MICROPROPAGATION AND SECONDARY METABOLITE STUDIES IN TAXUS SPP. AND WITHANIA SOMNIFERA (L.) DUNAL.

A THESIS SUBMITTED TO THE UNIVERSITY OF PUNE FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY

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CERTIFICATE

This is to certify that the work incorporated in the thesis entitled **"Micropropagation and Secondary Metabolite Studies in *Taxus* spp. and *Withania somnifera* (L.) Dunal."** submitted by Mrs. Anjali Abhay Kulkarni was carried out under my supervision at Plant Tissue Culture Division, National Chemical Laboratory, Pune. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

Place: Pune (Dr. K. V. Krishnamurthy)

Date: Research Guide

Key to Abbreviations

Abbreviation	Full form
2,4-D	2,4-Dichlorophenoxyacetic acid.
2-iP	6-(γ,γ-dimethylallylamino)-purine or N^6-(2-isopentyl)-adenine.
°C	Degree Celcius (Temperature)
AC	Activated charcoal.
ANOVA	Analysis of variance
B5	Gamborg et al. Medium (1968).
BAP	N^6 Benzylaminopurine.
BM	Basal medium.
CH	Casein hydrolysate.
CM	Coconut milk.
DCR	Gupta and Durzan's Medium (1985).
DW/dw	Dry weight
EDTA	Ethylenediaminetetraacetic acid.
FW/fw	Fresh weight.
GA3	Gibberelic acid.
HPLC	High pressure liquid chromatography.
IAA	Indolyl-3-acetic acid.
IBA	Indolyl-3-butyric acid.
KIN/K	Kinetin, 6-furfurylaminopurine.
LM	Litvay's Medium (1981).
µE	Microeinstein.
mcg/µg	Microgram.
µL	Microlitre.
µM	Micromete.r
MLM	Modified Litvay's Medium by Anjali A. Kulkarni (1997).
MR	Methyl red test.
MS	Murashige and Skoog's Medium (1962).
NA	Nutrient agar.
NAA	1-Naphthaleneacetic acid.
NB	Nutrient broth.

Contd…..

Key to Abbreviations Contd.

Abbreviation	Full form
PDA	Potato-dextrose-agar.
P	Picloram, 4-amino-3,5,6-trichloropyridine-2-carboxylic acid.
PR	Propagation medium.
PVP	Polyvinylpyrrolidone
RH	Relative humidity.
rpm	Revolutions per minute
SH	Schenk and Hildebrandt's Medium (1972).
SD	Standard deviation.
SE	Standard error.
TDZ	Thidiazouron, N-phenyl-N'-1,2,3-thidiazol-5-ylurea.
TLC	Thin layer chromatography.
UV	Ultraviolet light
UV-VIS	Ultraviolet-Visible (Spectrum of wavelength)
VP	Voges-Proskauer reaction.
v/v	Volume by volume (Concentration)
w/v	Weight by volume (Concentration)
WPM	Lloyd and McCown's Woody Plant Medium (1981).
YEB	Yeast, beef extract medium (1995).

SYNOPSIS

Since the dawn of civilization upto the twenty-first century, plants have been the most important sources of drugs for the mankind. One of the most exciting discoveries during this long journey has been the isolation of a variety of chemicals from plants, which have potent anti-cancer activities. Cancer is a major killer disease all over the world and more than six million new cases are reported every year.

"Taxol", a novel diterpenoid, isolated first from the bark of *Taxus brevifolia* Nutt. (Taxaceae) has been the most promising anti-cancer agent isolated in recent years. It is also reported from all the known spp. of *Taxus* including the Himalayan Yew: *Taxus baccata* ssp. *wallichiana* Zucc. Pilg. These plants are also rich in a variety of other related molecules namely **"Taxanes"** with different degrees of cytotoxicity and anti-tumor activities.

The plants have very low content of **taxol** (0.04-0.1% on dry weight basis) in mature organs. These trees are very slow growing and their seeds have a long dormancy period of two years. In addition, they are becoming endangered due to overexploitation. Hence the tissue culture work was started in the Himalayan Yew with the aim to explore the possibility of standardizing a micropropagation protocol because of its extreme slow growth and non-availability of the planting material as well as to assess **taxane** production *in vitro*.

"Withaferin-A", a withasteroid/steroidal lactone, first isolated *from Withania somnifera* (L.) Dunal has anti-tumor activity in a number of systems. The plant belonging to Solanaceae is used extensively in Ayurvedic system of medicine. It also has a number of tropane alkaloids effective against arthritis, rheumatism, fevers, fatigue etc. Plant tissue culture work was initiated with the aim to micropropagate selected superior chemotypes from various explants and to assess for the possible presence of **Withaferin-A** in tissue culture raised plants, callus cultures and hairy root cultures.

The present thesis is divided into six chapters and an annexure followed by summary and a list of references.

CHAPTER 1 GENERAL INTRODUCTION.

This chapter deals with an overview of developments in plant tissue culture, both in angiosperms and gymnosperms with special reference to medicinal plants. *In vitro* studies carried out in *Taxus* spp. and *Withania* spp. so far have also been described.

CHAPTER 2 MATERIALS AND METHODS (GENERAL).

Compositions of various plant tissue culture and bacteriological media used has been included in this chapter as also the various techniques used during the course of this work.

CHAPTER 3 CONTAMINATION CONTROL.

This chapter deals with bacterial and fungal contaminants and phenolic oxidation observed during the course of this work and measures undertaken to control and overcome these problems.

- % data for contaminants observed in *Taxus* spp. and *Withania* spp.
- Identification of contaminant bacteria based on morphological and biochemical characters.
- Comparison with reported data using a specially developed software program for identifying the contaminating bacteria upto the genus level.
- Identification of fungi based on vegetative and reproductive structures.
- Antibiogramme for bacteria and determination of MIC and MBC of effective antibiotics.
- Determination of effective anti-fungal agents.
- Development of a pre-treatment solution with both anti-fungal and anti-bacterial agents to overcome contamination problem in *Taxus* spp. and data for reduction in contamination with this solution.
- Steps undertaken to reduce contamination in *Withania* spp. cultures.

CHAPTER 4 STUDIES ON CALLUS INDUCTION FROM VARIOUS EXPLANTS OF *TAXUS* SPP. AND ANALYSIS OF TAXANE CONTENT OF *IN VITRO* AND *IN VIVO* TISSUES.

