screened for the presence of *BAPT* gene and 5 showed positive results. Presence of both these genes suggested that these endophytic isolates may produce taxol. Based on preliminary molecular screening, the different taxol producing fungal isolates were selected and characterized morphologically as well as on the basis of ITS rDNA sequence analysis. These fungi were identified as *Fusarium redolens, Microdiplodia sp, Paraconiothyrium brasiliense, Fusarium tricinctum* and *Gibberella avenacea*.

4.2 Biochemical screening of taxol production by endophytic fungi

In order to detect the taxol producing capability of selected endophytic isolates, *Fusarium redolens* (TBPJ-B), *Microdiplodia* sp. (TBPJ-A), *Paraconiothyrium brasiliense* (TBPJ-13), *Fusarium verticillioides* (B-7) and *Phomopsis* sp. (C-1), extracts of all the five species with positive results in the primary screening were put through chromatographic analysis (TLC, HPLC and LC-MS) for the detection of fungal taxol. Briefly, all five endophytes were individually cultured in S7 liquid medium (Appendix II) for 3 weeks at 28º as still cultures (Fig. 4.15 a, b). After 21 days of incubation, the fungal mycelium was separated from broth by filtration through cheese cloth. Both mycelium and fermentation broth were processed separately and extraction was done with DCM (Fig. 4.16). DCM fractions were evaporated under reduced pressure by rotary evaporator and organic extracts re-dissolved in chloroform were purified by column chromatography.

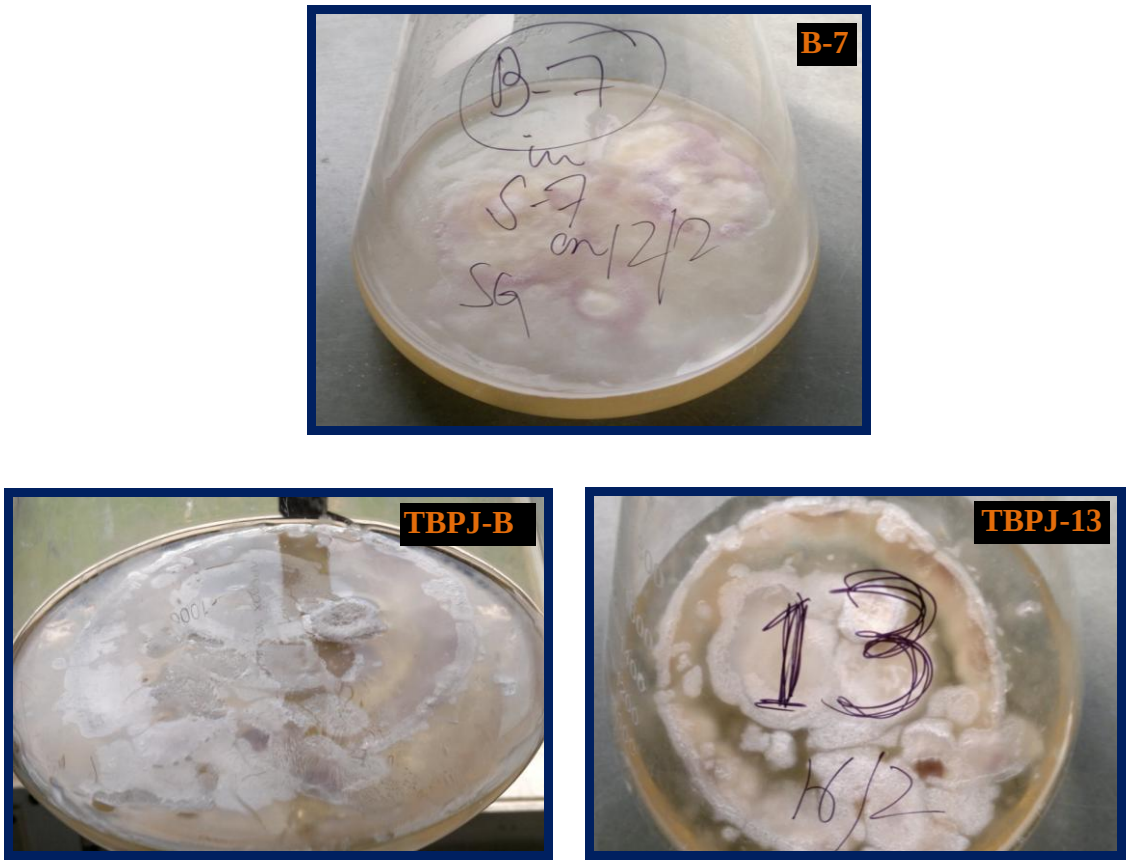


Fig. 4.15a Fungal isolates grown in S7 media for 21 days for taxol detection

Fig. 4.15b Fungal isolates grown in S7 media (for 21 days)

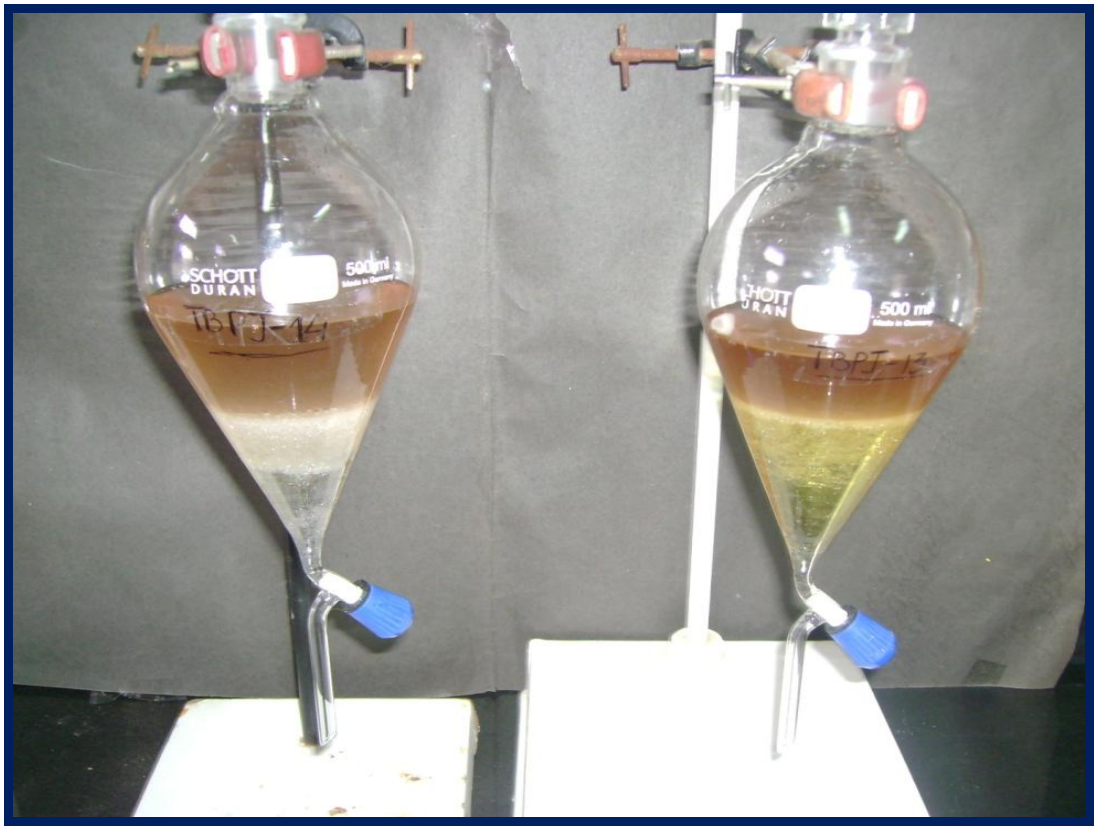


Fig. 4.16 DCM extraction of fermentation broth and mycelia extract

4.2.1 Thin layer chromatography (TLC)

In order to gain first insight into the presence of taxol in the extracts of 5 fungal endophytes (TBPJ-13, B-7, TBPJ-A, TBPJ-B and C-1) obtained after column chromatography, TLC was employed. Fungal extract samples along with standard taxol were equidistantly loaded on silica gel TLC plate, samples allowed to air dry and then the plate was introduced in TLC chamber having chloroform:methanol as the mobile phase. After the mobile phase had covered around 80% of TLC plate through capillary action it was carefully removed, air dried completely and sprayed on with vanillin-sulphuric acid spray reagent. After reacting with the spray reagent on gentle heating, the fungal taxol and standard taxol appeared as bluish spots, which after 24 h turned grey in color (Fig. 4.17). Authentic taxol which was used as positive control gave a Rf value of 0.36, corresponding to which the taxol in the fungal extracts yielded the same intense blue color reaction with the spray reagent.

TLC plates with fungal samples and standard taxol were also viewed under UV illumination at a wavelength of 254 nm. The active principle on thin layer plate appeared as dark bands.TLC thus provided the initial confirmation for the presence of taxol in the organic extracts of fungal isolates (combind result of medium filtrate and mycelium extract).

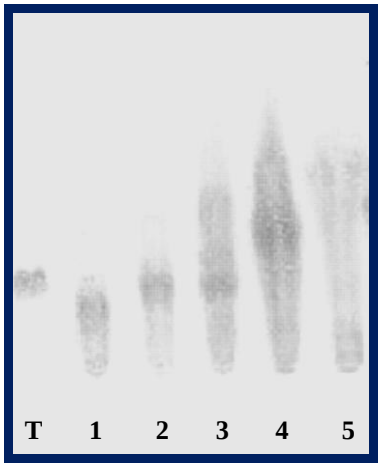


Fig. 4.17 Thin layer chromatography; compounds obtained after silica gel column chromatography. Lane **T** standard taxol; **1-5** fractions obtained from the gradient of chloroform, acetonitrile (**1:** TBPJ-B**, 2:** TBPJ-A**, 3:** TBPJ-13**, 4:** B-7**, 5:** C-1)

4.2.2 High performance liquid chromatography (HPLC; Perkin Elmer)

After the initial confirmation for the presence of taxol in fungal extracts through TLC, all extracts were further subjected to HPLC analysis. HPLC was performed to authenticate the presence of taxol in the fungal extracts and if present, then to quantify the amount in each sample. Fungal extract samples in pure HPLC grade methanol, obtained after processing culture filtrate and mycelium from 21 day old culture in S7 semi synthetic medium was subjected to HPLC. Before loading samples for HPLC detection, all fungal extracts were filtered through sterile 0.2 µm syringe filter individually (Millipore, USA). Purified fungal extracts as well as standard taxol were subjected to HPLC detection. 20 µL of purified extract was injected for each fungal endophyte in triplicates and peak detected at a wavelength range of 232 nm. HPLC chromatogram analysis of fungal extracts and authentic taxol gave a single peak under the same chromatographic conditions when eluted from a reverse phase C18 column (Fig. 4.18). The HPLC peak positions and peak shapes of taxol from fungal extracts had same retention time as that of standard taxol i.e. retention time of standard taxol 13.27 min and fungal taxol; C-1: 13.17 min, TBPJ-13: 13.30 min, B-7: 13.14 min, TBPJ-A: 13.08 min and TBPJ-B: 13.05 min (Fig. 4.18). Standard curve prepared by using peak values of different concentrations of authentic taxol served as the basis for quantification of taxol produced by the endophytic fungi. Among the 5 taxol-producing fungi, TBPJ-B had the highest taxol yield of 66.25 µg/L, in comparison with those of TBPJ-A which produced 27.40 µg/L, B-7 produced 23.47 µg/L, TBPJ-13 produced 19.60 µg/L and C-1 produced 11.03 µg/L of taxol in S-7 semi synthetic liquid medium (Table 4.7). Quantitative HPLC analysis of the fungal taxol showed that TBPJ-B (*Fusarium redolens*) produced the maximum amount of taxol compared to other isolates. Thus, HPLC was successfully implied for the detection and quantification of taxol in all five fungal extracts.

Table 4.7 Detection and quantification of fungal taxol by HPLC

Sl. No.	Sample codes	Samples	Retention time (in min)	Amount of taxol (µg/L)
1	Standard taxol			
	(a) 0.1 µg/µl		13.27	
	(b) 0.01 µg/µl	Authentic taxol	13.27	
	(c) 0.001 µg/µl		13.27	
2	C-1	*Phomopsis* sp.	13.17	11.03 ± 0.52^{e}
3	TBPJ-13	*Paraconiothyrium brasiliense*	13.30	19.60 ± 1.3^{d}
4	B-7	*Fusarium verticillioides*	13.14	23.47 ± 2.1^{c}
5	TBPJ-A	*Microdiplodia* sp.	13.08	27.40 ± 2.43^{c}
6	TBPJ-B	*Fusarium redolens*	13.05	66.25 ± 3.9^{a}

Values sharing a common letter within the column are not significant at ($P<0.05$). All values are mean ± SD (n = 3)

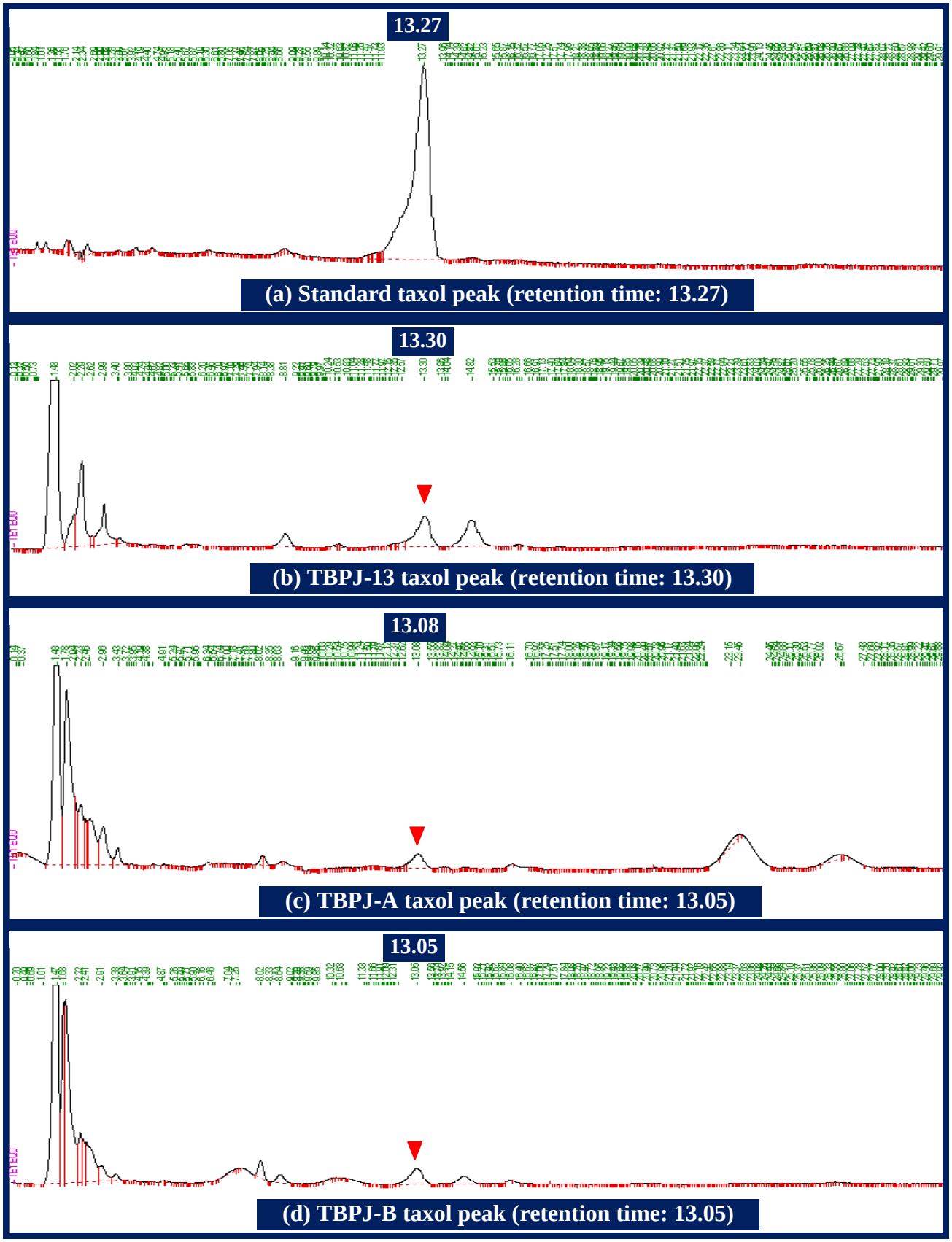


Fig. 4.18 Identification and quantification of taxol by HPLC **(a)** elution profile of standard taxol, **(b–d)** elution profile of taxol from the extracts of fungal isolates: **(b)** TBPJ-13; **(c)** TBPJ-A; **(d)** TBPJ-B

4.2.3 Liquid chromatography mass spectroscopy (LC-MS; Waters)

Convincing evidence for the identity of the fungal taxol was obtained by high resolution LC-MS and was carried out using Waters Acquity HPLC-MS apparatus (Fig. 4.19). The parameters followed for LC-MS analysis are as described in table 4.8.

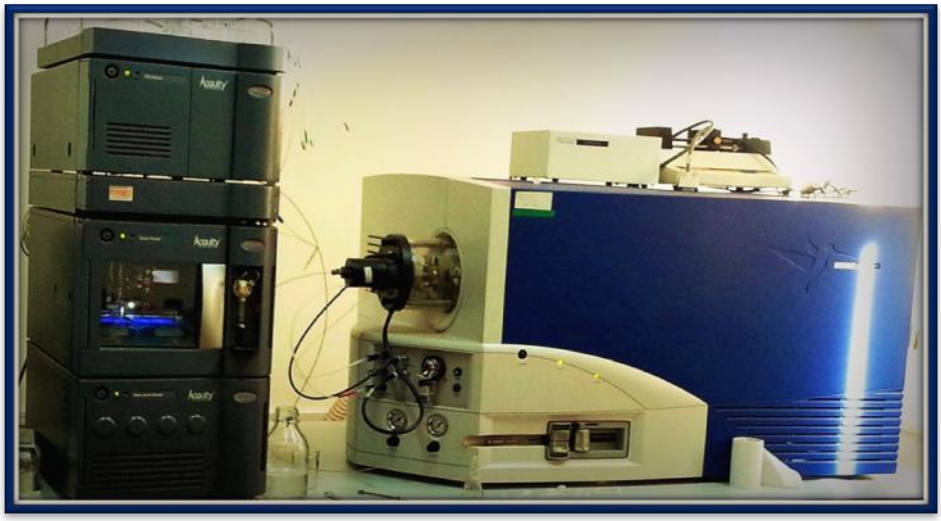


Fig. 4.19 Set up for Liquid chromatography mass spectroscopy

Table 4.8 Parameters followed for LC-MS analysis

Mobile phase	Methanol:Acetonitrile:Water
Sample dissolved in	Methanol
Absorbance	232 nm
Spray flow	2 µL/min (autosampler)
Spray voltage	2.2kV (loop injection method)
Run time	15 min
Detector	Photodiode Array(PDA)
Column	Reverse phase C18
Column temperature	25°C
Data type	Accurate Mass

The elution profile of HPLC portion in LC-MS clearly showed that the authentic taxol and taxol in fungal extracts had the same retention time i.e. retention time of standard taxol was 3.43 min. while those of fungal extracts was: 3.44 min for TBPJ-B, 3.42 min for TBPJ-13, 3.43 min for TBPJ-A (Fig. 4.20). Characteristically, mass spectra of authentic taxol yielded an $(M+H)^+$ peak at *m/z* 854.7 and an $(M+Na)^+$ peak at *m/z* 876.8 (Fig. 4.21). By comparison, the fungal taxol also yielded a peak MH^+ at *m/z* 854.3 and MNa^+ at *m/z* 876.3 with characteristic fragments at *m/z* 569, 551, 509, 286 and 268 (Fig. 4.21; Table 4.9). Major fragment ions observed in the mass spectrum of taxol can be placed into three categories which represents the major portions of the molecule (McClure et al. 1992). Spectral peaks of fungal taxol gave *m/z* ratios similar to the molecular ions of standard taxol, verifying that the 5 endophytic strains can produce taxol *in vitro*.