Here callus induction is reported from different explants on different basal media supplemented with various combinations of plant growth regulators. Callus induction is confirmed by histology. The taxane contents (Taxol and 10-DAB, 10-deacetyl baccatin-III) are analyzed by TLC and HPLC in different callus lines and are compared against the contents of the explants of the parent trees (*in vivo* tissues) determined by TLC and HPLC.

CHAPTER 5 *IN VITRO* STUDIES ON MICROPROPAGATION FROM MATURE AND JUVENILE EXPLANTS OF *TAXUS* SPP.

- The present chapter describes precocious germination of embryos on various basal media and the effect of light and darkness, culture vessel, cold treatment, developmental stage, locations and growth regulators.
- The chapter also describes accelerated, precocious primary sprouting from axillary and apical buds of mature explants as influenced by different basal media and growth regulator combinations, light conditions etc. It also describes growth of separated sprouts on various subculture media.

CHAPTER 6 DIRECT REGENERATION OF SHOOTS FROM VARIOUS EXPLANTS OF *WITHANIA* SPP. AND ANALYSIS OF WITHAFERIN-A PRODUCTION THEREFROM.

- This chapter summarizes the results of direct regeneration of shoots from various explants such as leaves, nodes, internodes, hypocotyls and embryos. Various parameter studies affecting the regeneration of plants from each of the explant are also reported. Direct regeneration of shoots is confirmed by histology.
- The present chapter also describes TLC and HPLC analysis of seedlings, multiple shoot and hairy root cultures of *Withania somnifera* .

ANNEXURE INDUCTION OF HAIRY ROOT CULTURES IN *WITHANIA* BY INFECTION WITH *AGROBACTERIUM RHIZOGENES*.

This annexure describes the preliminary results obtained in induction of hairy root cultures with different strains of *Agrobacterium rhizogenes* and their growth studies. The analysis of hairy roots for withanolide production is described in the previous chapter.

SUMMARY.

This part of the thesis summarizes the findings of the present investigation and its future implications.

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This part of the thesis lists all the references together.

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CHAPTER 1

General Introduction.

Preamble

In recent years a lot of attention has been devoted to novel molecules derived from natural sources that exhibit a range of clinical and pharmacological activities. This has led to a flurry of research activities in phytochemicals, synthesized by various plants, growing in diverse habitats and displaying a range of habits.

In our laboratory a number of plants producing a number of anti-cancer and anti-tumor metabolites were identified and detailed investigations were undertaken with respect to morphogenesis, *in vitro* culture and production of secondary metabolites and their chemical elucidation.

The present thesis describes the work on morphogenesis, genetic transformation and production of anti-cancer compounds *in vitro* of two plants namely, *Taxus baccata* ssp. *wallichiana* Zucc. Pilg. and *Withania somnifera* (L.) Dunal.

Taxus baccata ssp. *wallichiana* Zucc. Pilg. is a woody and endangered Himalayan Gymnosperm producing the most important anti-cancer diterpenoid **Taxol** and a range of other clinically important and structurally related **Taxanes** (Baloglu and Kingston, 1999 and references therein). *Withania somnifera* (L.) Dun. is a herbaceous Solanaceous plant producing anti-tumor **Withanolides** and a range of **Tropane Alkaloids** having anti-arthritic, anti-rheumatic and anti-depression properties (Malhotra et al., 1961; Uma Devi et al., 1992, 1993, 1995).

Because of their diverse habits, habitats and taxonomic differences both these plants responded differently *in vitro*. **Hence the present thesis has beeen organized as follows for better clarity, reading and understanding:**

1. **Chapter 1**: General Introduction describes History of Plant Tissue Culture, Morphogenesis and Regeneration *in vitro* (PART A) and Secondary Metabolite Production by *in vitro* Cultures (PART B). It also describes the overall objectives of the present thesis in brief.
2. **Chapter 2**: Describes the General Methodology of plant tissue culture techniques followed for both the plant species. The specific techniques, media and conditions used for each of the plant species are described in their respective chapters.

3. **Chapter 3**: Outlines the strategy employed for Control of Contamination and Phenolic Browning. Since the problems of bacterial and fungal contamination and phenolic browning are common for both the plant species, the procedure followed to evolve a control strategy for contamination and phenolic browning, applicable for the explants of both *Taxus* spp. and *Withania* spp. is written as a **"Common"** chapter.
4. **Chapter 4**: Deals with the work done on *Taxus* spp. including an Introduction, Literature Survey related to undifferentiated *in vitro* cultures of yews and **Taxol** Production therefrom and Objectives in detail. It also includes Callus Formation, Nutrient Media Optimization, Suspension Cultures and Analysis of **Taxane** Production in *in vitro* Cultures of *Taxus baccata* ssp. *wallichiana* Zucc. Pilg.
5. **Chapter 5**: Includes an Introduction, Literature Survey related to precocious germination and regeneration studies in *in vitro* cultures of yews and Objectives in detail. It also details Micropropagation Studies on *Taxus baccata* ssp. *wallichiana* Zucc. Pilg. with Mature and Juvenile Explants. Results obtained with Precocious Germination and Endosperm Culture have also been described here. The results obtained have also been extrapolated to plant material collected from different locations in the Himalayas.
6. **Chapter 6**: Outlines the work done on *Withania somnifera* (L.) Dun. including Literature Survey and Objectives pertinent to the species. It also includes Direct Shoot Regeneration from Various Explants of *Withania somnifera* (L.) Dun. and Analysis of **Withanolide** Production therefrom.
7. **Annexure**: Describes the preliminary results obtained on Hairy Root Production from various explants of *Withania somnifera* (L.) Dun. Culture of Hairy and Seedling Roots in Liquid Culture and Analysis of **Withanolide** Production therefrom forms the contents of this chapter.
8. **Bibliography.**
9. **Summary:** Describes concisely the results obtained during the course of the present work.

PART A. GENERAL INTRODUCTION TO PLANT TISSUE AND CELL CULTURE TECHNIQUES.

In the last forty years the flow of biological discovery has swelled from a trickle into a torrent, driven by a number of new methodologies developed in Plant Tissue Culture, Recombinant DNA Technology, Monoclonal Antibodies and Microchemical Instrumentation (Opportunities in Biology, 1989). Great advances are seen in health care with development of new therapeutic drugs and improved methods of diagnosis. This is true especially for ailments such as cancer, AIDS, Alzheimer's.

Biological research has been transformed from a collection of single discipline endeavors into an interactive science with bridges between a number of traditional disciplines. This synergism has made biology the "sunrise field" of the new millenium.