Table 4.9 LC-MS analysis of purified fungal taxol

Sl. No.	**Sample Codes**	**LC-MS**		
		Liquid chromatography retention time (in min**)**	**Mass spectroscopy**	
			Characteristic ions [$(M+H)^+$ at *m/z* 854 & (M+Na) at *m/z* 876**]**	**Specific ions (***m/z***)**
1	Authentic taxol (conc. 0.1 µg/µl)	3.43	854, 876	569, 551, 509, 286, 268
2	TBPJ-13	3.42	854	569, 509, 286
3	TBPJ-A	3.43	854, 876	569, 551, 286
4	TBPJ-B	3.44	854, 876	569, 551, 509, 286

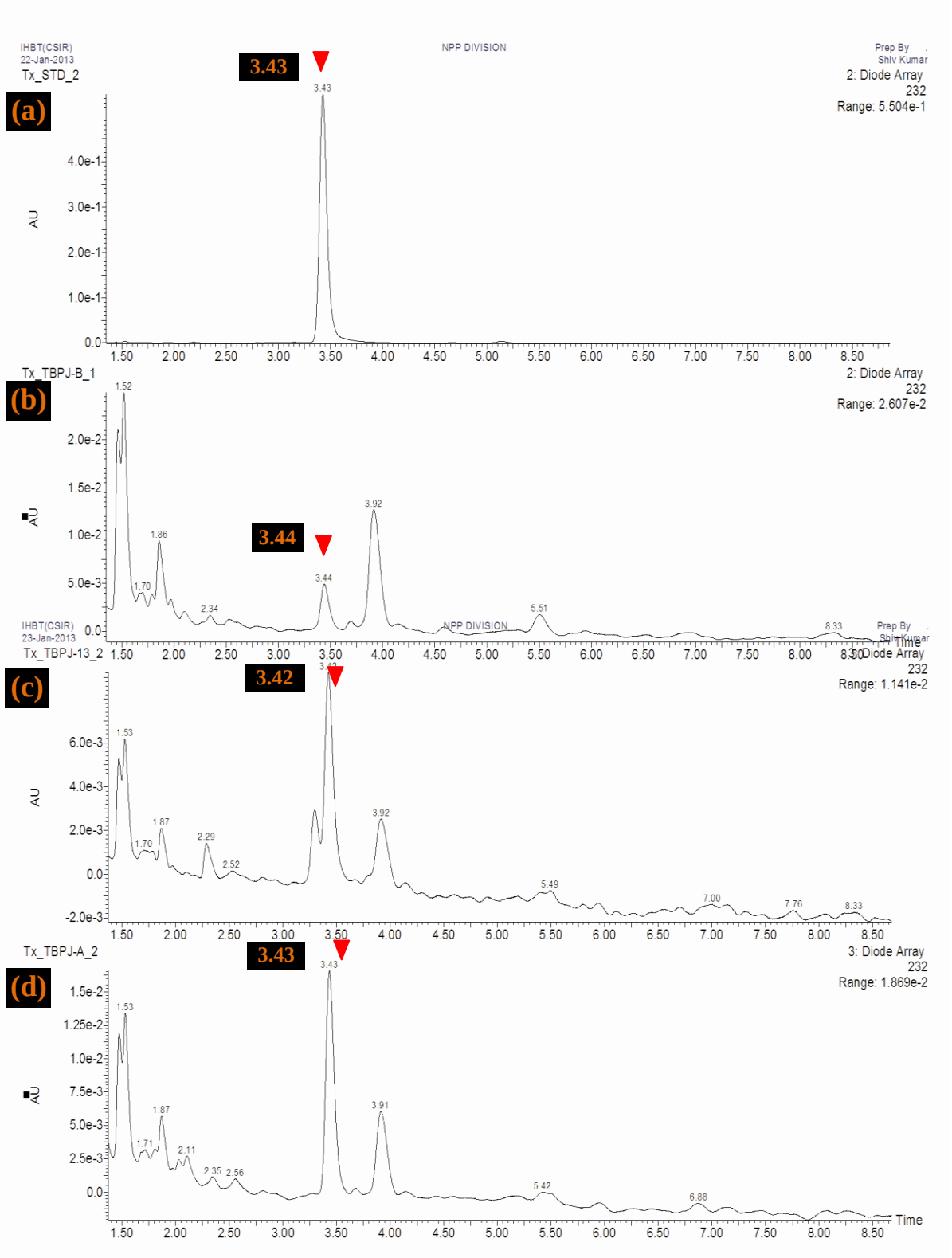


Fig. 4.20 HPLC portion in LC-MS **(a)** elution profile of standard taxol, **(b–d)** elution profile of taxol from the extracts of fungal isolates: **(b)** TBPJ-B; **(c)** TBPJ-13; **(d)** TBPJ-A

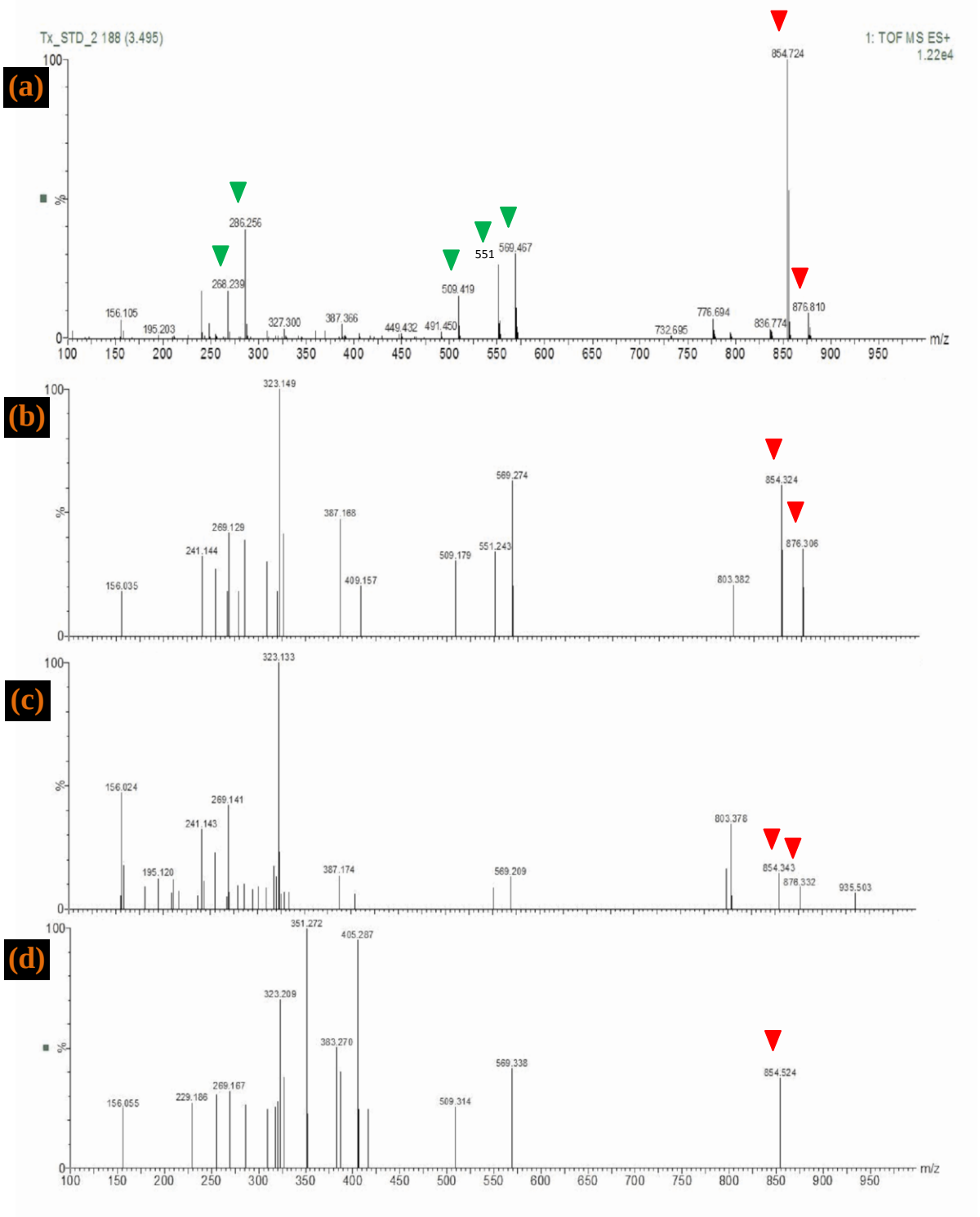


Fig. 4.21 Mass spectra of **(a)** standard taxol and taxol isolated from fungal strain **(b)** TBPJ-B; **(c)** TBPJ-A; **(d)** TBPJ-13. Characteristic ions at *m/z* 854 $(M+H)^+$ and *m/z* 876 $(M+Na)^+$ were recorded along the characteristic fragmentation pattern; specific ions were recorded at *m/z* 569, 551, 509, 286, and 268 both in standard and fungal taxol

Conclusions and salient findings

TLC and HPLC analysis depicted that fungal extracts from *Fusarium redolens*, *Microdiplodia* sp., *Paraconiothyrium brasiliense*, *Fusarium verticillioides* and *Phomopsis* sp. had similar peaks of taxol as was in the case of standard taxol. *F. redolens* produced significantly higher amount of taxol compared to other fungal isolates. The results were further verified by LC-MS which confirmed the production of taxol by all these endophytes. So, based on this study, we have been successful in isolating novel and promising strains of taxol producing endophytic fungi from *Taxus baccata*.

4.3 Anti tumorous activity of fungal taxol

4.3.1 Potato disc tumor induction assay

Results of chromatographic techniques mentioned in the previous section clearly showed presence of fungal taxol in the extracts of all five fungal endophytes obtained from the bark of *Taxus baccata* (Himalayan yew). The very next task was to test whether the fungal taxol bears the same antineoplastic activity as that of standard paclitaxel. So to test the anti-tumorigenic activity of fungal taxol an immunoassay "Potato disc tumor induction assay" was performed.

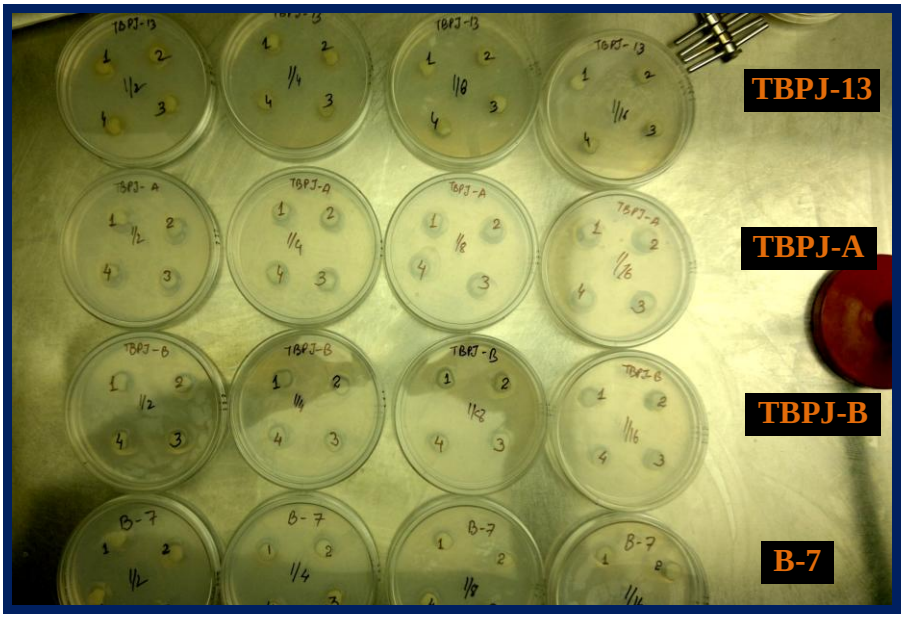


Fig. 4.22 Potato disc tumor induction assay with taxol producing fungal endophytes

Agrobacterium tumefaciens strain MTCC 431 was used as the tumor inducing agent. Potato discs placed aseptically in Petri plates containing 15% water agar were overlaid with 50 µL of appropriate fungal extract/water/bacterial mix (Fig. 4.22). After 20 days of incubation at room temperature, the potato discs were stained with Lugol's reagent. Starch in the potato tissue took up the strain and appeared dark brown in color, but tumors produced by *A. tumefaciens* did not take up the stain and appeared creamy to orange (Mc Laughlin and Rogers 1998). In this study three internal control treatments were used (Table 4.10). *A.*

tumefaciens with DMSO induced at least 10 tumors per potato disc (Fig. 4.23 A-D). DMSO alone did not induce any tumor. These results clearly showed that DMSO as a solvent neither interfered with the activity of the bacterium nor induced tumor itself (Table 4.10). Authentic taxol served as positive control and inhibited tumor production at all the tested concentrations (Fig. 4.23 E). Fungal taxol from 5 endophytes (TBPJ-B, TBPJ-A, TBPJ-13, B-7 and C-1) also inhibited tumor formation the same way the authentic taxol did (Fig. 4.23 F). These results justified that all fungal extracts tested in this study had similar antineoplastic activity as that of paclitaxel and thus inhibited tumor formation at all tested concentrations in potato disc tumor induction assay.

Viability test conducted to check whether there was any effect of the standard drug (paclitaxel) and drug in the fungal extracts on the viability of the bacterium (*A. tumefaciens*). The bacterial viability was determined by incubating standard paclitaxel and fungal extract individually with 1×10^9 colony-forming units (CFU) of *A. tumefaciens* suspension (in PBS; three tubes per test). Paclitaxel was used at a concentration of 0.001 mg/ml and fungal extract was used undiluted. Bacterial suspension and standard paclitaxel/fungal extract where given an exposure time of 3, 6, 9 and 12 h. After specified time intervals 100 µL of solution from each tube was transferred to fresh YEM medium and incubated for 24 h. Growth of bacterium was determined spectrophotometrically by taking readings at O.D. 600 nm (Table 4.11). The results of the viability test in the present study depicted that action of drugs tested is on the formation of tumors and not on the bacterial viability.

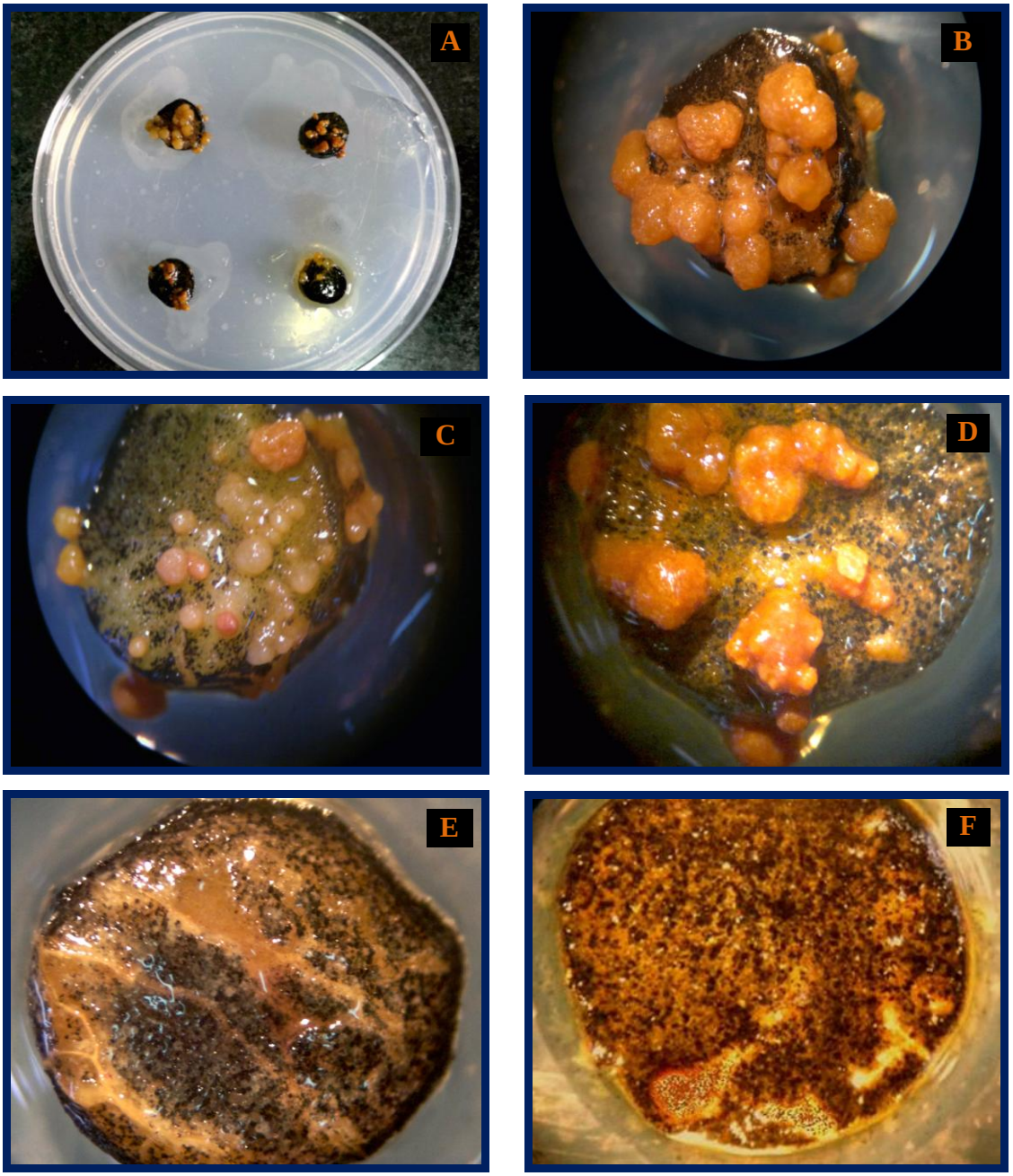


Fig. 4.23 Potato disc tumor induction assay. **(A-D)** Tumor formation was observed on potato disc treated only with *Agrobacterium tumefaciens*, and no tumor formation was observed on potato disc treated with **(E)** *A. tumefaciens* + standard taxol or **(F)** *A. tumefaciens* + fungal extract (5 endophytic fungi, respectively)

Table 4.10 Potato disc tumor induction assay by *Agrobacterium tumefaciens* at different concentrations of standard taxol and fungal extracts

Sl. No.	Potato discs treated with	Tumors produced	Results
1	DMSO or pure methanol (**Solvent control**: without *Agrobacterium* or fungal extract)	**-**	No effect of solvent control on potato discs
2	*Agrobacterium tumefaciens* (**tumor initiation control**: *Agrobacterium* used without drug)	**+**	Tumor formation observed (5-7 tumors produced per disc)
3	*A. tumefaciens* + DMSO or Methanol	**+**	No effect of solvent control on the viability of bacterium and tumor induction (5-7 tumors produced per disc)
4	Taxol (1mg/ml stock) (a) 0.1 µg/µl (b) 0.01 µg/µl (c) 0.001 µg/µl	**-**	No tumor development at all tested concentrations in the positive control
Different concentrations of fungal extract of sample no. 5-9 used were: undiluted sample, ½ dilution, ¼ dilution, 1/8 dilution, 1/16 dilution.			
5	TBPJ-13	**-**	
6	TBPJ-A	**-**	
7	TBPJ-B	**-**	No tumors developed at all tested concentrations; fungal extract having antitumor effect
8	B-7	**-**	
9	C-1	**-**	

- ve indicates no formation of tumors; **+ ve** indicates formation of visible tumors; assay was performed in triplicates.