The whole gamut of new discoveries in biology and allied sciences can be grouped together under a single umbrella term of **"Biotechnology"** . It can be defined as **a manipulation of biological systems (organisms or biological processes) via modern technology to solve practical problems in agriculture, environment, medicine and industry**. It includes clonal propagation via tissue culture, protoplast fusion, gene cloning, DNA recombination, genetic engineering, mutant induction, *in vitro* genetic selection, molecular marker aided genome analysis and gene mapping (Huang et al., 1993). Enhanced production of pharmacologically important secondary metabolites *in vitro* can also be included here.

Higher plants are the ultimate and renewable solar-powered biochemical factories manufacturing both primary and secondary metabolites from air, water and minerals. This has led man to manipulate and modify the plants so as to suit his growing requirements of food, industrial raw material, drugs, pesticides, flavors and fragrances. Since the earliest primitive farming, man has been continuously exploiting plants towards his own needs, by carrying out experiments for plant improvement (Pauls, 1995). But a large number of techniques developed over the last 150 years have actually laid the foundations of the modern science in agriculture. Since the last fifty years, development in agriculture has been greatly aided, in general, by the development of **"Biotechnology"** as mentioned earlier and **"Plant Tissue Culture"** and **"Genetic Engineering"** in particular (Brar and Khush, 1994). These techniques can contribute to various crop-improvement stages like

germplasm manipulations, genotype selection, stabilization, variety testing, variety increase, proprietary protection and crop production (Pauls, 1995).

Since plants are immovable, environment has a very strong impact on every aspect of plant form and function. As a consequence, plant development shows a high degree of plasticity. Environmental stimuli are used as cues to rapidly modify and influence developmental programming to provide flexibility in response throughout the growth cycle of plant. The entire technology of **"Plant Tissue Culture"** is based on this ability of plant cells to be influenced by their surroundings and differentiate to give rise to a range of organs dependent on the culture conditions (Bowles and Leyser, 1994). **"Plant Tissue Culture" is the maintenance and propagation of plant parts, as small as a single cell, in axenic culture under controlled environmental conditions** (Pauls, 1995). The theoretical basis of tissue culture, in general, was established as early as 1838-1839 in Schlieden and Schwann's **Cell Theory**. Haberlandt (1902) first attempted to prove this theory experimentally using monocotyledonous plants and though his attempt was unsuccessful, he elaborated the concept of **Totipotency** that refers to **the potential of an individual cell to regenerate a whole plant**. This period was followed by many unsuccessful attempts at plant tissue culture. But Kotte (1922) and Robbins (1922a, b) independently achieved *in vitro* culture of pea and maize roots in synthetic media under sterile conditions. Although these root cultures could not be maintained indefinitely, they proved that it was easier to maintain cultures having meristematic cells. White (Tomato roots: 1934a, Spontaneous tumor callus in tobacco: 1939a), Gautheret (Carrot tissue: 1939) and Nobecourt (Carrot tissue: 1939) first achieved the prolonged and indefinite growth of plant tissues *in vitro*. White also observed that root meristems are free of viruses during the course of these studies (White 1934b). Gautheret (1942, 1955) described the phenomenon of callus **habituation** where, as the cultures age they can grow in nutrient media free of plant growth regulators, especially auxins.

During the period between 1951-1961, successful **Callus Cultures** were raised starting from normal or tumor tissues from a number of plants belonging to Dicots, Monocots and Gymnosperms (Gautheret, 1985 and references therein).

The demonstration of **hormonal regulation of growth, differentiation and organ formation** in plants (Skoog and Miller, 1957), the **regeneration of plantlets from callus cultures** (Reinert, 1958) and **plantlet regeneration from cell suspension cultures**

(Steward et al., 1958) paved the way for the real exploitation of this knowledge towards the benefit of mankind.

The formation of full plants of tobacco from isolated cells in microculture (Vasil and Hildebrandt, 1965) and formation of a somatic embryo from a single plated cell of carrot (Backs-Hussemann and Reinert, 1970) finally led to the unequivocal demonstration of totipotency of single and isolated plant cells more than 60 years after Haberlandt's theory of totipotency. The concept of totipotency also holds true for haploid pollen cells which was proven by the demonstration of formation of somatic embryos from microspores of *Datura*: **'Androgenesis'** (Guha and Maheshwari, 1964). Due to the voluminous amount of work done on these aspects *in vitro* on a broad range of plants, detailed review of literature is beyond the scope of the present thesis.

'Regeneration' is defined as the ability of single protoplasts, single cells or tissues to develop complete plants. **When the organogenetically formed shoots are rooted, it can be termed as regeneration**. The regenerative response *in vitro* occurs in two different patterns viz. **'Somatic embryogenesis'** and **'Organogenesis'**. The former leads to the production of a bipolar structure containing a root/shoot axis with a closed independent vascular system while the latter leads to the production of unipolar structures such as a root or a shoot primordium, whose vascular system is often connected with the parent plant (Thorpe, 1993). These responses are due to the two properties of the plant cells namely, **'Autonomy'** and **'Totipotency'**.

In tissue cultures, the meristematic region at the initiation of organogenesis originates either from the reproduction of previously existing meristems or is produced *de novo* from other component cells in the tissue (Endress, 1994). External manipulations such as selection of explant (type of organ, physiological and ontogenetical age of the explant, season for obtaining explant, size of explants, position of explant on mother plant, state of mother plant), changes in chemical components like media, plant growth regulators and other additives like amino acids, coconut milk (CM), casein hydrolysate (CH), oligosaccharides and various physical factors (physical form of the medium, pH of the medium, humidity, light, temperature) induce explant cells in the right physiological state to respond and undergo organized development. These structural changes are results of preceding biochemical and biophysical events that ultimately reflect selective gene activity (Thorpe, 1980).

Organogenesis *in vitro* can follow either of the two paths:

1. Indirect organogenesis: Primary explant is induced to form callus prior to undergoing *de novo* organogenesis. The resultant plants may show variations due to the involvement of the callus phase.
2. Direct organogenesis: No intervening callus phase is involved and the regenerants may be identical to the parent plant.

The key feature of *de novo* organogenesis is the formation of a 'meristemoid' arising from vacuolated parenchyma cells. The cells of the meristemoid are small, isodiametric, thin-walled with prominent nuclei and with extensive network of plasmodesmata. This is the first step of **'Cellular Differentiation'**. It is followed by **'Acquisition of Competence'**, **'Cellular Determination'** and finally **'Cellular Differentiation'** giving rise to organs (Christianson and Warnick, 1985). The whole process is high-energy intensive where accumulated starch and carbohydrates from the media are used (Thorpe, 1980).

Refined tissue culture procedures have made it possible to introduce foreign DNA and cloned genes into cultured cells, protoplasts and plant organs from diverse biological systems and to regenerate **transgenic** plants in more than 50 plant species, across Gymnosperms, Dicots and Monocots, with altered genetic properties. The first *Agrobacterium tumefaciens* transformed transgenic plant expressing engineered foreign genes was Solanaceous *Nicotiana tabacum* (Horsch et al., 1984) and the successive plant generations have also expressed the introduced gene. The first transgenic conifer was *Larix decidua* obtained via *Agrobacterium rhizogenes* mediated transformation (Huang et al., 1993).

Agrobacterium rhizogenes mediated transformation was also used for the production of **Hairy Roots** that can synthesize a number of secondary metabolites of the parent plant at a level comparable to or more than *in planta*. First report of such a result appeared for *Hyoscyamus muticus* (Flores and Filner, 1985). Hairy roots have also been shown to produce novel secondary metabolites, which are not produced by the parent plant.

Most of the current interest in medicinal plants has stemmed from the fact that in the recent past, a number of promising drugs have been developed from these plants based on the traditional knowledge and the new insights gained by modern methods of research. Hence cataloguing of medicinal plants and their uses, constant review of their natural status (endangered or otherwise) and exploitation of their natural biodiversity have proved to be important tools in their detailed and varied investigations. Use of Plant Tissue and Cell Culture can be an invaluable aid in the constant search for new drugs from as yet unexplored or underexplored plants.

PART B. SECONDARY METABOLITE PRODUCTION *IN VITRO*.

Introduction

Plants synthesize a bewildering array of chemical compounds with a variety of physiological roles, starting from air, water, minerals and sunlight as the energy source. Various compounds produced by plants can be broadly grouped into two categories namely, **"Primary Metabolites"** and **"Secondary Metabolites"**. The distinctions between them are summarized in **Table B 1.1**.

Table B 1.1 Primary and Secondary Metabolism: Two Functional Levels with Different Characteristics (Modified from Endress, 1994; Hartmann, 1996).

No.	Primary Metabolism (Growth & Development of an Individual)	Secondary Metabolism (Interaction of an Individual with the Environment)
1.	Indispensable for growth and development.	Dispensable for growth and development.
2.	Not related to competition between individuals.	Useful for competition among individuals and for population survival as a whole.
3.	Uniform.	Variable.
4.	Universal.	Universal.
5.	Conservative.	Diverse.
6.	Constant.	Adaptive.
7.	Less genetic variation.	High genetic variation.
8.	High volume, low value compounds.	Low volume, high value compounds.
9.	Constitutive production.	Constitutive as well as inducible production.
10.	Relatively simpler structures and so could be synthesized in laboratories.	Highly complex structures with many chiral centers and so difficult to synthesize in the laboratories.

The secondary metabolites are also referred to as **"Natural Products"**. It is believed that more than 100,000 different structures of secondary metabolites maybe synthesized by organisms, to a tune of 10^9 tons per year (Bowles and Leyser, 1994). Out of these, more than 80% are found in plants (Harborne, 1993). They are used either as medicines/pharmaceuticals, foods, neutraceuticals (foods as well as medicines used for preventive and curative treatments), flavors, colors, spices or fragrances by humans while in plants they constitute a chemical response to pollinators and distributors, to competitors and herbivores, to symbionts and pathogens and to stress. They are also believed to detoxify substances accumulated in the primary metabolism and to provide chemical signals to coordinate metabolism of multicellular organisms. They are believed to coordinate activities of different individuals of the same species (Endress, 1994). Many of the social, political and economic changes throughout the history of the mankind have been driven by the desire to gain or exploit economically important plants like spices, rubber, coffee, tea, tobacco, cocoa and opium poppy producing bioactive molecules.

Although secondary metabolism was first recognized in 1873 (Sachs, 1873), its function was elucidated only in 1888 (Stahl, 1888). But upto the 1950s, secondary metabolites were regarded as end products of deluxe metabolism and relegated to the rank of 'waste products'. It was only during the 1960s, that their eco-relations were discovered (Kurz and Constabel, 1998). More recently, biotechnologies have become indispensable in efforts to experimentally widen our knowledge of the ontogeny, metabolism, function and production of secondary metabolites.

Chemical diversity in plants is not incidental but is brought about by specifically organized and controlled biosynthetic pathways, well integrated into the metabolism of plants. This is also the expression of plant genome under developmental control. Most of the secondary metabolites are formed via a few biogenetic routes, leading to one or a few key metabolites, from which numerous derivatives are formed by simple enzymatic transformations. The biosynthesis usually occurs in an organ in a tissue-specific manner and is often temporally restricted during the development. It is often accompanied by specific translocation and storage behavior (Hartmann, 1996; Kurz and Constabel, 1998).

The enormous diversity of secondary metabolites found in nature results from three main features such as a high degree of chemical freedom, strictly regulated metabolic pathways and enzyme optimization (Hartmann, 1996).

As commodities, secondary metabolites are increasingly in great demand. This demand is increasingly becoming acute because of their non-availability, owing to adverse climatic conditions, pests, political instability in cropping areas, misuse and overexploitation. Many medicinally important plants are already threatened in their natural habitats. E.g. *Lithospermum erythrorhizon*, *Hydrastis canadensis*, *Cephaelis ipecacuanha*, *Rauwolfia serpentina*, *Podophyllum peltatum*, *Artemisia genipi*, *Taxus* spp. There is a growing need to preserve these plants either *in vivo* or *in vitro* because of the uniqueness of each species. and their extinction may lead not so much in the loss of an individual secondary metabolite, but rather, in the specific bouquet of all the metabolites presented by each unique species (Kurz and Constabel, 1998). Up till now, 47 major drugs have been produced from the tropical forests but it is believed that around 328 potential, major drugs and 125,000 flowering trees of potential pharmacological importance may be still hidden in the unexplored depths of these forests. Every year the market for herbs-based drugs is increasing at the rate of 12-15% (http://www.phytochemistry.freeserve.co.uk).

Plant tissue and cell culture technologies in this context are seen as a savior in channelizing the resources of the nature for the benefit of the mankind by conservation of elite, endangered plants and eco-friendly production of drugs and drug intermediates. Cryopreservation of germplasm would help in maintaining the genetic diversity of the endangered population and improved cell and tissue culture technologies would help in producing the active compounds *in vitro* with better productivities without cutting down the natural resources. Recombinant DNA technologies would also supplement plant cell technologies towards this end.