Table 4.11 Growth of *Agrobacterium tumifaciens* on YEM medium (OD at 600 nm) as a function of incubation time in phosphate buffered saline (0.001 mg/ml of paclitaxel and undiluted fungal extract was used)

Exposure time (in hours)	Growth			
	Negative control (Bacterium inoculum)	**Positive control** (DMSO +Bacterium)	**Paclitaxel** (Taxol)	**Fungal extract**
3	0.20 ± 0.03^{d}	0.16 ± 0.031^{c}	0.18 ± 0.03^{c}	0.16 ± 0.02^{c}
6	0.33 ± 0.03^{c}	0.25 ± 0.032^{c}	0.25 ± 0.04^{c}	0.23 ± 0.04^{c}
9	0.45 ± 0.04^{b}	0.40 ± 0.025^{b}	0.37 ± 0.05^{b}	0.35 ± 0.03^{b}
12	0.58 ± 0.07^{a}	0.54 ± 0.070^{a}	0.53 ± 0.06^{a}	0.47 ± 0.06^{a}

Values sharing a common letter within the column are not significant at (P<0.05). All values are mean ± SD (n = 3)

Conclusions and salient findings

In this part of work, the anti-tumourogenic activity of fungal taxol was assessed in comparison to the standard taxol. Potato disc tumour induction assay depicted that fungal taxol from all the endophytes of present study successfully inhibited tumor formation in potato discs just like authentic taxol while it did not affect bacterial viability in any case. Current work proved the significant potential of fungal taxol without any negative effect on the bacterial viability.

4.4 Process optimization for taxol production from endophytic fungus *Fusarium redolens*

As it was envisaged that fungal taxol is novel and promising alternative for producing large amounts of the drug in very efficient and economical way in short periods of time, an attempt was made to enhance the production of taxol from efficient taxol producing endophyte *Fusarium redolens* by optimizing the media components through Response Surface Methodology. The different parameters to be optimized were as under:

Process optimization for taxol production from endophytic fungus *Fusarium redolens*	
Sr. No.	**Parameters**
1.	Radial Growth
2.	Growth kinetics (No. of days)
3.	Media-to-flask volume (V_m/V_f)
4.	Optimal pH
5.	Optimal temperature
6.	Carbon sources (Sucrose/Glucose/Fructose)
5.	Nitrogen sources (NH_4NO_3/Peptone/$CaNO_3$)
6.	**Plackett-Burman Design:** Screening of important factors for biomass and taxol production
7.	**Response Surface Methodology:** Determining the optimal concentrations of selected significant factors (independent variables)

4.4.1 One-factor-at-at-a-time (OFAT)

4.4.1.1 Standardization of basic parameters

(a) Estimation of radial growth

This factor was studied to determine how much time does *F. redolens* takes on PDA plate to cover the maximum media surface with mycelia. The results (Table 4.12) showed that maximum radial growth of the fungus on PDA was observed on 7th day of culturing i.e. the fungal mycelium covered approximately 8.9 cm media surface and figure 4.24 is the graphical representation for the same. Statistical analysis revealed that time period had significant effect on growth of the fungus. Growth was lowest on day 1 while it was found to be highest on 7th day.

(b) Estimation of fungal biomass (Growth kinetics)

To study the growth kinetics of *F. redolens*, the fungus was grown in S7 liquid medium in 250 ml flasks, incubated at 28º for varying time intervals (in days). The mycelial growth and product profile for the fungus showed that the biomass increased exponentially till the experimental period of 20 days after which it started decreasing (Fig. 4.25). On the other hand taxol production also increased from 5.02-67.72 µg/L in 20 days before falling down to 60 µg/ L at 30 days (Table 4.13). The growth and secondary metabolite production remained almost static after 20 days of incubation. It was found that time period had significant effect on biomass and taxol production. Highest taxol production (67.72 µg/L) and biomass production (12.8 gm/250 ml) was obtained on day 20th of growth cycle (Fig. 4.25).

(c) Optimization of medium volume (*Vm*) to flask volume (*Vf*) ratio

While optimizing the ratio of *Vm* to *Vf*, it was observed that the biomass increased from 4.4 gm/250 ml at *Vm*/*Vf* ratio of 0.10 to a reasonably high value of 12.5 gm/ 250 ml at *Vm*/*Vf* ratio of 0.20, which thereafter decreased to 7.2 gm/250 ml at *Vm*/*Vf* ratio of 0.30 (Fig. 4.26). On the other hand taxol production (as combined effect of mycelium and culture filtrate)

continuously increased from 14-70 µg/L with an increase in *Vm*/*Vf* ratio from 0.10-0.20, which thereafter decreased to 60.9 µg/L at *Vm*/*Vf* = 0.30 (Table 4.14). Statistical analysis revealed that *Vm*/*Vf* had significant effect on biomass as well as taxol production. Hence the optimal value of *Vm*/*Vf* for successful development of S7 medium for maximum biomass and taxol production was found to be 0.20.

(d) Estimation of optimal pH and temperature for biomass and taxol production

The initial pH of 6.5 of S7 medium was found to be optimal for growth (12.7 gm/250 ml) and taxol production (69.4 µg/L) by *F. redolens* (Fig. 4.27). Neutral pH also supported the growth and taxol production of the strain (Table 4.15). No growth was observed at pH <3.0 and pH >11.0. Although pH had significant effect on biomass and taxol production, but an insignificant effect was observed in pH range of 7.5-8.5.

Maximum biomass (12.9 gm/ 250 ml) and taxol production (68.12 µg/L) by the fungus was recorded at incubation temperature of 25ºC (Fig.4.28) compared to low (15ºC) and high (35ºC) temperatures. Growth was almost ceased at < 10 ºC and > 40 ºC (Table 4.16). Statistical analysis revealed that biomass and taxol production varied significantly over the temperature range.

Table 4.12 Estimation of radial growth of *Fusarium redolens*

No. of days	Growth (cm)
1	1.3 ± 0.06^{e}
2	2.8 ± 0.09^{d}
3	3.5 ± 0.14^{c}
4	5.0 ± 0.18^{bc}
5	7.2 ± 0.26^{b}
6	8.4 ± 0.35^{a}
7	8.9 ± 0.42^{a}

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 3)

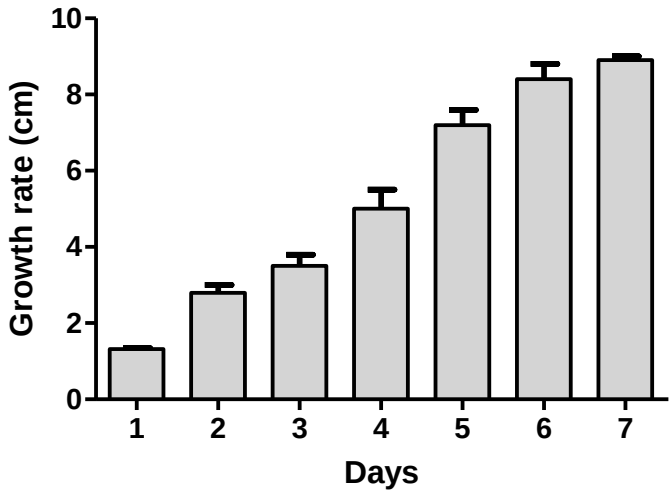


Fig. 4.24 Estimation of radial growth of taxol producing endophytic fungus *Fusarium redolens.* Bars represent mean ± SD (n = 3)

Table 4.13 Effect of different time intervals on biomass and taxol yield in taxol producing endophytic fungus *Fusarium redolens*

Time (days)	Biomass FW (gm)	Taxol * (µg/L)
0	0.94 ± 0.03^{fg}	5.02 ± 0.11^{i}
5	1.9 ± 0.08^{f}	11.5 ± 1.9^{fg}
10	6.2 ± 0.73^{d}	26.43 ± 2.4^{e}
15	9.2 ± 0.86^{b}	54.86 ± 2.8^{bc}
20	12.8 ± 0.94^{a}	67.72 ± 3.4^{a}
25	10.8 ± 0.87^{b}	64.4 ± 2.7^{a}
30	7.2 ± 0.79^{c}	60.9 ± 3.1^{b}

* Taxol production from mycelium and culture filtrate

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 3)

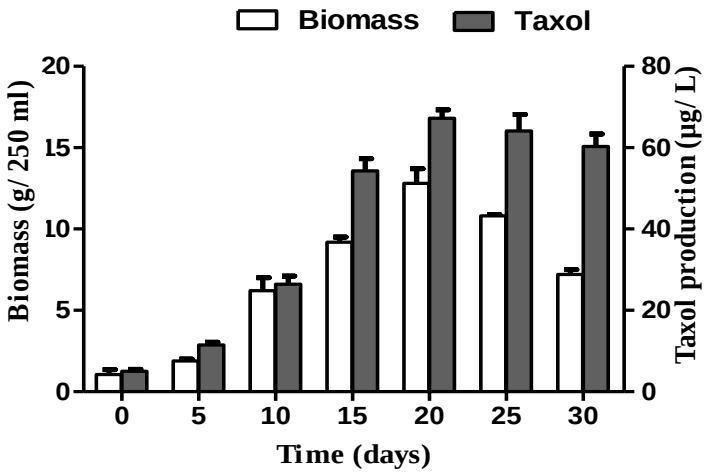


Fig. 4.25 Effect of different time intervals on biomass and taxol yield in endophytic fungus *Fusarium redolens.* Bars represent mean ± SD (n = 3)

Table 4.14 Effect of medium to flask volume ratio on biomass and taxol yield in endophytic fungus *Fusarium redolens*

Medium to flask volume (V_m/V_f)	Amount of liquid medium (250 ml flask)	Biomass FW (gm/250ml)	Taxol* (µg/L)
0.10	25	4.4 ± 0.2^{fg}	14 ± 0.8^{fg}
0.15	37.5	8.7 ± 0.7^{c}	48 ± 2.3^{c}
0.20	50	12.5 ± 1.0^{a}	70 ± 2.8^{a}
0.25	62.5	10.9 ± 0.8^{b}	59 ± 2.6^{b}
0.30	75	7.2 ± 0.6^{d}	25 ± 1.3^{de}

* Taxol production from mycelium and culture filtrate

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 3)

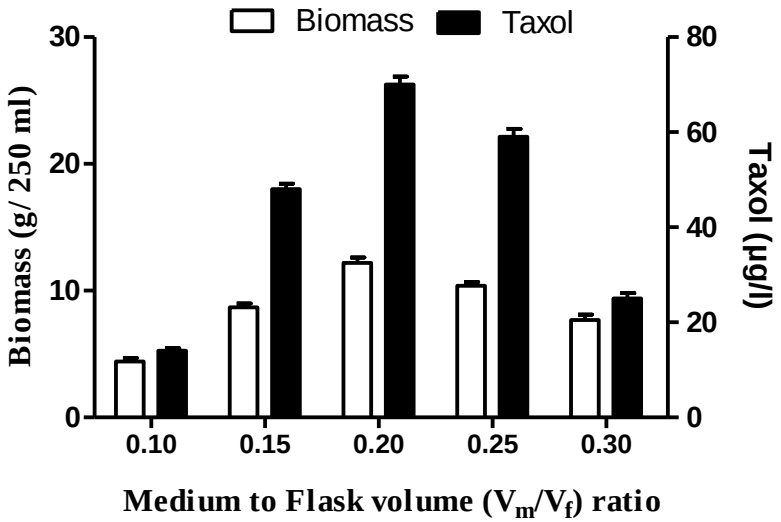


Fig. 4.26 Effect of medium/flask volume ratio on biomass and taxol yield in endophytic fungus *Fusarium redolens*. Bars represent mean ± SD (n = 3)

Table 4.15 Effect of different pH on biomass and taxol yield in endophytic fungus *Fusarium redolens*

pH range	Biomass FW (gm/250ml)	Taxol * (µg/L)
3.5	4.8 ± 0.31^{f}	13.47 ± 1.01^{f}
4.5	7.2 ± 0.90^{d}	28.78 ± 1.9^{d}
5.5	10.0 ± 0.82^{b}	54.32 ± 2.5^{b}
6.5	12.7 ± 0.96^{a}	69.4 ± 3.1^{a}
7.5	10.8 ± 0.91^{b}	47.12 ± 2.6^{c}
8.5	10.5 ± 0.87^{b}	31.83 ± 2.2^{d}
9.5	8.8 ± 0.72^{c}	18.71 ± 1.3^{ef}

* Taxol production from mycelium and culture filtrate

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 3)

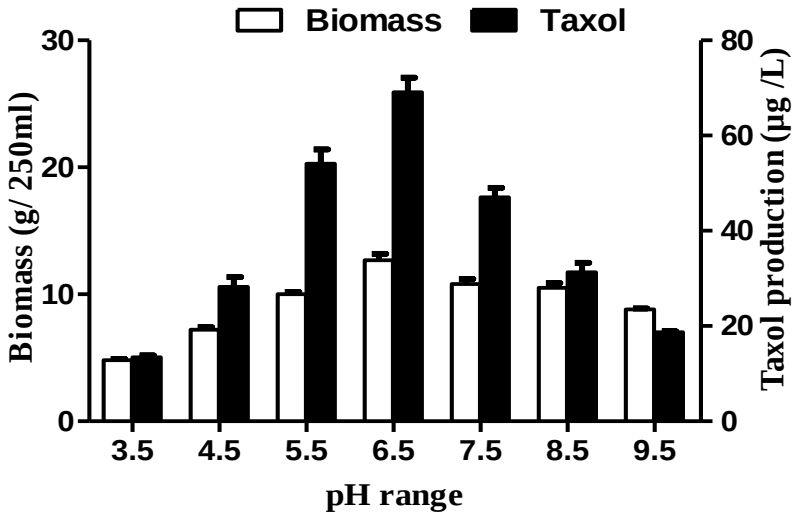


Fig. 4.27 Effect of different pH on biomass and taxol yield in endophytic fungus *Fusarium redolens.* Bars represent mean ± SD (n = 3)

Table 4.16 Effect of different temperature on biomass and taxol yield in endophytic fungus *Fusarium redolens*

Temperature range (ºC)	Biomass FW (gm/250 ml)	Taxol* (µg/L)
5	0.5 ± 0.02^{f}	1.7 ± 0.08^{g}
15	6.2 ± 0.3^{bc}	6.78 ± 0.49^{f}
25	12.9 ± 1.01^{a}	68.12 ± 2.9^{a}
35	7.5 ± 0.7^{b}	9.4 ± 0.52^{f}

* Taxol production from mycelium and culture filtrate

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 3)

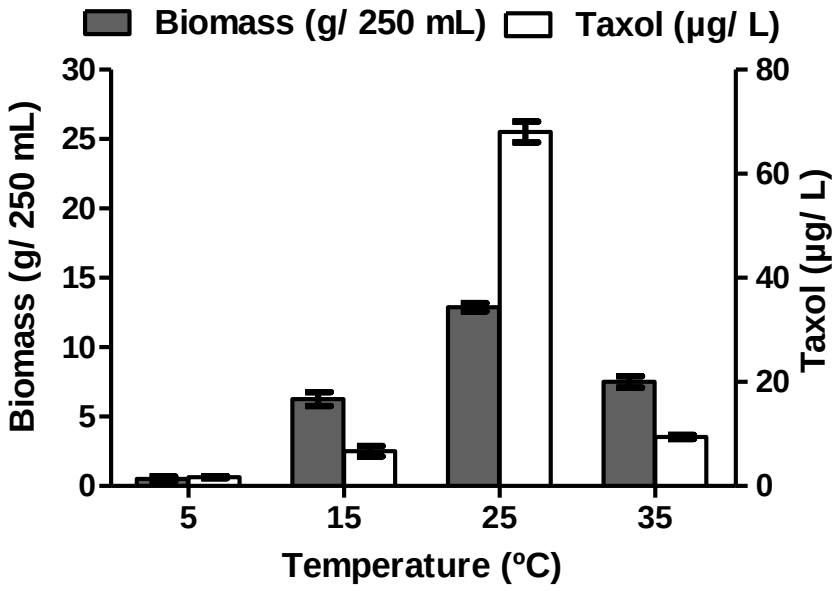


Fig. 4.28 Effect of different temperature on biomass and taxol yield of taxol producing endophytic fungus *Fusarium redolens.* Bars represent mean ± SD (n = 3)

4.4.1.2 Medium optimization

Effect of carbon and nitrogen sources on biomass and taxol production

Higher biomass and taxol production was observed in sucrose amended medium (80 gm/L) compared to other carbon sources. ANOVA test revealed that carbon source had significant effect on fungal biomass as well as taxol production. Among the carbon sources, medium supplemented with sucrose showed significantly higher (P<0.05) fresh weight, dry weight as well as taxol production compared to glucose and fructose (Table 4.17). Although some statistical difference was found in taxol production in fructose amended media, but relatively lesser biomass and taxol was produced in both fructose and glucose amended media. Least taxol production was noticed in glucose amended media. Maximum taxol yield of 133 µg/L was observed when the concentration of sucrose was 8% (w/v). The biomass increased with the increasing concentration of sucrose, suggesting that higher concentration of sucrose did not affect the growth of the fungus (Fig. 4.29, 4.30).

Maximum biomass and taxol production was observed in NH_4NO_3 amended medium (8 gm/L) compared to other nitrogen sources (Table 4.18). ANOVA test revealed that nitrogen source had significant effect on fungal biomass as well as taxol production. Culture amended with NH_4NO_3 showed significantly higher (P<0.05) fresh weight, dry weight as well taxol production (Table 4.18). Although some statistical difference was found in taxol production in peptone amended media, but relatively lesser biomass and taxol was produced in both $CaNO_3$ and peptone amended media. Least taxol production was noticed in $CaNO_3$ amended media. Maximum taxol yield of 145 µg/L was observed when the concentration of NH_4NO_3 was 0.8% (w/v) (Fig. 4.31, 4.32).

Table 4.19 summarizes the optimized media components using OFAT approach.