Large-scale production of phytochemicals with plant cell and tissue culture technologies has the following advantages (Modified from Zafar et al., 1992):

1) Independence from environmental factors like climate, pests, geographical and seasonal constraints.
2) Defined production systems as and when required.

3) More consistent product quality and yield.
4) Potential for fast growth rates.
5) Continuous and homogeneous supply of plant material in a uniform physiological state.
6) Reduction in land use for 'cash crops'.
7) Freedom from political constraints.
8) Process isolation from related metabolic pathways leading to higher yields.
9) Magnification and accentuation of chemical reactions under growth control.
10) Use of improvement strategies for better yields and cost-benefit ratios by growth medium and microenvironment manipulations.
11) Use of recombinant DNA technologies for yield improvement.
12) Use of genetic transformation with *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* to achieve high product yields in the absence of growth regulators but with proper organ differentiation.
13) Production of novel compounds *in vitro*, which are absent in the *in vivo* parent plant material.
14) Possibility of having continuous production of metabolites that are normally transiently made in the life cycle of the parent plant (e.g. flower colors).
15) Possibility of having an active synthetic tissue for a metabolite normally accumulated in a non-active tissue like bark.
16) Understanding of complex metabolic pathways involved in the synthesis of secondary products and identification of the key enzymes.
17) Possibility of metabolic engineering at the cellular level for better productivities.
18) Ability for large-scale cultivation of cells, organs and even entire plants in bioreactors for easier and higher product recoveries.

Despite a number of advantages listed above, plant cell and tissue culture technologies also suffer from some drawbacks (Zafar et al., 1992):

1. Since plant cells are much larger in size, they have a bigger surface area and hence lower metabolic activity resulting in slower growth rates as compared to smaller sized microbial cells.
2. Plant cells tend to grow together as cell clumps rather than single cells posing some problems for their cultivation in bioreactors.

3. The yield of secondary metabolites *in vitro* cannot be predicted beforehand. Many-a-times the compounds of interest are not produced *in vitro* or if produced are present in extremely low quantities.
4. High genetic and epigenetic instability of cell cultures can lead to loss of phytochemical production capacities of cells.
5. Differentiation-related products are not produced in unorganized tissues that are easier to manipulate *in vitro*.
6. Culture conditions may trigger new pathways producing novel but useless products.
7. Empirical methods developed for cell cultures of a particular plant cannot be extrapolated to a wide range of plants and culture systems.

There are four main *in vitro* approaches for production of secondary metabolites:

1. Callus Cultures

Callus cultures consist of an undifferentiated, proliferating mass of cells usually arising on wounds of differentiated tissues and cells. These can be initiated either from the source tissue synthesizing the secondary compound of interest or from other tissues like embryo. Generally the plants which accumulate relatively high yields of specific secondary metabolites, give rise to tissue cultures producing high levels of secondary metabolites and vice versa. This is because the capacity for the biosynthesis of secondary metabolites is genetically determined (Lindsey and Yeoman, 1985). Cell division usually occurs in parenchymatous cells by dedifferentiation. During this process adult cells temporarily revert to juvenile state (**Rejuvenation**) and hence show intense growth and division activity. The degree of dedifferentiation is different in different cells derived from the same explant (Lindsey and Yeoman, 1985 and references therein). Normally juvenile and hence physiologically the most active tissues give better callus formation. The exogenous plant growth regulator requirement (Type, concentration, auxin to cytokinin ratio) for callus formation depends upon the genotype and endogenous hormone content of the tissues (Pierik, 1987).

During callus formation there are two distinct phases namely, the **"wound response"** and the **"growth response"**. The former phase is characterized by a rapid increase in metabolic activity when the explant is cultured on the growth medium but *does not* lead to callus formation. The latter phase results in cell division and is dependent

upon an exogenous supply of auxin. The cell structure also undergoes changes such as increased numbers of mitochondria and polyribosomes, disappearance of storage reserves like starch etc. Thus both the **"wound response"** and the **"growth response"** are characterized by distinct periods of m-RNA activity and the levels of t-RNA and r-RNA are regulated independently (Lindsey and Yeoman, 1985 and references therein).