Table 4.17a Effect of different concentrations of carbon sources on fresh weight, dry weight and taxol yield in *Fusarium redolens*

Conc. (%)	Fresh Weight (FW)			Dry Weight (DW)			Taxol*		
	Sucrose	**Glucose**	**Fructose**	**Sucrose**	**Glucose**	**Fructose**	**Sucrose**	**Glucose**	**Fructose**
4	16.31 ± 0.86^{aA}	10.71 ± 1.02^{bC}	13.03 ± 0.76^{cB}	1.52 ± 0.18^{dA}	0.91 ± 0.03^{dC}	1.21 ± 0.03^{cB}	120.3 ± 4.3^{aA}	89.0 ± 4.7^{aC}	98.25 ± 3.6^{cB}
6	16.9 ± 0.93^{aA}	11.90 ± 1.07^{aC}	14.68 ± 0.82^{bB}	1.67 ± 0.22^{cA}	0.94 ± 0.08^{cdC}	1.15 ± 0.02^{cB}	127.0 ± 3.8^{aA}	93.2 ± 4.3^{aC}	$107.0 \pm 5.1b^{cB}$
8	17.9 ± 1.02^{aA}	12.93 ± 1.04^{aC}	15.8 ± 1.03^{aB}	1.89 ± 0.31^{aA}	1.36 ± 0.09^{aC}	1.51 ± 0.07^{aB}	133.0 ± 4.5^{aA}	98.6 ± 4.9^{aC}	120.2 ± 6.1^{aB}
10	17.2 ± 0.94^{aA}	12.5 ± 0.92^{aC}	15.4 ± 0.92^{abB}	1.85 ± 0.29^{aA}	1.08 ± 0.05^{bC}	1.43 ± 0.04^{bB}	130.16 ± 4.1^{aA}	96.16 ± 3.7^{aC}	115.85 ± 5.2^{abB}
12	17.0 ± 0.79^{aA}	12.1 ± 0.81^{aC}	15.25 ± 0.87^{abB}	1.71 ± 0.24^{aA}	0.95 ± 0.03^{cC}	1.40 ± 0.05^{bB}	128.7 ± 3.2^{aA}	91.32 ± 3.1^{aC}	110.5 ± 4.3^{abB}

* Taxol production as a combined effect of mycelium and culture filtrate; Values sharing a common letter in the column (lower case) and rows (uppercase) are not significant ($P < 0.05$); All values are mean ± SD (n = 3)

Table 4.17b Two-way ANOVA results for carbon sources

Source of Variation	Fresh weight					Dry weight					Taxol				
	df	*SS*	*MSS*	*F*	*P -value*	*df*	*SS*	*MSS*	*F*	*P -value*	*df*	*SS*	*MSS*	*F*	*P -value*
Carbon sources	2	190.2	95.08	337.5	***	2	3.480	1.740	1093	***	2	8872	4436	190.1	***
Conc. (%)	4	22.96	5.739	20.37	***	4	0.8664	0.2166	136.1	***	4	1123	280.8	12.03	***
Interaction	8	1.652	0.2065	0.7331	ns	8	0.1314	0.01642	10.32	***	8	177.1	22.14	0.9489	ns
Residual (error)	30	8.451	0.2817			30	0.04773	0.001591			30	700.1	23.34		

df: degree of freedom; *SS*: sum-of-squares; *MSS*: mean square; ns: not significant; *** $P < 0.0001$

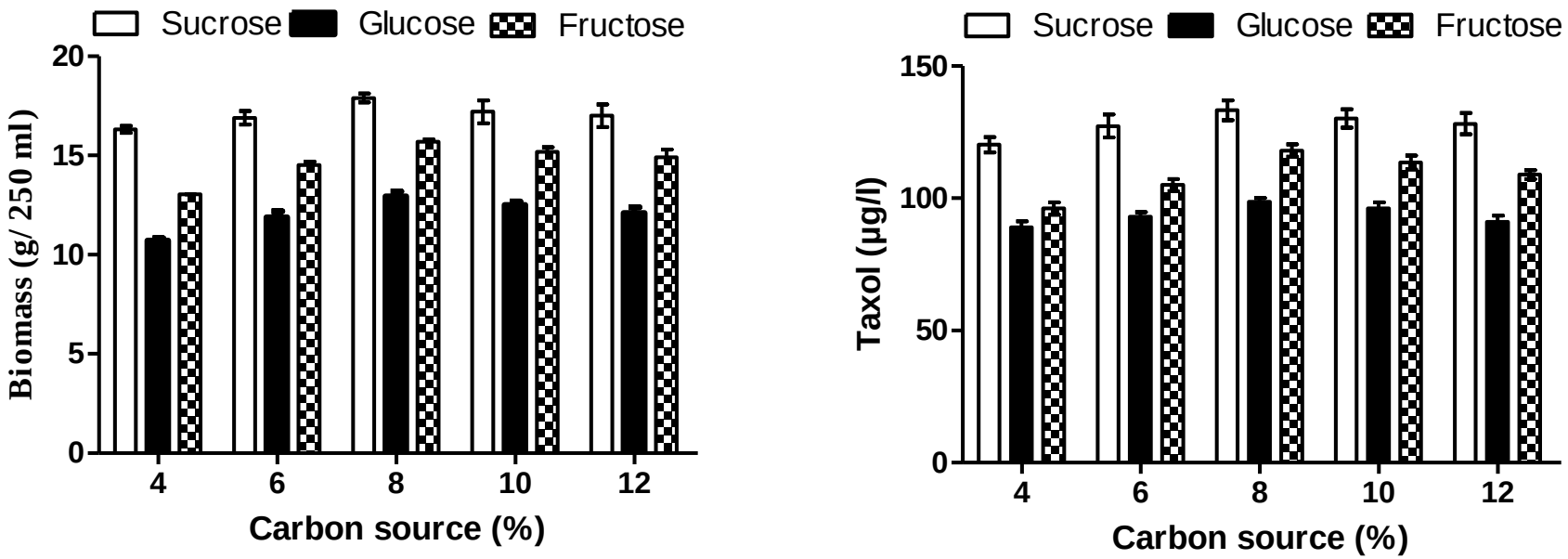


Fig. 4.29, 4.30 Effect of different carbon sources on biomass and taxol yield in endophytic fungus *Fusarium redolens.* Bars represent mean ± SD (n = 3)

Table 4.18a Effect of different concentrations of nitrogen sources on fresh weight, dry weight and taxol yield in *Fusarium redolens*

Conc. (%)	Fresh Weight (FW)			Dry Weight (DW)			Taxol*		
	NH_4NO_3	Peptone	$CaNO_3$	NH_4NO_3	Peptone	$CaNO_3$	NH_4NO_3	Peptone	$CaNO_3$
0.4	15.37 ± 1.3^{cA}	14.56 ± 1.7^{cA}	13.12 ± 1.4^{cA}	1.61 ± 0.08^{bA}	1.54 ± 0.09^{bA}	1.39 ± 0.03^{dB}	114 ± 3.1^{cA}	95 ± 3.3^{cB}	89 ± 3.9^{cB}
0.6	17.5 ± 1.5^{bA}	17.7 ± 1.3^{aA}	16.75 ± 1.3^{aA}	2.07 ± 0.09^{aA}	1.86 ± 0.07^{aB}	1.74 ± 0.09^{aC}	132 ± 3.9^{bA}	121 ± 3.9^{aAB}	111 ± 3.4^{aB}
0.8	18.3 ± 1.2^{aA}	16.83 ± 1.6^{bAB}	15.43 ± 1.6^{bB}	2.10 ± 0.04^{aA}	1.84 ± 0.10^{aB}	1.65 ± 0.08^{bC}	145 ± 5.2^{aA}	107 ± 2.7^{bB}	97 ± 3.5^{bB}
1.0	18.7 ± 1.7^{aA}	17.9 ± 1.1^{aA}	13.57 ± 1.2^{cB}	2.05 ± 0.09^{aA}	1.8 ± 0.04^{aB}	1.37 ± 0.06^{dC}	124 ± 4.3^{cdA}	106 ± 3.5^{bB}	84 ± 4.1^{cC}

* Taxol production as a combined effect of mycelium and culture filtrate; Values sharing a common letter in the column (lower case) and rows (uppercase) are not significant ($P < 0.05$); All values are mean ± SD (n = 3)

Table 4.18b Two-way ANOVA results for nitrogen sources

Source of Variation	Fresh weight					Dry weight					Taxol				
	df	*SS*	*MSS*	*F*	*P -value*	*df*	*SS*	*MSS*	*F*	*P -value*	*df*	*SS*	*MSS*	*F*	*P -value*
Nitrogen sources	2	48.54	24.27	37.45	***	2	1.245	0.6227	353.0	***	2	7024	3512	90.96	***
Conc. (%)	3	56.92	15.64	24.13	***	3	0.8203	0.2734	155.0	***	3	2811	937.1	24.27	***
Interaction	6	18.69	3.115	4.806	**	6	0.2047	0.03412	19.35	***	6	949.1	158.2	4.097	**
Residual (error)	24	15.55	0.6481			24	0.04233	0.001764			24	926.7	38.61		

df: degree of freedom; *SS*: sum-of-squares; *MSS*: mean square; ns: not significant; ** $P < 0.01$; ******* $P < 0.0001$

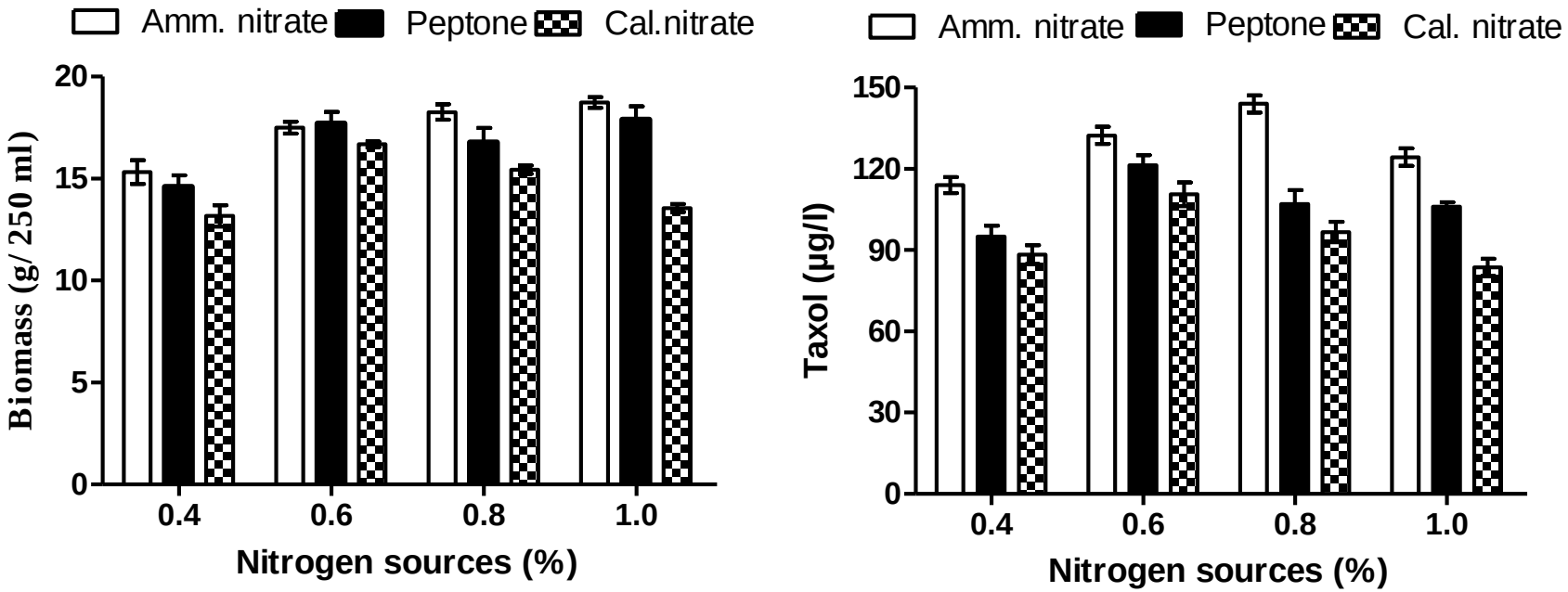


Fig. 4.31, 4.32 Effect of different Nitrogen sources on biomass and taxol yield in endophytic fungus *Fusarium redolens*. Bars represent mean ± SD (n = 3)

Table 4.19 Summary of the optimized media components using OFAT approach

Sr. No.	Parameters	Optimized value
1	Radial growth	7^{th} day (8.9 cm)
2	Growth Kinetics	20 days
3	Media-to-flask volume	50 ml / 250 ml
4	Temperature	25ºC
5	pH	6.5
4	Sucrose (carbon source)	80 g/L
5	Ammonium nitrate (nitrogen source)	8 g/L

4.4.2 Plackett-Burman design

Plackett-Burman design results showed that KH_2PO_4, $MgSO_4$, $ZnSO_4$, NaOAc and vitamin B1 are significant model terms (Table 4.20, 4.21). NaOAc, $MgSO_4$ and $ZnSO_4$ were shown as the important components which gave main contribution on taxol production, while, $ZnSO_4$ had a negative effect (Fig. 4.33). The same effect for the three micronutrients (NaOAc, $MgSO_4$, $ZnSO_4$) was confirmed from the high positive *t* values (or regression coefficients) calculated for these components in the ANOVA test by Design-Expert version 8.0.7.1 software (Stat-Ease Corporation, Minneapolis, MN). Among the eight micronutrients studied under PB design, $ZnSO_4$ had a negative effect on taxol production (as indicated by the negative *t* value and studied effect) and factors with "Prob>F" (*p* value) more than 0.1 i.e. $FeCl_3$, $MnCl_2$ and phenylalanine did not have a significant effect on taxol production. These

four micronutrients were included at a fixed concentration level in the optimized medium. Although the PB design could be successfully used for reasonable prediction of the significant level of different variables (micronutrients) affecting the response (taxol production), some of the significant interactive effects of the chosen variables (two-factor interaction) were confounded in its complex structure. Because of this reason, the actual main effects of these variables may have been influenced. Hence, the significant components NaOAc and $MgSO_4$ whose interactive effects were demonstrated by PB design were further modelled more precisely by RSM.

Table 4.20 Plackett–Burman experimental design matrix with taxol production

Trial	A	B	C	D	E	F	G	H	Taxol (µg/L)
1	1	1	-1	1	1	1	-1	-1	**156.7**
2	-1	1	1	-1	1	1	1	-1	**143.3**
3	1	-1	1	1	-1	1	1	1	**189.1**
4	-1	1	-1	1	1	-1	1	1	**106.3**
5	-1	-1	1	-1	1	1	-1	1	**129.3**
6	-1	-1	-1	1	-1	1	1	-1	**173.6**
7	1	-1	-1	-1	1	-1	1	1	**75.2**
8	1	1	-1	-1	-1	1	-1	1	**158.8**
9	1	1	1	-1	-1	-1	1	-1	**124.7**
10	-1	1	1	1	-1	-1	-1	1	**118.0**
11	1	-1	1	1	1	-1	-1	-1	**89.4**
12	-1	-1	-1	-1	-1	-1	-1	-1	**81.6**

A: $FeCl_3$, **B**: KH_2PO_4, **C**: $MnCl_2$, **D**: $MgSO_4$, **E**: $ZnSO_4$, **F**: NaOAc, **G**: Thiamine (Vit. B1), **H**: Phenylalanine

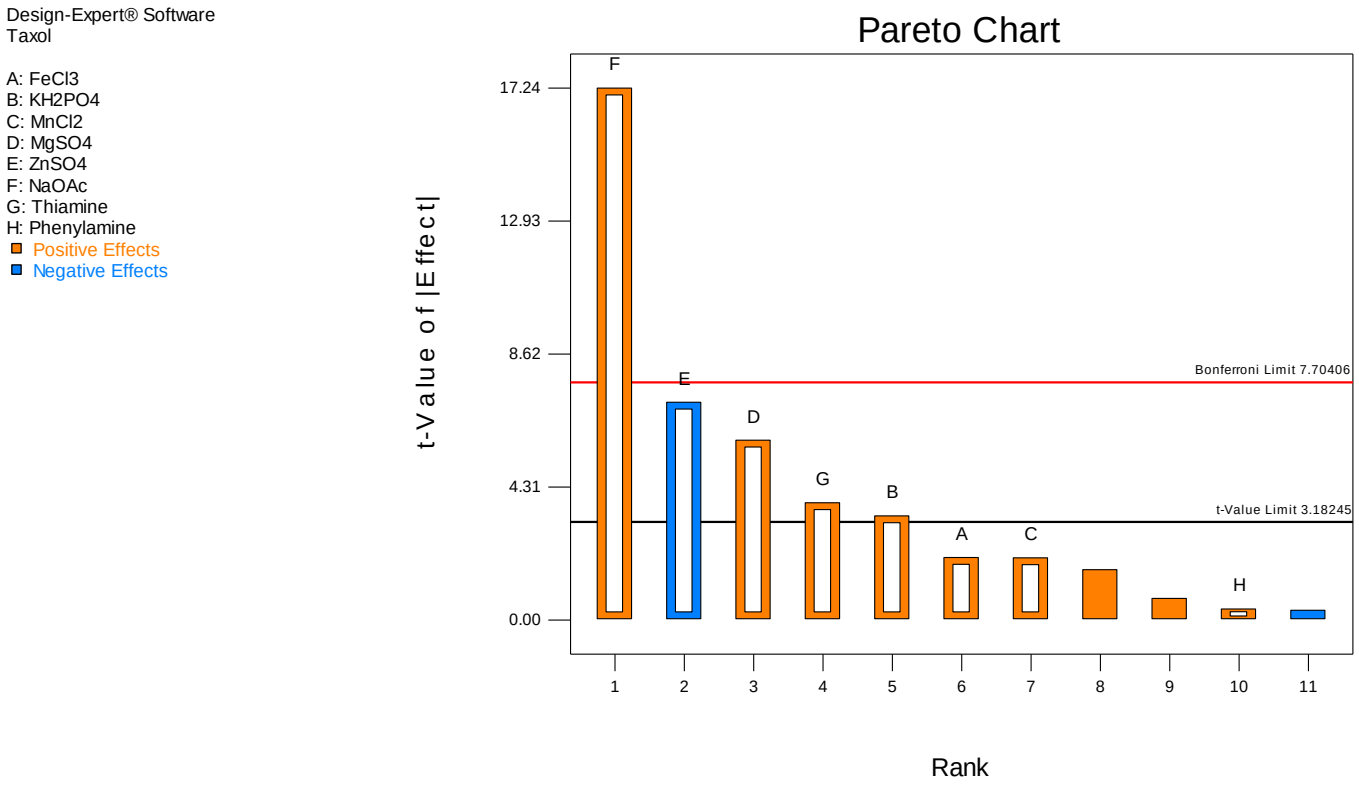


Fig. 4.33 Pareto chart showing the effects of different media components on taxol yield in *Fusarium redolens*

Table 4.21 Statistical data for the determination of variable significance in the Plackett-Burman design experiment

Symbol	Components	t Coefficient	Studied effect	Contribution (%)	Prob > F (p-value)*#
A	**$FeCl_3$**	2.9	6.97	0.98	0.1358
B	**KH_2PO_4**	3.3	11.6	2.72	0.0433 **(5)**
C	**$MnCl_2$**	2.97	6.93	0.97	0.1371
D	**$MgSO_4$**	6.7	20.03	8.12	0.0101 **(3)**
E	**$ZnSO_4$**	-7.3	-24.27	11.92	0.0058 **(2)**
F	**NaOAc**	17.24	59.27	71.08	0.0004 **(1)**
G	**Thiamine (Vitamin B1)**	4.2	13.07	3.46	0.0320 **(4)**
H	**Phenylalanine**	0.95	1.23	0.031	0.7436
	Model	-	-	-	0.0039 **(significant)**

***** Values of **"Prob > F"** less than **0.0500** indicate model terms are significant.

Values greater than **0.1000** indicate the model terms are not significant

4.4.3 Response surface methodology

Various combinations of the components were used and corresponding taxol yields (experimental and predicted) were recorded (Table 4.22). The amounts of remaining components in all assemblies were the same as those in basal medium. The response for taxol production was analyzed by linear multiple regression and graphical analysis using the Design-Expert version 8.0.7.1 software (Stat-Ease Corporation, Minneapolis, MN). The mathematical model incorporating the interactive effect of NH_4NO_3, $MgS0_4$ and NaOAc proposed for taxol production was as:

$$\textbf{Taxol} = +195.54 - 4.45A - 10.19B - 1.96C - 1.48B - 15.98AC - 3.35BC - 23.12A^2 - 18.94B^2 - 12.10C^2$$

A, B and C are the symbols representing concentrations of NH_4NO_3, $MgS0_4$ and NaOAc, respectively.

ANOVA of linear regression model equation demonstrated that the model equation was highly significant, as evident from value of "Model Prob>F" less than 0.0001 (Table 4.24). In this case A, B, AC, A^2, B^2 and C^2 are significant model terms. The "Lack of Fit Prob> F-value" of 0.0614 implies that the lack of fit is insignificant. The goodness of fit of model was tested by the determination coefficient (R^2). In the present study, R^2 value of this model is 0.9772, so it is reasonable to use the regression to analyse the trends in the responses.

The 3D response surface and 2D contour plots generated during data analysis are graphical representation of regression equation. The 2D contour plots in Fig. 4.35 illustrates the interaction of the concentrations of most significant effectors; NH_4NO_3, $MgSO_4$ and NaOAc on taxol production. From the study of these response surface contour plots,

maximum taxol production was obtained when the concentration of NH_4NO_3, $MgSO_4$ and NaOAc were 6.25, 0.63 and 1.25 g/l, respectively.

Table 4.22 Experimental recipe and response in Box-Behnken experimental design protocol for medium optimization

Trials	Coded values and actual values (g/L)			Taxol (µg/L)	
	NH_4NO_3	$MgSO_4$	NaOAc	Experimental	Predicted
1	-1 (2.5)	0 (0.63)	-1 (0.5)	145.7	150.76
2	-1 (2.5)	0 (0.63)	1 (2)	179.8	178.79
3	1 (10)	0 (0.63)	-1 (0.5)	172.8	173.81
4	1 (10)	0 (0.63)	1 (2)	143.0	137.94
5	0 (6.25)	-1 (0.25)	-1 (0.5)	178.1	173.30
6	0 (6.25)	-1 (0.25)	1 (2)	174.8	176.07
7	0 (6.25)	1 (1)	-1 (0.5)	160.9	159.62
8	0 (6.25)	1 (1)	1 (2)	144.2	149.00
9	-1 (2.5)	-1 (0.25)	0 (1.25)	166.9	166.64
10	1 (10)	-1 (0.25)	0 (1.25)	156.9	160.69
11	-1 (2.5)	1 (1)	0 (1.25)	153.0	149.21
12	1 (10)	1 (1)	0 (1.25)	137.0	137.36
13	0 (6.25)	0 (0.63)	0 (1.25)	198.1	195.54
14	0 (6.25)	0 (0.63)	0 (1.25)	192.9	195.54
15	0 (6.25)	0 (0.63)	0 (1.25)	196.6	195.54
16	0 (6.25)	0 (0.63)	0 (1.25)	192.3	195.54
17	0 (6.25)	0 (0.63)	0 (1.25)	197.8	195.54

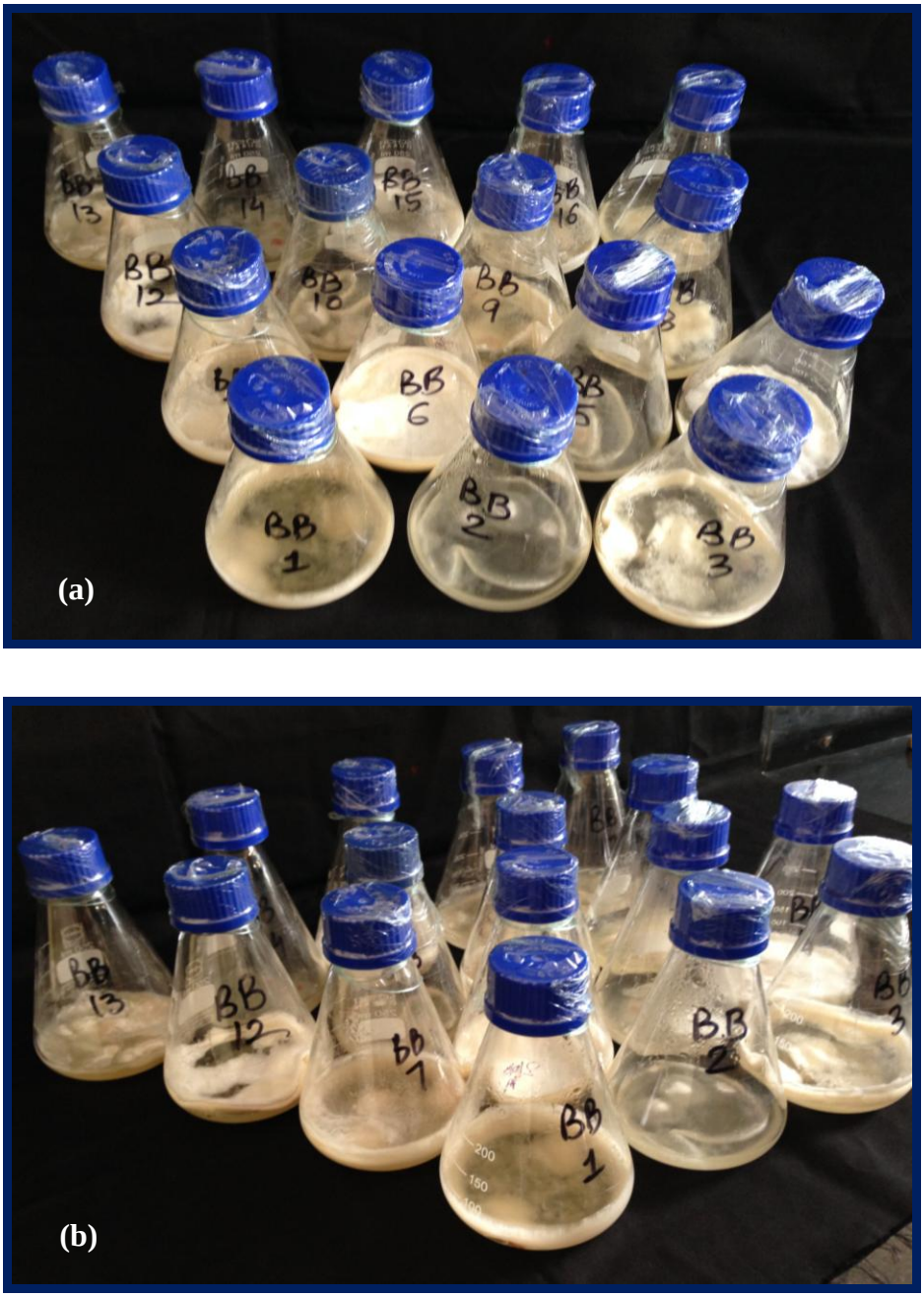


Fig. 4.34 a, b Fungal growth (*Fusarium redolens*) as per seventeen experiments formulated using Box-Behnken design

Table 4.23 Comparison of mycelial growth (Fresh weight) and taxol production of *Fusarium redolens* as per experimental recipe in Box-Behnken experimental design protocol for medium optimization

Trials	Fresh weight (mycelial growth in gm: 50 mL medium in 250 mL flask as per Box-Behnken experimental design)	Taxol (µg/L)
1	16.20	150.76
2	21.00	178.79
3	20.77	173.81
4	14.03	137.94
5	20.12	173.30
6	20.89	176.07
7	17.50	159.62
8	15.72	149.00
9	18.94	166.64
10	17.87	160.69
11	15.90	149.21
12	14.20	137.36
13	25.12	195.54
14	24.89	195.54
15	24.76	195.54
16	24.83	195.54
17	24.90	195.54

Table 4.24 Regression coefficients and their significance for response surface model

Source	Sum of squares	df	Mean square	*F*-value	Prob > *F*
Model	6943.04	9	771.45	**33.39**	<0.0001 **(significant)**
A	158.42	1	158.42	6.86	0.0345
B	830.28	1	830.28	35.94	0.0005
C	30.81	1	30.81	1.33	0.2861
$\mathbf{A^2}$	8.70	1	8.70	0.38	<0.0001
$\mathbf{B^2}$	1020.80	1	1020.80	44.19	<0.0001
$\mathbf{C^2}$	44.89	1	44.89	1.94	0.0013
AB	2250.67	1	2250.67	97.42	0.5588
AC	1511.21	1	1511.21	65.41	0.0003
BC	615.95	1	615.95	26.66	0.2060
Lack of fit	131.50	3	43.82	5.79	0.0614

A-NH_4NO_3; **B**-$MgSO_4$; **C**-NaOAc

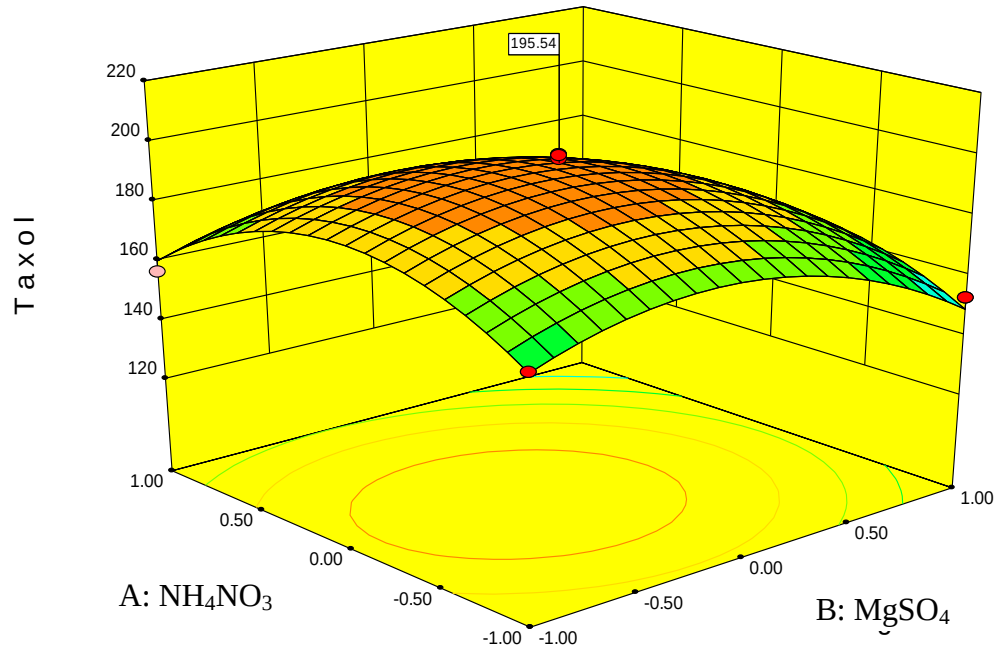
The model **F-value** of **33.39** implies the model is significant

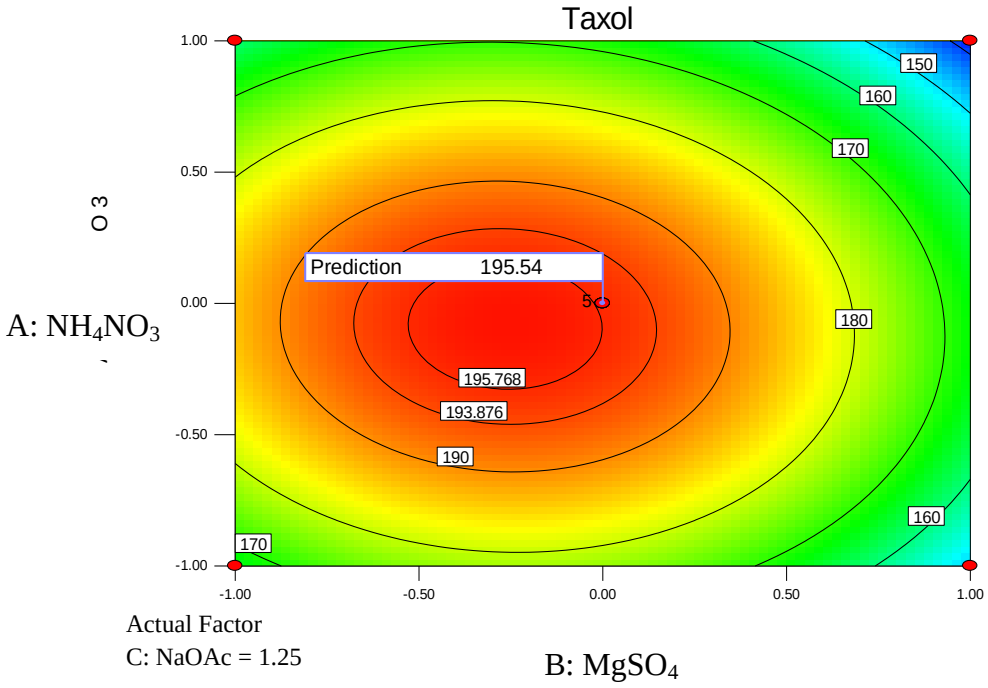
Values of **"probability > F"** less than **0.0500** indicate model terms are significant

In this case **A, B, AC,** $\mathbf{A^2}$**,** $\mathbf{B^{2,}}$ $\mathbf{C^2}$ are significant model terms

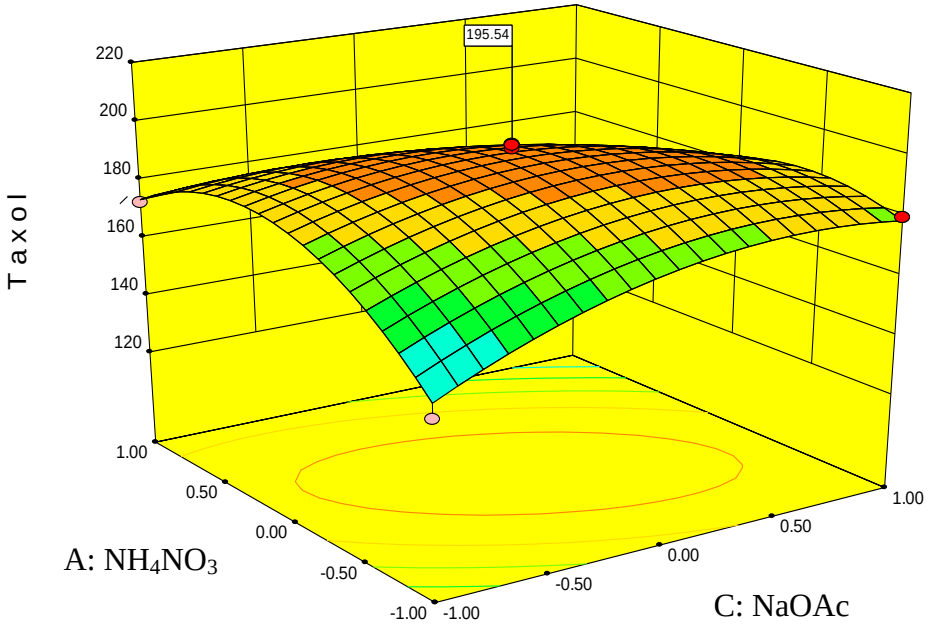
Values greater than **0.1000** indicate the model terms are not significant

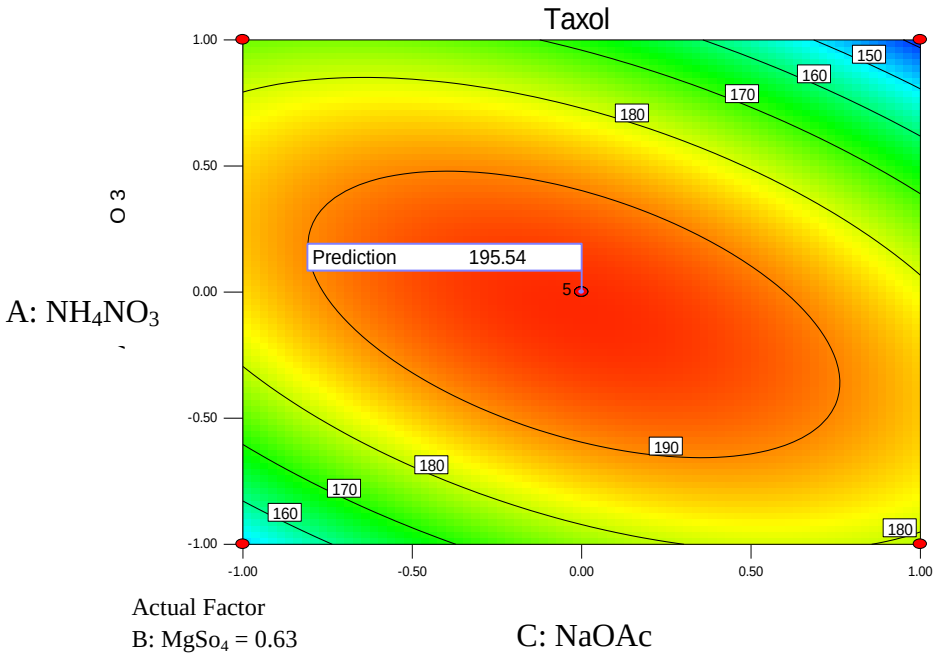
(A)


195.54
220
200
180
160
140
120
Taxol
1.00
0.50
0.00
-0.50
-1.00
A: NH4NO3
B: MgSO4


Taxol
Prediction 195.54
150
160
170
180
190
193.876
195.768
A: NH4NO3
B: MgSO4
Actual Factor
C: NaOAc = 1.25

(B)


195.54
220
200
180
160
140
120
Taxol
1.00
0.50
0.00
-0.50
-1.00
A: NH4NO3
C: NaOAc


Taxol
1.00
0.50
0.00
-0.50
-1.00
O 3
Prediction 195.54
150
160
170
180
190
A: NH4NO3
Actual Factor
B: MgSo4 = 0.63
C: NaOAc

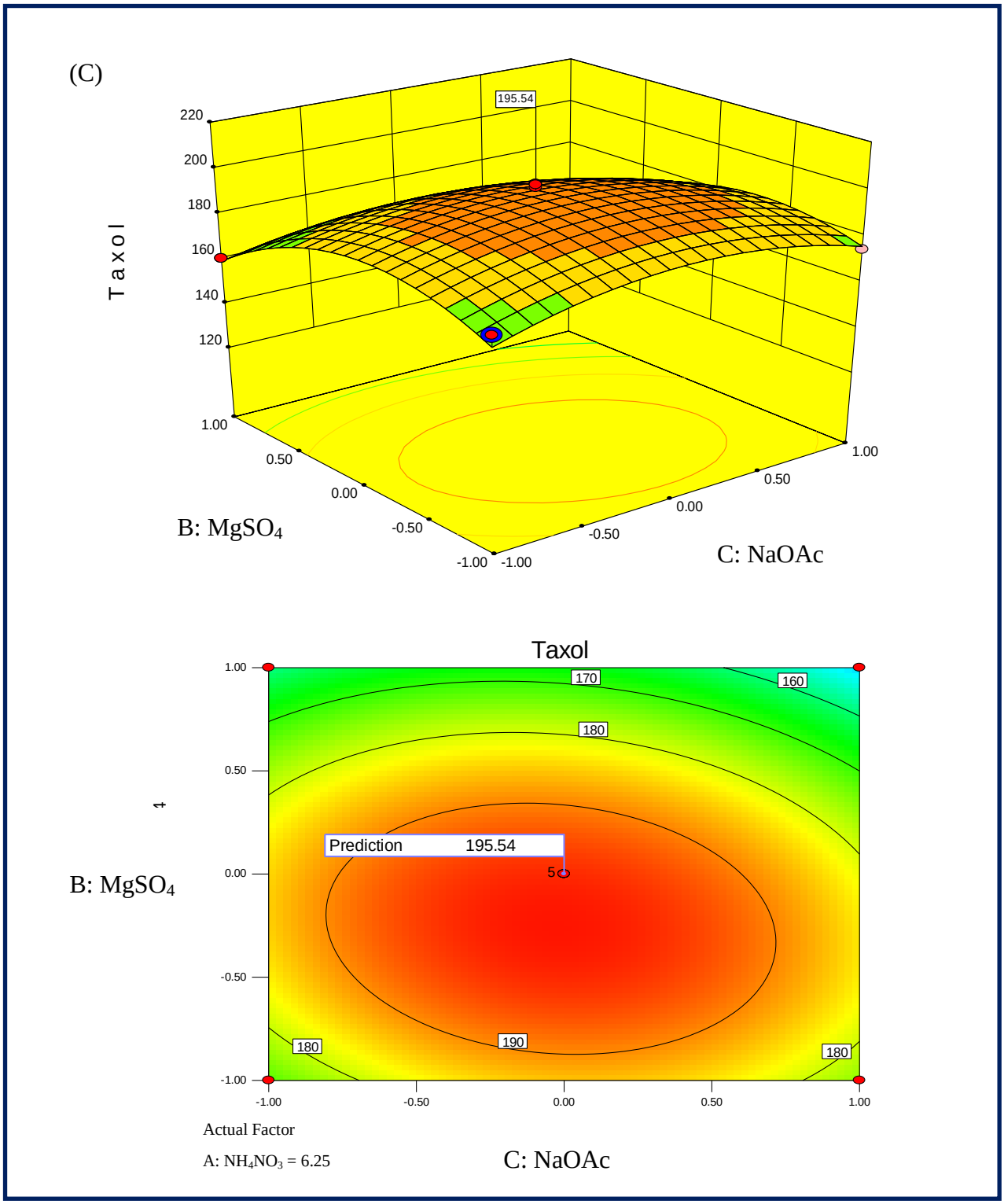


Fig. 4.35 3D surface and contour plots showing the effect of different variables on taxol production. **(A)** effect of NH_4NO_3 and $MgSO_4$ on taxol production; **(B)** effect of NH_4NO_3 and NaOAc on taxol production; **(C)** effect of $MgSO_4$ and NaOAc on taxol production