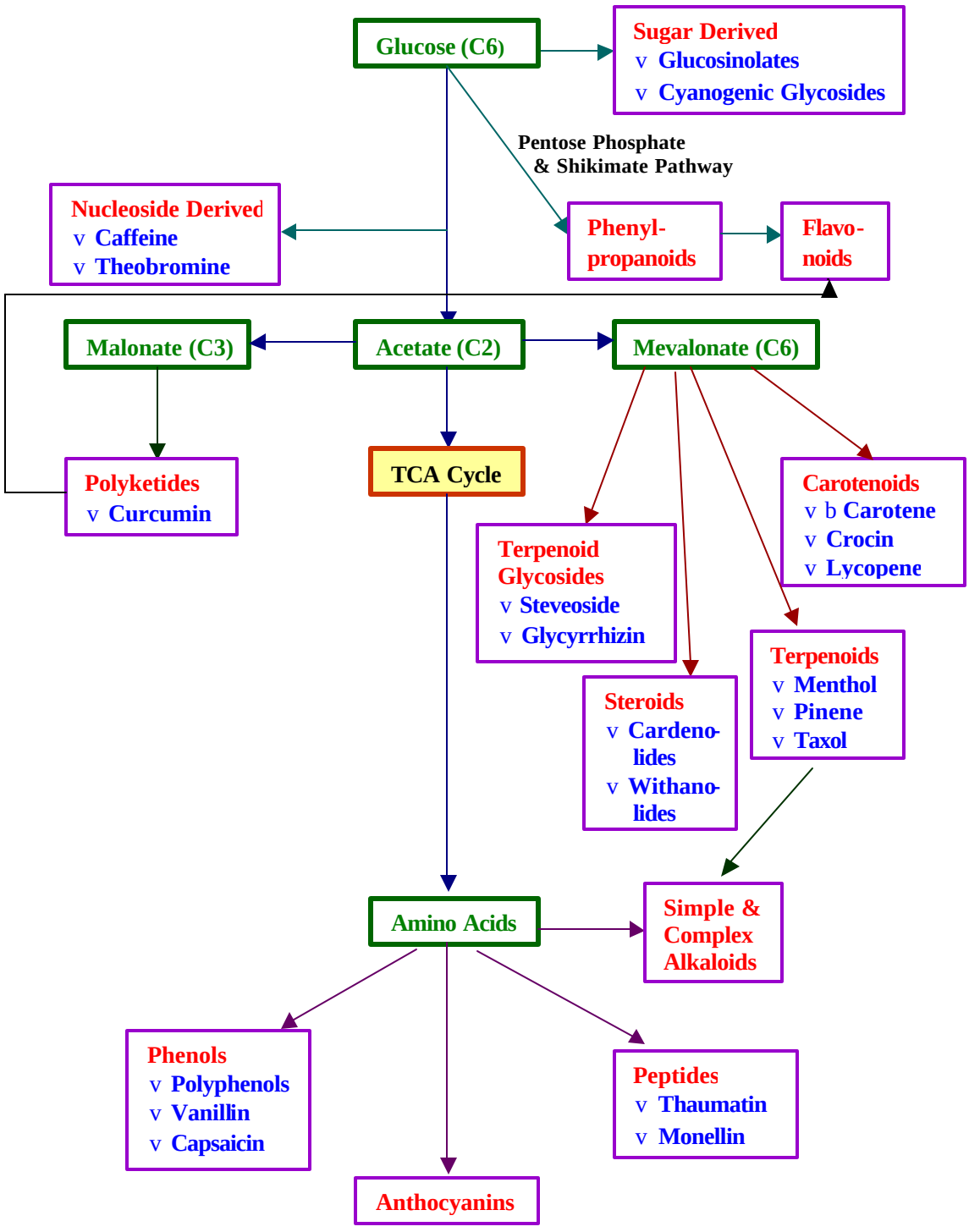
Due to the presence of diffusion gradients of nutrients, oxygen and other factors, the growth of callus is not always uniform and rapid and hence has less possibility for scale-up. But the chemical gradients, cell-to-cell contact and small degrees of differentiation seem to favor secondary metabolite production of some classes (Lindsey and Yeoman, 1985). Callus induction requires an environment where some of the cells can divide and proliferate. The strongest induction factor is the growth/nutrient medium supplemented with plant growth regulators (Gibson et al., 1995). With prolonged culture, some of the callus cells may undergo some degree of differentiation and may change the spectrum of secondary metabolite synthesis. For many years, callus cultures have been employed in the study of secondary metabolism *in vitro*. In many of the callus cultures, conditions for callus induction and growth (**Growth Medium**) are not conducive for secondary metabolite production and the tissues need to be transferred to a new medium with a different composition (**Production Medium**). Production methods tend to be contingent upon defining conditions that allow maximum product accumulation rate.

Table B 1.2 lists different classes of secondary metabolites produced by callus and cell suspension cultures *in vitro* while **Flow Chart B 1.1** depicts the mode of formation of these compounds.

Table B 1.2 Groups of Natural Products So Far Isolated from Callus and Cell Suspension Cultures of Higher Plants (Stöckigt et al., 1995).

I Phenyl-propanoids	II Alkaloids	III Terpenoids	IV Quinones	V. Steroids
1. Antho-cyanins	1. Acridines	1. Carotenoids	1. Anthra-quinones	1. Steroidal lactones
2. Coumarins	2. Betalaines	2. Monoterpenes	2. Benzo-quinones	2. Steroidal glycos ides
3. Flavonoids	3. Quinolizidines	3. Sesquiterpenes	3. Naphtho-quinones	
4. Hydroxy-cinnamoyl derivatives	4. Furo-quinolines	4. Diterpenes		
5. Isoflavonoids 6. Lignans	5. Harringtonines 6. Isoquinolines	5. Triterpenes		
7. Phenalinones	7. Indoles			
8. Proantho-cyanidins	8. Purines			
9. Stilbenes 10. Tannins	9. Pyridines 10. Tropane Alkaloids			
	11. Indole Alkaloids			
	12. Isoquinoline Alkaloids			

Flow Chart B 1.1 Schematic Biosynthetic Pathways for Secondary Metabolites (Modified from Edwards and Gatehouse, 1999).



Tables B 1.3 and **B 1.4** list some of the callus cultures derived from a number of plant species and producing secondary metabolites. **Table B 1.3** specifically represents secondary products synthesized in callus cultures *in vitro* in concentrations equal to or more than in intact plants while **Table B 1.4** presents data on production of some important antitumor compounds *in vitro* by cell and callus cultures.

Table B 1.3 Secondary Products derived from Callus Cultures *in vitro* in Concentrations Equal to or More than in Intact Plants (Doran, 1989; Heble, 1993; Endress, 1994).

No.	Plant Species	Metabolite	Callus Cultures (% dry wt.)	Intact Plant (% dry wt.)
1.	*Ammi visnaga*	Visnagin	0.31	0.1
2.	*Cassia tora*	Anthraquinones	0.33	0.21
3.			6.0	0.6
4.	*Catharanthus roseus*	Ajmalicine	2.2	0.30
5.		Serpentine	1.8	0.5
6.	*Coleus blumei*	Rosamarinic Acid	27.0	3.0
7.	*Coptis japonica*	Berberine	13.0	7.5
8.	*Dioscorea deltoides*	Diosgenin	7.8	2.0
9.	*Gallium aparine*	Anthraquinones	3.8-5.7	0.28
10.	*Lithospermum erythrorhizon*	Shikonin	14-23	1.5
11.	*Morinda citrifolia*	Anthraquinone	18.0	2.2
12.	*Nicotiana tabacum*	Nicotine	5.0	2.1
13.		Glutathione	0.22 $g.l^{-1}$ of suspension medium.	0.1
14.		Ubichinone-10	0.19	0.003
15.	*Panax ginseng*	Ginsenosides	27.0	4.5
16.	*Rubia fruticosa*	Anthraquinones	20.0	2.5
17.	*Stefania cepharantha*	Biscoclaurine	2.3	0.8
18.	*Trypterygium wilfordii*	Triptolide	0.05	0.001

Table B 1.4 Antitumor Compounds Produced from Cell and Callus Cultures of Higher Plants (Modified from Misawa and Nakanishi, 1988).

No.	Plant Species	Metabolite	Cultured Cells Yield (% dry weight)	Reference
1.	*Baccharis megapotamica*	Baccharine	-	-
2.	*Brucea antidysenterica*	Bruceantin	5.8×10^{-2}	Misawa et al. (1983)
3.	*Camptotheca acuminata* and *Nothapodytes foetida*	Camptothecin	2.5×10^{-4}	Misawa et al. (1974)
4.	*Catharanthus roseus*	Vinblastine and Vincristine	5.0×10^{-4}	
5.	*Cephalotaxus harringtonia*	Harringtonine, Homo-harringtonine	5.5×10^{-8}	Misawa et al. (1985)
6.	*Maytenus bucchnanii*	Maytansine	5.0×10^{-7}	Misawa et al. (1985)
7.	*Ochrosia elliptica*	Ellipticine	2.7×10^{-4}	Kouadio et al. (1985)
8.	*Podophyllum peltatum* and *Podophyllum hexandrum*	Podophyllotoxin	7.1×10^{-1}	Kadkade (1982)
9.	All *Taxus* spp.	Taxol	See **Chapter 4, Table 4.3** for the details.*	
10.	*Trypterygium wilfordii*	Tripdiolide	1.0×10^{-2}	Misawa et al. (1983)
11.	*Withania somnifera*	Withanolide I, Withanolide G	1.3-17.2, 1.0-31.7	Roja et al. (1991)*

* New data added based on the literature survey carried out during the course of the present work.

2. Suspension Cultures

When callus is suspended in a liquid growth medium, the cells disperse producing cell suspension cultures that can have faster and uniform growth rates coupled with secondary metabolite production. With their relatively faster growth, ease of manipulation and comparative homogeneity, suspension cultures are the most widely employed systems in the studies of secondary metabolism (Parr, 1989). They represent various degrees of cellular aggregation and generally, the more aggregated, slower growing cultures display the highest productivities (Lindsey and Yeoman, 1985). They can be 'induced' by media manipulations and elicitors to produce compounds of interest. They are also amenable for growth in fermentors. But these cultures show genetic and biochemical heterogeneity.

Street (1977) has described the growth and development of a cell culture as comprising a succession of physiological states, each characterized by distinctive structural and biochemical features.

Normally plant cells tend to grow in aggregates of 2-20 cells and can rarely grow as fine cell suspensions. Because of this aggregation, cells in the center tend to be morphologically, physiologically and chemically distinct from the cells at the periphery (Gibson et al., 1995). Cell aggregation can be reduced by choice of an appropriate culture medium having suitable osmotic agents such as sorbitol and low concentrations of cell wall degrading enzymes (King et al., 1973). Such a finely dispersed culture grows in a generalized form of sigmoidal curve (Street, 1977) and goes through a number of phases such as **Lag Phase** (absence of cell division or very low rate of cell division, increase in protein synthesis and increase in the levels of energy-generating systems of cells depending upon the nutrient medium and plant growth regulator composition, low levels of secondary metabolites), **Log Phase** (a short period of exponential cell division followed by a steady rate of cell division, increase in average cell size, increase in nucleic acid contents, increase in primary metabolism which can be made to divert into secondary metabolism by certain media and culture environment manipulations in some species) and **Stationary Phase** (period of decelerating cell divisions due to exhaustion of an essential nutrient, cessation of cell divisions and hence absence of growth, decrease in respiration, RNA synthesis, protein synthesis). If at the stationary phase, the nutrient medium composition is changed, differentiation may occur which may in turn lead to increase in

secondary metabolite production (Observed in many Solanaceous plants). In some plant species like *Acer pseudoplatanus*, King (1980) observed that phenols continued to accumulate even with declining cell divisions and lowered cell aggregations. So he suggested that the changing growth rate, rather than organization per se, affected the expression of secondary metabolism. In such suspension cultures, cultural conditions undergo changes continuously leading to uncoupling of growth and secondary metabolite accumulation. This uncoupling could be modified by nutrient medium composition (King et al., 1973). They further suggested that the gross changes occurring in the metabolic activity and cell composition resulted due to independence of the control mechanism for cell division and for the synthesis of individual metabolites.

The origin of rapidly dividing and friable cell suspension cultures appears to be the result of a random, critical event occurring in the initial phase of explant exposure to culture medium. This event is probably more a function of the species and the nature of the explant than of the experimental conditions (King, 1980). Such friable cell lines exhibit following characters:

- High friability.
- Homogeneous cell type.
- Cells more or less isodiametric.
- Cells highly cytoplasmic.
- Cells with distinct nuclei.
- Cells with many starch grains.
- Cultures with limited cytodifferentiation. Presence of a few tracheids or pigmentation.
- Cell doubling times of 24-72 hrs.
- No apparent regeneration capacity.
- Auxotrophic for several common metabolites.
- Easily converted to hormone autotrophy (**Habituation**).
- Growth promoted by high CO_2 levels.
- Susceptibility to ethylene.
- Abnormal ploidy levels, often aneuploidy.

A list of suspension cell cultures producing secondary metabolites is given in **Tables B 1.5** and **B 1.6**. **Table B 1.5** specifically lists phytochemical accumulated in high levels in cell suspension cultures while **Table B 1.6** lists some examples of novel secondary metabolites synthesized only by plant cell cultures and not by the parent plants.

Table B 1.5 Secondary Metabolites of Pharmaceutical Significance Accumulated in High Levels by Plant Cell Cultures (Zafar et al., 1992 and references therein; Stöckigt et al., 1995 and references therein).

No.	Plant Species	Metabolite	Yield ($g.L^{-1}$)
1.	*Coptis japonica*	Berberine	7.0
2.	*Coleus blumei*	Rosamarinic acid	5.6
3.	*Lithospermum erythrorhizon*	Shikonin	4.0
4.	*Berberis wilsoniae*	Latrorrhizin	3.0
5.	*Morinda citrifolia*	Anthraquinones	2.5
6.	*Rauwolfia serpentina*	Ajmaline	2.0
7.	*Linum flavum*	Coniferin	2.0
8.	*Rauwolfia serpentina*	Raucaffricine	1.6
9.	*Nicotiana tabacum*	Cinnamoylputrescine	1.5
10.	*Galium molugo*	Shikimic acid	1.2

Table B 1.6 Novel Secondary Metabolites (Hitherto Unknown in Intact Plants) Produced in Plant Cell Cultures (Zafar et al., 1992 and references therein; Kokate, 1995 and references therein; Stöckigt et al., 1995).