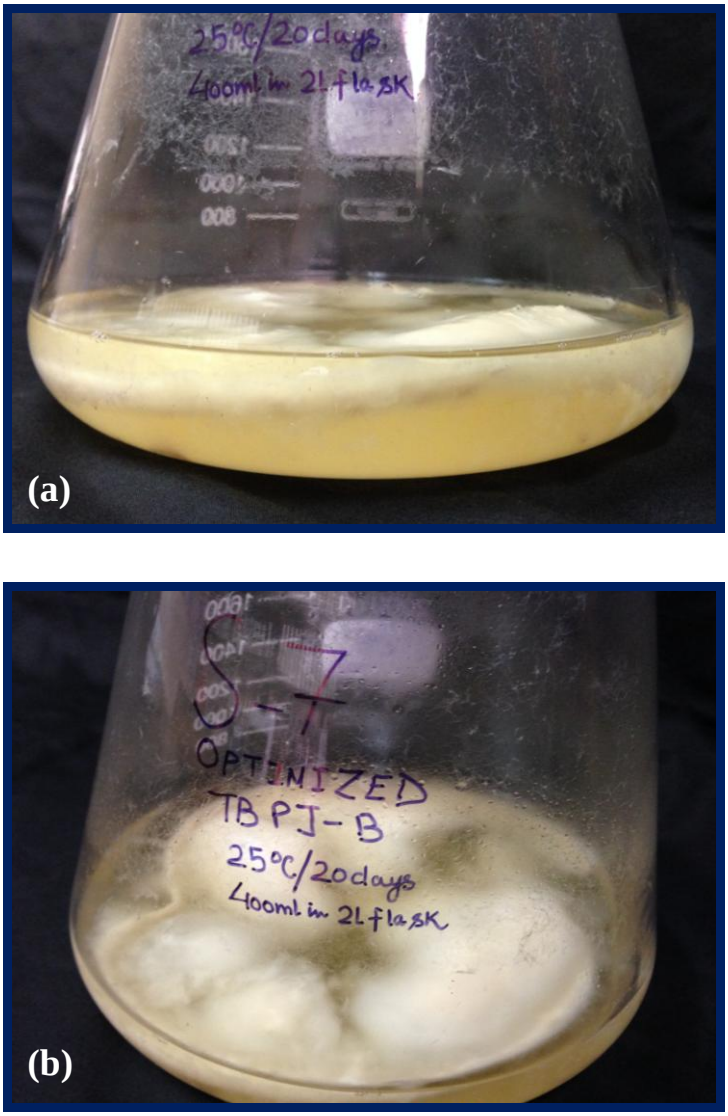


Fig. 4.36 a, b Growth of *Fusarium redolens* in optimized S7 medium

In order to verify the RSM predicted results, an experiment was performed under the optimized nutrients levels (Fig. 4.34). The values obtained (experimental) were compared with the predicted values. The predicted response in Box-Behnken experimental design for taxol production gave a value of 195 µg/L, while the actual experimental value was 198 µg/L, suggesting that experimental and predicted values were in good agreement. Further relation of taxol production with biomass was also noticed in this case also. Highest amount of taxol was found in the case of higher biomass favouring positive correlation between the two (Table 4.23).

Conclusions and salient findings

The current study has culminated in production of three times higher taxol production by application of statistical tool; Response surface methodology. The physical and chemical factors which affect the production of taxol in *Fusarium redolens* were firstly varied one at a time and after that, Plackett-Burman design was implied to screen multiple variables which affect the production of taxol. NH_4NO_3, $MgSO_4$ and NaOAc were found to be the most significant factors that affected the taxol yield. Based on the results of Box-Behnken design, the optimized media and conditions for maximum taxol production comprised sucrose, NH_4NO_3, $MgSO_4$, NaOAc incubated at 25^oC for 7 days with the initial pH of 6.5 which resulted in 3 folds increase in the production of taxol in the optimized media. Based on the promising results of this study, enhancement of taxol yield by application of statistical tools including RSM seems to be a promising solution for production of these highly demanding commercial drugs.

5. Discussion

5.1 Isolation and characterization of taxol producing endophytic fungi

Taxol is the best known and most studied affiliate of the taxane diterpenoids, or taxoids and has been utilized in chemotherapy for many types of cancers since 1970s. Traditional practices of extracting taxol from the bark of *Taxus* species are inefficient and environmentally costly. For example, while 1 kg of taxol treats just five hundred patients; the production of 1 Kg of taxol demands 10 tons of bark or 300 trees. For this reason, seeking new ways of obtaining taxol is the key to protect this limited, endangered resource and minimizing the cost of drug therapy. In this regard, scientists all over the world have focused on endophytic fungi, which are extensively found in almost all kinds of plants including *Taxus* species, that can produce physiologically active compounds, such as taxanes, which are the same or analogous with those obtained from their hosts (Lu et al. 2000, Strobel et al. 2004). This constitutes a new approach to resolve resource limitation and an alternative taxol source. Indeed, it represents a sumptuous opportunity to find new and interesting microorganisms among *Taxus* species in different settings and ecosystems.

In the present study, Northern Himalayan yew was selected as the source for isolating endophytic fungi as to date no endophytes having taxol-generating capability have been reported from *T. baccata* subsp. *wallichiana* growing in this region. This yew species ranges widely in the temperate Himalayan region and in the hills of Meghalaya, Nagaland and Manipur and most of its growth takes place in moist and damp environments. Endophytes associated with *T. baccata* (Himalayan yew) may have certain biological links with its host for attaining the capability of producing taxol. Being endosymbiotic in nature, several endophytic fungi may also inherit taxol-producing capability and this may be considered as a reason for the transfer of genes involved in taxol biosynthesis to endophytes harbouring in

different parts of yew trees. Taxol, a known wonder cancer drug holds many properties such as stability, less side-effects, and higher effectiveness and has always been in surplus demand in the market owing to its broad spectrum of antitumor action (Wani et al. 1971). In order to fulfil the increasing demand of pure drug, scientists have always been searching for efficient endophytic strains from *Taxus* sp. and species other than *Taxus* that have the capability of generating a good amount of taxol. Current work is one more step towards this quest.

Number of (around 60) endophytic fungi harbouring in the bark of *Taxus baccata* L. subsp. *wallichiana* (Zucc.) tree were isolated in this study. In contrast to the biochemical screening (traditional) method, the molecular marker-based screening was also used as it is a rapid, economical and efficient alternative for the screening of taxol-producing endophytic fungi (Zhang et al. 2008; Mirjalili et al. 2012). The main advantage of this method is that it is not dependent on the production of taxol; rather, it indicates the existance of some key genes required for taxol biosynthesis in the fungal genome. The taxol biosynthetic pathway in yew trees involves 19 enzymatic steps (Croteau et al. 2006), and we chose the two main end step genes *dbat* (involved in the formation of Baccatin III) and *bapt* (involved in phenylpropanoid side chain formation at C13) involved in taxol biosynthesis, for primary screening of taxol-producing endophytes by molecular screening. The use of gene-specific PCR amplification for screening the isolated endophytes made it feasible to screen all the isolates, which would otherwise have been very laborious and practically time consuming by biochemical screening method. Eight out of the 60 fungi had positive hits of *dbat* gene and had approximately 200 bp amplified fragments of this gene. Presence of *DBAT* gene is essential for taxol biosynthesis but cannot be relied completely because some fungi having *DBAT* gene may produce baccatin III, but not taxol. So, eight fungi harbouring *DBAT* gene were screened for the presence of the *BAPT* gene. Five fungal isolates (TBPJ-B, TBPJ-A, TBPJ-13, B-7 and C-1) showed amplification of 530 bp fragment of *BAPT* gene, suggesting that all of them may

produce taxol. Currently, *DBAT and BAPT* genes have been mostly used as molecular probes for the primary screening of taxol producing microorganisms (Zhou et al. 2007; Zhang et al. 2008), thus designing appropriate degenerate primers for amplification of more target genes, e.g. the final acylation step in taxol biosynthesis, taxoid C13-side-chain N-benzoyltransferase (DBTNBT), may be a better choice for screening. When the amplified DNA fragments of *BAPT* gene from all 5 endophytic isolates were analyzed and aligned with the protein sequences of *BAPT* gene of various *Taxus* species, the characterization gave a new understanding to the controversial hypothesis of horizontal gene transfer (HGT). It showed that evolutionary trajectory of taxol gene clusters amidst microbial and plant origin might be coexisting. Although HGT in fungi are predominantly reported (Fitzpatrick 2012), the ultimate plausibility of microbial taxol gene cluster by HGT hypothesis should be revisited and further investigated because approximately 20 genes ramified in the taxol biosynthesis make HGT rather improbable. Moreover, taxol-producing endophytic fungi have been isolated from plants which themselves are incapable of producing taxol (Li et al. 1996; Kumaran et al. 2009), implying that taxol biosynthesis in fungi may not be attained from HGT. In nature, gibberellin biosynthetic pathways in fungi and higher plants have evolved independently and not by HGT (Tudzynski 2005; Hedden et al. 2001). We thus presumed that taxol biosynthetic cluster might be repeatedly originated during evolution.

The polymerase chain reaction (PCR) provides a rapid and highly sensitive method for the primer-mediated enzymatic amplification of specific target sequences resulting in the exponential increase of target DNA copies. Molecular identification has increasingly been used as supplementary tool for the traditional systematic classification. Genomic DNA from all the 5 selected endophytic fungi was subjected to amplification of ITS region using ITS1 and ITS4 universal primers. Resultant PCR products were sequenced and sequence analysis revealed that fungi belong to *Fusarium, Gibberella, Microdiplodia and Paraconiothyrium*

species. Out of the two most widely used methods for molecular phylogenetic analyses [Maximum parsimony (MP) and Maximum Likelihood (ML)] MP was used to check the clades of these endophytes based on sequence analysis. The phylogenetic tree was reconstructed using ITS sequences of TBPJ-B, TBPJ-A, TBPJ-13, B-7 and C-1 wherein strain TBPJ-B clustered with *Fusarium redolens* (KC924920), B-7 clustered with *Fusarium tricinctum* (KF010839), C-1 with *Gibberella avenacea* (KF010838), TBPJ-A clustered with *Microdiplodia* sp. G16A (KF010841) and TBPJ-13 with *Paraconiothyrium brasiliense* (KF010840). All the isolates were also studied based on their morphology and microscopic characters. Phylogenetic analysis of the ITS region showed a reasonable degree of correlation with the morphological classification schemes of species within the genus.

In the past two decades, several endophytic microorganisms isolated from different geographical settings have been reported to produce taxol through biochemical or molecular marker screening (Flores-Bustamante et al. 2010), the majority of which belongs to *Alternaria* sp., *Alternaria taxi*, *Alternaria alternata*, *Aspergillus* sp., *Cladosporium* sp., *Fusarium lateritium*, *Monochaetia* sp., *Ozonium* sp., *Pestalotiopsis microspora*, *Pestalotiopsis guepinii*, *Pithomyces* sp., *Taxomyces andreanae*, *Trichoderma* sp., *Tubercularia* sp., *Phomopsis* sp., *Penicillium*, *Botrytis taxi* etc. (Stierle et al. 1993; Gangadevi and Muthumary 2008; Zhao et al. 2008, 2009; Zhou et al. 2010). Endophytic fungi capable of taxol production obtained from the bark of *Taxus baccata* subsp. *wallichiana* in the northern Himalayan regions of India represented a phylogenetically diverse array of fungal taxa, including some frequent and some rare genera, confirming that a few species are frequent colonizers and a majority of groups are rare inhabitants in woody plants of temperate to sub-tropical regions (Tejesvi et al. 2005).

5.2 Biochemical screening of taxol production by endophytic fungi

In order to screen the presence of taxol by different endophytic fungi, extracts of all the five representative species *Fusarium redolens* (TBPJ-B), *Microdiplodia* sp. (TBPJ-A), *Paraconiothyrium brasiliense* (TBPJ-13), *Fusarium verticillioides* (B-7) and *Phomopsis* sp. (C-1) with positive results in the primary screening were put through chromatographic analysis by TLC, HPLC and LC-MS for the detection of fungal taxol. The first insight into the presence of taxol in the extracts of the 5 fungal endophytes (TBPJ-13, B-7, TBPJ-A, TBPJ-B and C-1) was done by thin layer chromatography. Authentic taxol was used as positive control. The active principle on development of the thin layer plate appeared as a dark band under UV illuminator at 254 nm. In order to further verify these results, HPLC analysis of the fungal extract and authentic taxol was studied. The results gave a single peak under the same conditions when eluted from a reverse phase C18 column. The HPLC peak positions and peak shapes of taxol from fungal extracts had same retention time as that of standard taxol. Mycelium from 21 day old culture in S-7 semi synthetic medium was subjected to HPLC to quantify the amount of taxol produced by the fungus. Among the 5 taxol-producing fungi, TBPJ-B had the highest taxol yield of 66.25 µg/L, in comparison with those of TBPJ-A which produced 27.40 µg/L, B-7 produced 23.47 µg/L, TBPJ-13 produced 19.60 µg/L and C-1 produced 11.03 µg/L of taxol in S-7 semi synthetic liquid medium. Quantitative HPLC analysis of the fungal taxol showed that TBPJ-B (*Fusarium redolens*) produced the maximum amount of taxol compared to other isolates. These results are comparable with previously reported taxol-producing fungi isolated from different geographical settings (Stierle et al. 1993; Gangadevi and Muthumary 2008; Zhao et al. 2008, 2010). There are a number of reports in the literature about *Fusarium* sp. capable of taxol production in range of 0.13-286 µg/L with strain improvement procedures; *F. mairei* (20 µg/L, Dai and Tao 2008), *F. solani* (*Taxus celebica*, 1.6 µg/L, Chakravarthi et al. 2008), *F. solani* Tax-3 (*Taxus chinensis*,

163.35 µg/L, Deng et al. 2009). *Fusarium* and some *Aspergillus* species have also been reported earlier to be capable of producing taxol *in vitro* (Caruso et al. 2000; Zhao et al. 2008; Deng et al. 2009), but *F. redolens* and *F. verticillioides* are the first ever reports of endophytic fungi capable of taxol production obtained from *T. baccata* subsp. *wallichiana*. *Paraconiothyrium brasiliense*, *Microdiplodia* sp. and *Phomopsis* sp. reported from other *Taxus* plants have not been acquired from the Himalayan yew until now, implying that yews growing in different geographical settings can harbour novel, highly diverse taxol-producing endophytic strains and that certain taxol-producing fungi seem to be host specific. These endophytes that possess the capability to produce such important secondary metabolites may succeed in occupying a niche within the plant tissue or even contribute to host defence against the invading pathogens (Liu et al. 2009; Chandra 2012).

Convincing evidence for the identification of the fungal taxol was also obtained by high resolution mass spectroscopy (MS). Characteristically, the authentic taxol yielded an $(M+H)^+$ peak at *m/z* 854.7 and an $(M+Na)^+$ peak at *m/z* 876.8. By comparison, the fungal taxol also yielded a peak MH^+ at *m/z* 854.3 and MNa^+ at *m/z* 876.3 with characteristic fragments at *m/z* 569, 551, 509, 286 and 268. Major fragment ions observed in the mass spectrum of taxol can be placed into three categories which represents major portions of the molecule (McClure et al. 1992). The peaks of fungal taxol gave *m/z* ratios similar to the molecular ions of standard taxol, verifying that the 5 endophytic strains can produce taxol *in vitro*.

In this study, based on morphological and molecular data as well as phytochemical analysis, *Fusarium redolens*, *Microdiplodia* sp., *Paraconiothyrium brasiliense*, *Fusarium verticillioides* and *Phomopsis* sp. were determined as novel and promising strains of taxol producing endophytic fungi. As mentioned above, quantitative HPLC analysis showed that the taxol content of all these taxol producing endophytes was in the range of previously reported fungi, but was found to be relatively lower than that of *Taxus* species, the high

growth rate and short generation time make these endophytes advantageous to continue investigation. Notably, the extraction yield from taxol producing fungi has been recently enhanced by the use of new biotechnological techniques, such as fungal strain improvement (Xu et al. 2006), recombinant techniques (Wang et al. 2007; Zhou et al. 2008; Wei et al. 2009) and microbial fermentation engineering (Xu et al. 2006; Gogoi et al. 2008; Zhao et al. 2011). Thus, in order to fulfill the commercial demand for taxol, further work was focussed on ameliorating taxol yield in fungi by optimization of culture conditions. In addition, the paucity of a complete taxol biosynthetic cluster (5 unknown enzymatic steps) is at present a bottleneck for basic and applied research. Genome sequencing and analysis of taxol-producing microorganisms thus could significantly expand the number of known taxol biosynthetic genes to illuminate the whole pathway and provide the basis for heterologous production. From an evolutionary adaptation point of view, endophytic microorganism harbouring discrete metabolic functions could achieve extraordinary success in occupying a alcove within plant tissues or even contribute to host defense against various invading pathogens (Liu et al. 2009). Hence, analysis of genes involved in taxol synthesis from the diverse fungi will significantly intensify our understanding of the co-evolutionary mechanism of the endophyte host (Mirjalili et al. 2012).

5.3 Antitumorous activity of fungal taxol

Other than chromatographic methods for the determination of taxol in biological matrices, an immunoassay to study the antitumor effect of fungal extracts was employed. The antimitogenic activity of fungal taxol was assessed by potato disc tumor induction assay. This assay is known for its simplicity and reliability by many researchers (Coker et al. 2003) and has been used in screening of antitumor agents irrespective of their mode of action. Authentic taxol served as positive control and inhibited tumor production at all the tested concentrations. Fungal taxol from 5 endophytes (TBPJ-B, TBPJ-A, B-7, TBPJ-13 and C-1)

also inhibited tumor formation the same way the authentic taxol did. It was justified as starch in the potato tissue took up the stain and appeared dark brown in color, but tumors produced by *A. tumefaciens* did not take up the stain and appeared creamy to orange (Mc Laughlin & Rogers 1998). As both fungal taxol and authentic taxol inhibited tumor formation in potato discs, it was authenticated that the fungal taxol has antitumorigenic activity. Bacterial viability test showed that the standard drug and the drug in the fungal extracts did not affect the viability and growth of the bacteria i.e., drug didn't hinder bacterial tumor causing ability. So, it was depicted that action of drugs tested is on the formation of tumors and not on the bacterial viability.

5.4 Process optimization for taxol production from endophytic fungus *Fusarium redolens*

As it was envisaged that fungal taxol is novel and promising alternative for producing large amounts of the drug in very efficient and economical way in short periods of time, an attempt was made to enhance the production of taxol from efficient taxol producing endophyte *Fusarium redolens* by optimizing the media components through One factor at a time (OFAT) and Response Surface Methodology analysis. Response surface methodology (RSM) allows a rapid screening of most significant factors affecting the production of any metabolite/ enzyme or other by products.

After initial screening of different variables by OFAT, Plackett-Burman design was employed to screen significant factors affecting Taxol production. This test showed that NaOAc, $MgSO_4$ and $ZnSO_4$ were most important components which gave main contribution on taxol production. Although the PB design could be successfully used for equitable prediction of the significant level of different variables (micronutrients) affecting the response (taxol production), some of the significant interactive effects of the chosen variables (two-factor interaction) were staggered in its complex structure. Because of this reason, the

actual main effects of these variables may have been influenced. Hence, the significant components NaOAc and $MgSO_4$ whose interactive effects were demonstrated by PB design were further modelled more precisely by RSM. From the study of response surface contour plots, maximum taxol production was obtained when the concentration of NH_4NO_3, $MgSO_4$ and NaOAc were 6.25, 0.63 and 1.25 g/l, respectively. The verification of RSM predicted results was done by performing experiment under the optimized nutrients levels for taxol production. The predicted response in Box-Behnken experimental design for taxol production gave a value of 195 µg/L, while the actual experimental value was 198 µg/L, suggesting that experimental and predicted values were in good agreement. From these results, the basic parameters optimized for biomass and taxol production by *F. redolens* were observed when the culture was grown at 25^{o}C for 7 days with the initial pH of 6.5. The growth and taxol production remained almost static after 20 days of incubation. Huang et al. 2001 also reported antitumor and antifungal activity of endophytic fungi when the cultures were incubated at 25ºC for 7 days. Taxol is a secondary metabolin whose synthesis is regulated by carbon and nitrogen sources, phosphate, trace elements and precursors (Parra et al. 2005). In the present study, sucrose served as best carbon source for taxol production compared to other carbon sources tested. Contrary, Xu et al. 2006 reported glucose as best carbon source for taxol production by *Fusarium maire*. Ammonium nitrate served as good source of nitrogen in this study. Strobel et al. 1996 reported ammonium nitrate and peptone as good nitrogen sources for taxol production by *Pestalotiopsis microspora*. NH_4NO_3 was also found to have significant effect on taxol production in case of *F. maire* (Xu et al. 2006). Nutrition plays a crucial role in the onset and intensity of secondary metabolism. To achieve high product yield, it is prerequisite to design appropriate production medium as there is relationship between media composition and biosynthesis of secondary metabolites (Wang et al. 2013). NaOAc, $MgSO_4$ and $ZnSO_4$ were shown as important components for taxol production in this

study. Sodium acetate as an activator was also reported with *Taxomyces andreanae* (Stierle et al. 1993). Similar results were also reported by Xu et al. (2006) who found enhancement of taxol yield from 20 µg/L to 225 µg/L in mutant strain of *F. maire*. Zhao et al. (2011) found enhancement of taxol production from 397 to 456 µg/ L while Xu et al. (2006) reported 31% increase in taxol production upon media optimization. Luo and He (2004) also found 2 times higher production of Paclitaxel upon optimizing the concentration of elicitors and precursors. The present study led to enhancement of fungal taxol up to three folds which are encouraging in terms of product yield for scale up studies and commercial exploitation of fungal taxol. Statistical analysis of coefficients in Plackett-Burman design experiments demonstrated that $MgSO_4$ and NaOAc are the major factors influencing the production of taxol. With the application of RSM, production of fungal taxol increased 3 folds compared to unoptimized medium. Our work has proved the effectiveness of statistical tools in bioprocess optimization for exploring scale up feasibility, augmenting the economic viability of the process. Further improvement in taxol production can be achieved by improvement of the fungal strains through genetic manipulations and/ or mutagenesis or by augmentation of elicitor and precursor molecules to this fungal strain.

In conclusion, the present study led to isolation of several novel endophytic fungi capable of producing the anticancerous drug taxol which is highly demandable and pharmaceutically important medicine. *DBAT* and *BAPT* genes were found to be reliable molecular markers for initial screening of taxol biosynthetic pathway in endophytic fungi. Taxol producing endophytes were found to belong to *Fusarium redolens, Microdiplodia sp., Paraconiothyrium brasiliense, Fusarium tricinctum* and *Gibberella avenacea*. Several of these fungal species have been found to be the first reports of taxol producing endophytes. Along with molecular screening, biochemical screening of fungal taxol by HPLC and LC MS further confirmed that all the isolates were highly efficient in producing taxol. Potato disc

tumour assay verified the efficacy of fungal taxol comparable to that of commercial paclitaxel drug which paved way for fulfilling the demand of this wonder drug by these alternative sources other than plant source. Statistical tool RSM further led to three times enhanced production of taxol in endophytic fungus *Fusarium redolens* by optimization of various media components, highlighting its usage for commercial applications. This study is a step forward in proving the potential of endophytes in production of highly demandable pharmaceutical drugs and paves way for replacement of conventional sources of these drugs by serving as effective alternatives.

6. Summary

Endophytes, the organisms which inhabit living tissues of plants have long been recognized as important and novel resources of bioactive products. Number of medicinal plants has been reported to be source of fungal endophytes with capability to produce several novel pharmaceutically important compounds. Paclitaxel, the world's first billion dollar anticancer blockbuster, has long been isolated from yew plants which are conventionally largest sources for this drug. But due to various shortcomings of harvesting huge quantities of these plants for retrieval of small amounts of taxol and to fulfill the large demand of this drug, alternative sources need to be investigated. The primary focus of the current work is to investigate the potential of endophytic fungi for production of this wonder drug.

Till now, not much work has been reported on taxol producing capability of fungal endophytes from *Taxus baccata* L. subsp. *wallichiana* (Zucc.) Pilger growing in northern Himalayan region of India. The present work was aimed to study the diversity of taxol-producing endophytic fungi associated with *Taxus baccata* subsp. *wallichiana*, screening endophytic fungi for taxol production using molecular markers, evaluating anticancerous activity of fungal taxol and optimization of media components and conditions for enhancing the taxol production.

In this work, endophytic fungi from bark of yew trees of Indian Himalayan Region (IHR) were isolated and screened for production of anticancerous drug, taxol. Bark samples of *T. baccata* subsp. *wallichiana* were collected from different locations of Northern Indian Himalayan Region (IHR); Bhadrewah (district Doda, Jammu and Kashmir), Shimla (Himachal Pradesh) and Almora (Uttrakhand). The bark pieces were surface disinfected, small pieces of inner bark were excised and placed on the surface of potato dextrose agar (PDA) medium. Petri plates were incubated at 28ºC for 10 days to allow the growth of

endophytic fungi. Total of 60 fungal morphotypes were isolated and the isolates were coded and stored in sterile distilled water as agar plugs.

Taxol producing endophytic fungi were firstly screened on the basis of essential genes involved in taxol biosynthetic pathway i.e. *DBAT* (10-deacetylbaccatin III-10-O-acetyl transferase) and *BAPT* (C-13 phenylpropanoid side chain-CoA acyltransferase). Both the genes served as reliable molecular markers for initial screening of taxol producing capability in endophytes. The analysis of individual endophytic fungal genome with regard to presence of potential genes involved in taxol synthesis revealed that 8 out of 60 isolated endophytes were harbouring *DBAT* gene and 5 endophytic isolates out of these 8 gave positive hits for *BAPT* gene. Thus, five efficient endophytic fungi were isolated from Himalayan yew bark harbouring *dbat* and *bapt* genes of taxol biosynthetic pathway. These cultures were designated as TBPJ-B, TBPJ-A, TBPJ-13, B-7 and C-1. Five fungal isolates showed amplification of a 530 bp fragment of the *bapt* gene, suggesting that all of them may produce taxol. Sequences of *bapt* gene of endophytic fungi were analyzed using BLASTx and aligned with the protein sequences of the *bapt* gene of various *Taxus* species. The partial protein sequences of the *bapt* gene of all 5 fungi showed high homology with protein sequences of the *bapt* gene of various yew species, confirming the presence of a taxol biosynthetic pathway in all the endophytic fungal strains.

The fungal isolates which showed positive results in molecular screening were characterized based on morphological characters. Morphological characteristics of the fungi like mycelia, conidiophores and conidia were microscopically examined. To authenticate the classical identification of the five fungal internal transcribed spacer (ITS) regions of the rDNA from genomic DNA was amplified using the universal primers, ITS1 and ITS4. BLAST search was performed to find the possible homologous sequences of newly sequenced taxa from the GenBank database. The phylogenetic tree was reconstructed using

the maximum parsimony method and the Kimura two parameter distance calculation by the MEGA5 software. Based on classical and molecular profiling, the isolates were identified as *Fusarium redolens* (TBPJ-B), *Fusarium tricinctum* (B-7), *Gibberella avenacea* (C-1), *Microdiplodia* sp. G16A (TBPJ-A) and *Paraconiothyrium brasiliense* (TBPJ-13).

Biochemical screening of fungal taxol was done by TLC, HPLC and LC-MS from extracts of fungal cultures in accordance to the literature which verified the efficacy of all isolates in production of taxol. The 5 fungal endophytes were grown in S7 medium for 3 weeks to detect production of taxol. The fungal mycelia of all the isolates were extracted 3 times in methanol individually, while the fermentation broths were extracted with three equal volumes of dichloromethane (DCM). The extracts of each fungal isolate were purified by column chromatography and quantified using HPLC. Among the 5 taxol producing fungi, *Fusarium redolens* produced significantly higher taxol yield of 66.25 µg/L, compared to other fungi. *Microdiplodia* species produced 27.40 µg/L, *Fusarium tricinctum* 23.47 µg/L, *Paraconiothyrium brasiliense* 19.60 µg/L and *Gibberella avenacea* produced 11.03 µg/L of taxol in S7 liquid medium. Characteristically, the authentic taxol yielded an $(M+H)^+$ peak at *m/z* 854.7 and an $(M+Na)^+$ peak at *m/z* 876.8. By comparison, the fungal taxol also yielded a peak MH^+ at *m/z* 854.3 and MNa^+ at *m/z* 876.3 with characteristic fragments at *m/z* 569, 551, 509, 286 and 268 by LC-MS analysis. The peaks of fungal taxol gave *m/z* ratios similar to the molecular ions of standard taxol, verifying that the 5 endophytic strains are able to produce taxol *in vitro*.

Anticancerous activity of the 5 endophytic fungal isolates of the present study was investigated by potato disc tumour assay in comparison to authentic paclitaxel using *Agrobacterium tumefaciens* as the tumor causing agent along with bacterial viability test. It was noticed that fungal taxol was equally effective in inhibiting tumour formation as that of the standard paclitaxel. This further paved way for use of endophytic fungus as an effective

and novel source for production of anticancer drug, taxol. On the other hand the bacterial viability tests showed that the standard drug and the drug in the fungal extracts did not affect the viability of the bacteria, i.e., the drug did not hinder bacterial tumor causing ability.

In order to meet the ever increasing demand of this wonder drug, there is need for enhancing the production of this anticancer agent. In this regard, optimization of different physical and nutritional parameters for enhanced production of taxol from *Fusarium redolens* was done by one-factor-at-a-time (OFAT) and response surface methodology (RSM) approach. *F. redolens* was selected for process optimization as it was capable of producing maximum amount of taxol compared to other isolates. Highest taxol production was found to be at incubation period of 20 days, medium to flask volume (*Vm/Vf*) ratio of 0.20, pH 6.5 and incubation temperature of 25ºC. The statistical optimization led to enhancement in taxol production from 66 µg/L to 198 µg/L when optimized concentrations of factors: NH_4NO_3 (6.25 g/L), $MgSO_4$ (0.63 g/L) and NaOAc (1.25g/L) were added to S-7 liquid medium along with 8% (w/v) sucrose as carbon source and 0.8% (w/v) ammonium nitrate as nitrogen source. Upon optimization, there was three fold increase in the production of taxol by this endophyte. RSM was found to be effective tool for ameliorating the production of fungal taxol in the present work.

In summary, the research presented in the current study investigated the potential of yet untapped endophytes as prolific source of pharmaceutically relevant natural products, with focus on paclitaxel. As the highly desirable search for sustainable and economically feasible sources of this excellent antitumour agent has tempted various authors to draw premature conclusions proclaiming endophytes to be independent taxane bio-factories, we contend that the answer to the issue of paclitaxel supply which might lie within the interplay between the plant hosts and their microbial inhabitants, under evolutionary and environmental control. While one has to be mindful that the problem we set out to address is

several orders of magnitude larger than those with which we are familiar, no one can deny the opportunities that present themselves in the era of modern functional genomics and systems biology. There is need for further research at the molecular level for a better understanding of the host endophyte interaction in order to explore the reasons for host-endophyte gene transfer.

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Appendix I

1. Chemicals and consumables

All chemicals used had at least pro analysis quality. Solvents for extraction were for synthesis grade, whereas solvents for chromatography were gradient grade or HPLC/LC-MS grade. Chemicals, media components, enzymes and consumables were purchased from the following companies: Hi-Media, Merck, Agilent Technologies, Applied Biosystems, Waters, BioRad, Yamato, Thermo scientific/Fermentas, Eppendorf, Invitrogen, Nikon, Millipore, New England Biolabs, Qiagen, Sigma, Whatman and Tarsons.

2. Vector

- The vector(s) used for cloning was pTZ 57R/T (Thermo scientific, USA).

3. Microorganisms

- For amplification of plasmid DNA or for expression of proteins *Escherichia coli* (*E. coli*) strain *E. coli* DH5α™ (Invitrogen) was used. Genotype DH5α™: F-80*lacZ*ΔM15Δ (*lac*ZYA-*arg*F), U169, *rec*A1, *end*A1, *hsd*R17 (rk-, mk+), *pho*A, *sup*E44λ-*thi*-1, *gyr*A96, *rel*A1.
- *Agrobacterium tumefaciens* (MTCC No. 431) was used as the tumor-causing agent in Potato disc tumor induction assay to check the antitumorigenic activity of fungal taxol.

4. Media and antibiotics

All media listed were prepared with double distilled water and sterilized for 15 min at 121ºC prior to usage. For production of solid media 1.5-2 % agar was added before sterilization.

(a)

Yeast Extract-Mannitol (YEM) medium	
Yeast extract	1 g/L
Mannitol	10 g/L
K_2HPO_4	0.5 g/L
Magnesium sulphate	0.2 g/L
Sodium chloride	0.1 g/L
pH 7.0 ± 0.2	

(b)

***Luria-Bertani* (LB) medium**	
NaCl	10 gm/L
Tryptone	10 gm/L
Yeast extract	5 gm/L
pH 7.0 ± 0.2	

(c)

Nutrient broth (NB)	
Peptone	5 g/L
Beef extract	1.5 g/L
Yeast extract	1.5 g/L
Sodium chloride	5 g/L
7.4 ± 0.2	

(d)

S7 medium	
Glucose	1 gm/L
Fructose	3 gm/L
Sucrose	6 gm/L
Sodium acetate	1 gm/L
Peptone	1 gm/L
Thiamine	1 mg/L
Biotin	1 mg/L
Pyridoxal	1 mg/L
Calcium pantothenate	1 mg/L
$MgSO_4$	3.6 mg/L
$CaNO_3$	6.5 mg/L
$Cu(NO_3)_2$	1 mg/L
$ZnSO_4$	2.5 mg/L
$MnCl_2$	5 mg/L
$FeCl_3$	2 mg/L
Phenylalanine	5 mg/L
Sodium benzoate	100 mg/L
Potassium dihydrogenphosphate (KH_2PO_4; 1M, pH 6.8)	1 mL

❖ **Antibiotic used**

Ampicillin was used as antibiotic with stock concentration of 100 mg/mL and 100 μg/mL as the working concentration in the media. Dependent on the experiment, antibiotic(s) was added after sterilization of medium and cooling to room temperature. Antibiotic solution was filter sterilized before use.

(e) LB/amp+ agar plates

Prepared LB broth as mentioned above, added agar (15 gm/L), autoclaved, and cooled to 50°C, added ampicillin 50 µg/ml, Poured plates and stored at 4°C.

(f) IPTG stock solution (0.1M)

1.2 gm IPTG, added water to 50 ml final volume, filtered and stored at 4°C.

(g) X-Gal (2ml)

100 mg 5-bromo,4-chloro,3-indolyl, D galactoside dissolved in 2ml N,N-dimethylformamide, covered with aluminum foil and stored at 20°C.

(h) LB plates with Ampicillin/IPTG/X-Gal

Prepared LB plates with ampicillin as above; 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-Gal spread over the surface of LB ampicillin plate and allowed to absorb for 30 minutes at 37°C prior to use.

(5) Reagents

(a)

1 M Tris HCl pH 8.0	
Tris	121 gm
Dissolve in about 700 mL of H_2O **Bring pH down to 8.0 by adding concentrated HCl (approx. 50 mL).** **Bring total volume to 1 L with dd H_2O**	

(b)

0.5 M EDTA	
EDTA	186.12 gm
Add about 700 ml H_2O	
NaOH pellets	16-18 gm
Adjust pH to 8.0 with a few more pellets, **EDTA won't dissolve until the pH is near 8.0** **Bring total volume to 1 L with dd H_2O.**	

(c)

TE buffer	
1M Tris HCl pH 8.0	10 ml
0.5M EDTA	2 ml
Bring total volume to 1 L with dd H_2O	

(d)

CTAB (cetyltrimethyl ammonium bromide) buffer (for DNA extraction)	
2% CTAB	20 gm CTAB
20 mM EDTA	40 mL 0.5 M EDTA
100 mM Tris-HCl pH 8.0	100 mL 1M Tris-HCl
1.4M NaCl	280 mL 5 M NaCl
Make up to 1 L with dd H_2O and autoclave	

(e)

CTAB (cetyltrimethyl ammonium bromide) buffer (for RNA extraction)	
CTAB	2 gm
2% PVP (Polyvinylpyrolidone K30)	2 gm
25 mM EDTA	5 mL 0.5M disodium EDTA
1M Tris-HCl pH 8.0	10 mL
2M NaCl	11.68 gm
Make up to 100 mL 0.1% DEPC water and autoclave	

(f) Plasmid extraction solutions

Plasmid Extraction Solution I	
Glucose	50 mM
Tris-HCl	25 mM (pH 8.0)
Na_2EDTA	10 mM (pH 8.0)
Filter sterilizes small batches of 100 mL and store at 4ºC. Glucose will caramelize if autoclaved.	

Plasmid Extraction Solution II (Prepare fresh)	
5M NaOH	0.4 mL
20% SDS	0.5 mL
Sterile distilled water	9.1 mL

Plasmid Extraction Solution III	
5M K*Acetate (Final concentration 3M)	60 mL
Glacial acetic acid (Final concentration 5M)	11.5 mL
Distilled water	28.5 mL

(g)

Agarose Gel Loading Dye (6X)	
Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol in water	30.0%

(h)

TBE Buffer (10x)	
Tris-HCl	0.09 M (pH 8)
Boric acid	0.9 M
EDTA	0.02 M (pH 8)

(6) Kit systems

Qiagen	QIAquick® Gel Extraction Kit
	QIAquick® PCR purification kit
Thermo scientific	InsTAclone PCR Cloning Kit
	RevertAid First Strand cDNA Synthesis Kit

Appendix II

Internal transcribed spacer (ITS) sequences of five fungal isolates

1. *Fusarium redolens* TBPJ-B 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

GenBank: KC924920.1

FASTA Graphics
Go to:

```
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DEFINITION  Fusarium redolens 18S ribosomal RNA gene, partial sequence;
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REFERENCE   1  (bases 1 to 567)
  AUTHORS   Garyali,S., Reddy,M.S. and Kumar,A.
  TITLE     Fusarium redolens, a new taxol producing endophytic fungus isolated
            from Taxus baccata subsp. wallichiana (Zucc.)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 567)
  AUTHORS   Garyali,S., Reddy,M.S. and Kumar,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (22-APR-2013) Department of Biotechnology, Thapar
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                     /isolation source="bark"
                     /host="Taxus baccata subsp. wallichiana"
                     /db_xref="taxon:48865"
                     /tissue_type="mycelium"
                     /country="India"
                     /PCR_primers="fwd_seq: tccgtaggtgaacctgcgg, rev_seq:
                     tcctccgcttattgatatgc"
     misc RNA        <1..>567
                     /note="contains 18S ribosomal RNA, internal transcribed
                     spacer 1, 5.8S ribosomal RNA, internal transcribed spacer
                     2, and 28S ribosomal RNA"
ORIGIN
        1 tgatcttccg taggtgaacc tgcggaggga tcattaccga gtttacaact cccaaacccc
       61 tgtgaacata ccttactgtt gcctcggcgg atcagcccgc tcccggtaaa acgggacggc
      121 ccgccagagg acccctaaac tctgtttcta tatgtaactt ctgagtaaaa ccataaataa
      181 atcaaaactt tcaacaacgg atctcttggt tctggcatcg atgaagaacg cagcaaaatg
      241 cgataagtaa tgtgaattgc agaattcagt gaatcatcga atctttgaac gcacattgcg
      301 cccgccagta ttctggcggg catgcctgtt cgagcgtcat ttcaaccctc aagccctcgg
      361 gtttggtgtt ggggatcggc gagcctttct ggcaagccgg ccccgaaatc tagtggcggt
      421 ctcgctgcag cctccattgc gtagtagtaa aaccctcgca actggaacgc ggcgcggcca
      481 agccgttaaa cccccaactt ctgaatgttg acctcggatc aggtaggaat acccgctgaa
      541 cttaagcata tcaataagcg gagggag
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2. *Microdiplodia* sp. TBPJ-A 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

GenBank: KF010841.1

FASTA Graphics
Go to:

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LOCUS       KF010841                752 bp    DNA     linear   PLN 22-SEP-2013
DEFINITION  Microdiplodia sp. TBPJ-A 18S ribosomal RNA gene, partial sequence;
            internal transcribed spacer 1, 5.8S ribosomal RNA gene, and
            internal transcribed spacer 2, complete sequence; and 28S ribosomal
            RNA gene, partial sequence.
ACCESSION   KF010841
VERSION     KF010841.1  GI:544169412
KEYWORDS    .
SOURCE      Microdiplodia sp. TBPJ-A
  ORGANISM  Microdiplodia sp. TBPJ-A
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Dothideomycetes; Dothideomycetes incertae sedis; Botryosphaeriales;
            Botryosphaeriaceae; Microdiplodia.
REFERENCE   1  (bases 1 to 752)
  AUTHORS   Garyali,S., Reddy,M.S. and Kumar,A.
  TITLE     Diversity of taxol producing endophytic fungi from the bark of
            Himalayan Yew and study of antimitotic activity of fungal taxol
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 752)
  AUTHORS   Garyali,S., Reddy,M.S. and Kumar,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-MAY-2013) Biotechnology, Thapar University, Patiala,
            Punjab 147004, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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                     /db xref="taxon:1394173"
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                     /collected_by="Sanjog Garyali"
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                     tcctccgcttattgatatgc"
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                     /note="contains 18S ribosomal RNA, internal transcribed
                     spacer 1, 5.8S ribosomal RNA, internal transcribed spacer
                     2, and 28S ribosomal RNA"
ORIGIN
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       61 cgcggccctc ggcgggagca acagctaccg tcgggcggta gaggtaacac tttcacgcgc
      121 cgcatgtctg aatccttttt ttacgagcac ctttcgttct ccttcggcgg ggcaacctgc
      181 cgttggaacc tatcaaaacc ttttttttgca tctagcatta cctgttctga tacaaacaat
      241 cgttacaact ttcaacaatg gatctcttgg ctctggcatc gatgaagaac gcagcgaaat
      301 gcgataagta gtgtgaattg cagaattcag tgaatcatcg aatctttgaa cgcacattgc
      361 gccccttggt attccatggg gcatgcctgt tcgagcgtca tctacaccct caagctctgc
      421 ttggtgttgg gcgtctgtcc cgcctctgcg cgcggactcg ccccaaattc attggcagcg
      481 gtccttgcct cctctcgcgc agcacattgc gcttctcgag gtgcgcggcc cgcgtccacg
      541 aagcaacatt accgtctttg acctcggatc aggtagggat acccgctgaa cttaagcata
      601 tcaataagcg gaggaaaaga aaccaacagg gattgcccta gtaacggcga gtgaagcggc
      661 aacagctcaa atttgaaatc tggctctctt tgggggtccg agttgtaatt tgcagaggat
      721 gctttggcat tggcggcggt ctaagttcct tg
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3. *Paraconiothyrium brasiliense* strain TBPJ-13 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

GenBank: KF010840.1

FASTA Graphics
Go to:

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LOCUS       KF010840                643 bp    DNA     linear   PLN 22-SEP-2013
DEFINITION  Paraconiothyrium brasiliense strain TBPJ-13 18S ribosomal RNA gene,
            partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA
            gene, and internal transcribed spacer 2, complete sequence; and 28S
            ribosomal RNA gene, partial sequence.
ACCESSION   KF010840
VERSION     KF010840.1  GI:544169411
KEYWORDS    .
SOURCE      Paraconiothyrium brasiliense
  ORGANISM  Paraconiothyrium brasiliense
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Dothideomycetes; Pleosporomycetidae; Pleosporales; Massarineae;
            Montagnulaceae; Paraconiothyrium.
REFERENCE   1  (bases 1 to 643)
  AUTHORS   Garyali,S., Reddy,M.S. and Kumar,A.
  TITLE     Diversity of taxol producing endophytic fungi from the bark of
            Himalayan Yew and study of antimitotic activity of fungal taxol
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 643)
  AUTHORS   Garyali,S., Reddy,M.S. and Kumar,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-MAY-2013) Biotechnology, Thapar University, Patiala,
            Punjab 147004, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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                     /strain="TBPJ-13"
                     /isolation_source="bark"
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                     (Himalayan Yew)"
                     /db xref="taxon:300254"
                     /country="India"
                     /collected_by="Sanjog Garyali"
                     /PCR_primers="fwd_name: ITS1, fwd_seq:
                     tccgtaggtgaacctgcgg, rev_name: ITS4, rev_seq:
                     tcctccgcttattgatatgc"
     misc RNA        <1..>643
                     /note="contains 18S ribosomal RNA, internal transcribed
                     spacer 1, 5.8S ribosomal RNA, internal transcribed spacer
                     2, and 28S ribosomal RNA"
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       61 gatcattatc tattccatga ggtgcggtcg cggccctcgg cgggagcaac agctaccgtc
      121 gggcggtaga ggtaacactt tcacgcgccg catgtctgaa tccttttttt acgagcacct
      181 ttcgttctcc ttcggcgggg caacctgccg ttggaaccta tcaaaacctt tttttgcatc
      241 tagcattacc tgttctgata caaacaatcg ttacaacttt caacaatgga tctcttggct
      301 ctggcatcga tgaagaacgc agcgaaatgc gataagtagt gtgaattgca gaattcagtg
      361 aatcatcgaa tctttgaacg cacattgcgc cccttggtat tccatggggc atgcctgttc
      421 gagcgtcatc tacaccctca agctctgctt ggtgttgggc gtctgtcccg cctctgcgcg
      481 cggactcgcc ccaaattcat tggcagcggt ccttgcctcc tctcgcgcag cacattgcgc
      541 ttctcgaggt gcgcggcccg cgtccacgaa gcaacattac cgtctttgac ctcggatcag
      601 gtagggatac ccgctgaact taagcatatc aataagcgga gga
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4. *Fusarium tricinctum* strain B-7 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

GenBank: KF010839.1

FASTA Graphics
Go to:

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LOCUS       KF010839                517 bp    DNA     linear   PLN 22-SEP-2013
DEFINITION  Fusarium tricinctum strain B-7 internal transcribed spacer 1,
            partial sequence; 5.8S ribosomal RNA gene and internal transcribed
            spacer 2, complete sequence; and 28S ribosomal RNA gene, partial
            sequence.
ACCESSION   KF010839
VERSION     KF010839.1  GI:544169410
KEYWORDS    .
SOURCE      Fusarium tricinctum
  ORGANISM  Fusarium tricinctum
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae;
            Fusarium; Fusarium tricinctum species complex.
REFERENCE   1  (bases 1 to 517)
  AUTHORS   Garyali,S., Reddy,M.S. and Kumar,A.
  TITLE     Diversity of taxol producing endophytic fungi from the bark of
            Himalayan Yew and study of antimitotic activity of fungal taxol
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 517)
  AUTHORS   Garyali,S., Reddy,M.S. and Kumar,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-MAY-2013) Biotechnology, Thapar University, Patiala,
            Punjab 147004, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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                     /isolation source="bark"
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                     (Himalayan Yew)"
                     /db_xref="taxon:61284"
                     /country="India"
                     /collected_by="Sanjog Garyali"
                     /PCR_primers="fwd_name: ITS1, fwd_seq:
                     tccgtaggtgaacctgcgg, rev_name: ITS4, rev_seq:
                     tcctccgcttattgatatgc"
     misc_RNA        <1..>517
                     /note="contains internal transcribed spacer 1, 5.8S
                     ribosomal RNA, internal transcribed spacer 2, and 28S
                     ribosomal RNA"
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       61 aaaacgggac ggcccgccag aggatccaaa ctcttgctgt tattgtaact tctgagtaaa
      121 acaaacaaat aaatcaaaac tttcaacaac ggatctcttg gttctggcat cgatgaagaa
      181 cgcagcaaaa tgcgataagt aatgtgaatt gcagaattca gtgaatcatc gaatctttga
      241 acgcacattg cgcccgccag tattctggcg ggcatgcctg ttcgagcgtc atttcaaccc
      301 tcaagccccc gggtttggtg ttggggatcg gcaagccttc tggcgagccg ccccctaaat
      361 ctagtggcgg tctcactgca gcctccattg cgtagtagct aacacctcgc aactggaacg
      421 cggtgcggcc atgccgttaa acccccaact tctgaatgtt gacctcggat caggtaggaa
      481 tacccgctga acttaagcat atcaataagc ggaggaa
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5. *Fusarium avenaceum* strain C1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

GenBank: KF010838.1

FASTA Graphics
Go to:

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LOCUS       KF010838                590 bp    DNA     linear   PLN 22-SEP-2013
DEFINITION  Fusarium avenaceum strain C1 18S ribosomal RNA gene, partial
            sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene,
            and internal transcribed spacer 2, complete sequence; and 28S
            ribosomal RNA gene, partial sequence.
ACCESSION   KF010838
VERSION     KF010838.1  GI:544169409
KEYWORDS    .
SOURCE      Fusarium avenaceum
  ORGANISM  Fusarium avenaceum
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae;
            Fusarium; Fusarium tricinctum species complex.
REFERENCE   1  (bases 1 to 590)
  AUTHORS   Garyali,S., Reddy,M.S. and Kumar,A.
  TITLE     Diversity of taxol producing endophytic fungi from the bark of
            Himalayan Yew and study of antimitotic activity of fungal taxol
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 590)
  AUTHORS   Garyali,S., Reddy,M.S. and Kumar,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-MAY-2013) Biotechnology, Thapar University, Patiala,
            Punjab 147004, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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                     /isolation_source="bark"
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                     (Himalayan Yew)"
                     /db xref="taxon:40199"
                     /country="India"
                     /collected_by="Sanjog Garyali"
                     /PCR_primers="fwd_name: ITS1, fwd_seq:
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                     tcctccgcttattgatatgc"
                     /note="teleomorph: Gibberella avenacea"
     misc RNA        <1..>590
                     /note="contains 18S ribosomal RNA, internal transcribed
                     spacer 1, 5.8S ribosomal RNA, internal transcribed spacer
                     2, and 28S ribosomal RNA"
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       61 ttacaactcc caaacccctg tgaacatacc ttaatgttgc ctcggcggat cagcccgcgc
      121 cccgtaaaac gggacggccc gccagaggac ccaaactcta atgtttctta ttgtaacttc
      181 tgagtaaaac aaacaaataa atcaaaactt tcaacaacgg atctcttggt tctggcatcg
      241 atgaagaacg cagcaaaatg cgataagtaa tgtgaattgc agaattcagt gaatcatcga
      301 atctttgaac gcacattgcg cccgctggta ttccggcggg catgcctgtt cgagcgtcat
      361 ttcaaccctc aagcccccgg gtttggtgtt ggggatcggc tctgccttct ggcggtgccg
      421 cccccgaaat acattggcgg tctcgctgca gcctccattg cgtagtagct aacacctcgc
      481 aactggaacg cggcgcggcc atgccgtaaa accccaactt ctgaatgttg acctcggatc
      541 aggtaggaat acccgctgaa cttaagcata tcaataggcc gggagggaag
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Partial DNA sequences encoding *BAPT* (baccatin III-aminophenylpropanoyl-13-O-transferase) of five fungal isolates

1. *Fusarium redolens* TBPJ-B baccatin III-aminophenylpropanoyl-13-O-transferase (*BAPT*) gene, partial cds

GenBank: KC924919.1

FASTA Graphics

Go to:

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LOCUS       KC924919                570 bp    DNA     linear   PLN 12-AUG-2013
DEFINITION  Fusarium redolens C-13 phenylpropanoid side chain-CoA
            acyltransferase gene, partial cds.
ACCESSION   KC924919
VERSION     KC924919.1  GI:529280071
KEYWORDS    .
SOURCE      Fusarium redolens
  ORGANISM  Fusarium redolens
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae;
            Fusarium.
REFERENCE   1  (bases 1 to 570)
  AUTHORS   Garyali,S., Reddy,M.S. and Kumar,A.
  TITLE     Fusarium redolens, a new taxol producing endophytic fungus isolated
            from Taxus baccata subsp. wallichiana (Zucc.)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 570)
  AUTHORS   Garyali,S. and Kumar,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-APR-2013) Department of Biotechnology, Thapar
            University, Bhadson Road, Patiala, Punjab 147004, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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                     /isolation source="bark"
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                     tcgccatctctgccatactt"
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     CDS             join(<1..342,462..>570)
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                     baccatin III with beta-phenylalanoyl-CoA as the acyl donor
                     to form N-debenzoyl-2'-deoxytaxol"
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                     /translation="PLSAIDNIARAFSNILLVYSASHRVSADPAKVIREALSKVLVYY
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      421 cagcatgctt cggttctctt tgttgaatgt ctttcatgca ggtaactcgt tttacatgtg
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2. *Microdiplodia* sp. TBPJ-A baccatin III-aminophenylpropanoyl-13-O-transferase (*BAPT*) gene, partial cds

GenBank: KF010845.1

FASTA Graphics

Go to:

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LOCUS       KF010845                569 bp    DNA     linear   PLN 22-SEP-2013
DEFINITION  Microdiplodia sp. TBPJ-A baccatin
            III-aminophenylpropanoyl-13-O-transferase (BAPT) gene, partial cds.
ACCESSION   KF010845
VERSION     KF010845.1  GI:544169419
KEYWORDS    .
SOURCE      Microdiplodia sp. TBPJ-A
  ORGANISM  Microdiplodia sp. TBPJ-A
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Dothideomycetes; Dothideomycetes incertae sedis; Botryosphaeriales;
            Botryosphaeriaceae; Microdiplodia.
REFERENCE   1  (bases 1 to 569)
  AUTHORS   Garyali,S., Reddy,M.S. and Kumar,A.
  TITLE     Diversity of taxol producing endophytic fungi from the bark of
            Himalayan Yew and study of antimitotic activity of fungal taxol
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 569)
  AUTHORS   Garyali,S., Reddy,M.S. and Kumar,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-MAY-2013) Biotechnology, Thapar University, Patiala,
            Punjab 147004, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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                     (Himalayan Yew)"
                     /db_xref="taxon:1394173"
                     /country="India"
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                     to form N-debenzoyl-2'-deoxytaxol"
                     /note="involved in taxol biosynthetic pathway"
                     /codon_start=1
                     /product="baccatin
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                     /protein_id="AGW01020.1"
                     /db_xref="GI:544169420"
                     /translation="PLSAIDNIARAFSNILLVYSASHRVSADPAKVIREALSKVLVYY
                     YPFAGRLRKKENGELEVECTGQGVLFVEAMADSDLSVLTDLDDYNPSFQQLLFSVPQD
                     ADIEDLHLFIVQVTRFTCGGFVVGANVYGSVCDGKGFGQFLQSMAEMA"
ORIGIN
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      121 ctggtttatt attacccttt tgctgggcgg ctcagaaaaa aagaaaatgg ggaacttgaa
      181 gtggagtgca cagggcaggg tgttctgttt gtggaagcca tggctgacag cgacctttca
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3. *Paraconiothyrium brasiliense* strain TBPJ-13 baccatin III-aminophenylpropanoyl-13-O-transferase (*BAPT*) gene, partial cds

GenBank: KF010844.1

FASTA Graphics

Go to:

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LOCUS       KF010844                569 bp    DNA     linear   PLN 22-SEP-2013
DEFINITION  Paraconiothyrium brasiliense strain TBPJ-13 baccatin
            III-aminophenylpropanoyl-13-O-transferase (BAPT) gene, partial cds.
ACCESSION   KF010844
VERSION     KF010844.1  GI:544169417
KEYWORDS    .
SOURCE      Paraconiothyrium brasiliense
  ORGANISM  Paraconiothyrium brasiliense
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Dothideomycetes; Pleosporomycetidae; Pleosporales; Massarineae;
            Montagnulaceae; Paraconiothyrium.
REFERENCE   1  (bases 1 to 569)
  AUTHORS   Garyali,S., Reddy,M.S. and Kumar,A.
  TITLE     Diversity of taxol producing endophytic fungi from the bark of
            Himalayan Yew and study of antimitotic activity of fungal taxol
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 569)
  AUTHORS   Garyali,S., Reddy,M.S. and Kumar,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-MAY-2013) Biotechnology, Thapar University, Patiala,
            Punjab 147004, India
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4. *Fusarium tricinctum* strain B-7 baccatin III-aminophenylpropanoyl-13-O-transferase (*BAPT*) gene, partial cds

GenBank: KF010842.1

FASTA Graphics

Go to:

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DEFINITION  Fusarium tricinctum strain B-7 baccatin
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ACCESSION   KF010842
VERSION     KF010842.1  GI:544169413
KEYWORDS    .
SOURCE      Fusarium tricinctum
  ORGANISM  Fusarium tricinctum
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae;
            Fusarium; Fusarium tricinctum species complex.
REFERENCE   1  (bases 1 to 569)
  AUTHORS   Garyali,S., Reddy,M.S. and Kumar,A.
  TITLE     Diversity of taxol producing endophytic fungi from the bark of
            Himalayan Yew and study of antimitotic activity of fungal taxol
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 569)
  AUTHORS   Garyali,S., Reddy,M.S. and Kumar,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-MAY-2013) Biotechnology, Thapar University, Patiala,
            Punjab 147004, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
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5. *Fusarium avenaceum* strain C1 baccatin III-aminophenylpropanoyl-13-O-transferase (*BAPT*) gene, partial cds

GenBank: KF010843.1
FASTA Graphics
Go to:

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LOCUS       KF010843                569 bp    DNA     linear   PLN 22-SEP-2013
DEFINITION  Fusarium avenaceum strain C1 baccatin
            III-aminophenylpropanoyl-13-O-transferase (BAPT) gene, partial cds.
ACCESSION   KF010843
VERSION     KF010843.1  GI:544169415
KEYWORDS    .
SOURCE      Fusarium avenaceum
  ORGANISM  Fusarium avenaceum
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            Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae;
            Fusarium; Fusarium tricinctum species complex.
REFERENCE   1  (bases 1 to 569)
  AUTHORS   Garyali,S., Reddy,M.S. and Kumar,A.
  TITLE     Diversity of taxol producing endophytic fungi from the bark of
            Himalayan Yew and study of antimitotic activity of fungal taxol
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 569)
  AUTHORS   Garyali,S., Reddy,M.S. and Kumar,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-MAY-2013) Biotechnology, Thapar University, Patiala,
            Punjab, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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