No.	Plant Species	Metabolite
1.	*Andrographis paniculata*	Paniculid A
2.	*Arnica montana*	Two polysaccharides stimulating excretion of tumor necrosis factor (TNF).
3.	*Aspidosperma quebracho blanco*	Aspidochibine, Rhazinilam derivative
4.	*Gardenia jasminoides*	Tarennosid
5.	*Morinda citrifolia*	5,6-dihydroxy-lucidin
6.	*Ochrosia elliptica*	Epchrosin, 5,6-dihydroxy-lucidin
7.	*Picralima nitida*	Pericine, Pericalline
8.	*Plagiorhegma dubium*	Dehydro-diconiferyl-alcohol-γ-β-D-glucoside
9.	*Podophyllum versipelle*	Podoverines A, B and C
10.	*Rauwolfia serpentina*	Raumaclines, Rauglucines
11.	*Ruta graveolens*	Rutacultin
12.	*Stephania cepharantha*	Armorine
13.	*Thuja occidentalis*	Ainokiol
14.	*Valeriana wallichii*	A series of new Valepotriates
15.	*Voacanga africana*	Voafrine A, B

Secondary product synthesis by cell suspension cultures is typically low in a non-manipulated medium. A number of approaches are available for enhancing the productivities of such cultures and are summarized below in **Table B 1.7** .

Table B 1.7 Factors Influencing Secondary Metabolite Production in Plant Cell Cultures (Modified from: Dorenburg and Knorr, 1995).

Strain Improvement	Selection
	Screening
Medium Variation	Nutrients
	Phytohormones
	Precursors
	Antimetabolites
Culture Conditions	Inoculum size
	Subculture Durations
	pH
	Temperature
	Light
	Agitation
	Headspace Gas Composition
	Aeration/Gaseous Exchange
Specialized Techniques	Elicitors
	Stress Immobilization
	Permeabilization
	Online, Continuous Recovery of Products
	Artificial Accumulation Sites Two-phase systems
	Two-stage systems
	Adsorption Technology
	Genetic Engineering
	Somatic Hybridization

3. Immobilized Cell Cultures

When free cells are entrapped in various matrices like alginate, foam etc. or when they are allowed to grow into porous supports like reticulated, polyurethane foam, they give rise to immobilized cell cultures (Parr, 1989). These systems combine the advantages of the afore-mentioned two systems and are increasingly being preferred, since the cell-to-cell contact is better and the synthesized product can be easily removed without cell destruction. Some of these techniques applied for in vitro cultures of Taxus spp. are described in **Chapter 4, Table 4.3**.

4. Differentiated Cultures

Secondary metabolites are also synthesized or stored in organized structures such as roots, shoots, stigmas, embryos, transformed roots (By use of *Agrobacterium rhizogenes*) and transformed shoots (By use of shooty mutant of *Agrobacterium tumefaciens*). The root cultures can easily be exploited in bioreactors (Parr, 1989). These cultures show biochemical and genetic stability and usually exhibit the full biosynthetic capacity associated with the organ *in planta*.

A list of differentiated cultures producing secondary metabolites is given in **Table B 1.8** for multiple shoot cultures and in **Tables B 1.9 and B 1.10** for hairy root cultures.

A. Multiple Shoot Cultures

The synthetic capacity of dedifferentiated tissue often differs substantially from that of fully differentiated tissues, both qualitatively and quantitatively. The differing synthetic capacities are usually a direct result of differences in enzyme profiles which mirror the organ-specific expression of biosynthetic genes (Endress, 1994). The differentiated cultures often show biochemical (Flores and Filner, 1985) and genetic (Aird et al., 1988) stability and hence offer a predictable and high-productivity system which does not require extensive optimization. For example, the accumulation of isoprenoids usually is dependent upon plastid differentiation, as many of the enzymes in the pathway are plastid-related. In other cases, formation of glandular hairs or other storage organs is necessary for metabolite production to proceed. For example, vindoline, a major alkaloid of *Catahranthus roseus* is scarcely produced in suspension cell cultures but is produced in

multiple shoot cultures derived from the same (Hirata et al., 1987). Callus cultures of *Taraxacum officinale* synthesize and accumulate α- and β-amyrins while differentiated tissues synthesize and accumulate taraxasterol and lupeol because these differentiated tissues develop laticifers where these compounds are stored (Akashi et al., 1994).

Secondary product formation is an expression of a particular state of cell differentiation, which can be influenced by a number of signals. In some cases, initiation of morphological differentiation represents such a triggering signal. Different classes of secondary compounds require different degrees of cell or tissue differentiation. Formation of physical and biochemical gradients due to cellular organization is also an important contributing factor.

Table B 1.8 represents some of the work done in various plant species on multiple shoot cultures that produce secondary compounds in amounts either equivalent to or greater than those present in the parent plant.

Table B 1.8 Secondary Metabolite Production in Multiple Shoot Cultures of Various Plant species (Charlwood et al., 1990).

No.	Plant Source	Class of Secondary Product	Secondary Product	Yield (x times) C.f. With Intact Plant
1.	*Atropa belladonna*	Tropane Alkaloids	Hyoscyamine, Scopolamine	0.07 X C. f. plant shoot
2.	*Datura innoxia*		Hyoscyamine, Scopolamine	0.07 X C. f. leaves
3.	*Catharanthus roseus*	Indole Alkaloids	Ajmalicine, Cataranthine, Vindoline	1-8 X C. f. plant
4.			3',4'-anhydrovinblastine	0.3 X C. f. leaves
5.			Vinblastine	Lower than leaves
6.			Catahranthine	5 X parent plant
7.	*Rauwolfia serpentina*	Indole Alkaloids	Ajmalidine, Ajmaline, Yohimbine	1.4 X C. f. young shoot

Contd…..

Table B 1.8 Contd.

No.	Plant Source	Class of Secondary Product	Secondary Product	Yield (x times) C. f. With Intact Plant
8.	*Cinchona ledgeriana, C. succirubra*	Quinoline Alkaloids	Quinine, Quinidine, Cinchonine	0.2 X C. f. leaves
9.			Cinchonidine	0.01 X C. f. bark
10.	*Papaver bracteatum*	Isoquinoline Alkaloids	Thebaine, Sanguinarine	Much lower than mature flower stems
11.	*P. somniferum*		Thebaine, Codeine, Morphine	Much lower than plant
12.	*Heimia salicifolia*	Quinolozidine Alkaloids	Vertine, Lyfoline, Lythrine, Demethylsubine I & II	-
13.	*Digitalis lanata*	Steroids	Digitoxin	0.017-0.4 X C. f. leaf
14.			Digoxin	0.01 X C. f. leaf
15.			Odoroside H, Odorobioside G, Verodoxin, Glucodogifucoside, Strospeside	Not present in adult plants
16.	*D. purpurea*		Digitoxin	0.1 X C. f. leaf
17.			Digoxin	0.2 X C. f. leaf
18.	*D. lutea*		Digitoxin	0.019 X C. f. leaf
19.			Digoxin	0.05 X C. f. leaf
20.	*D. mertonensis*		Digitoxin	0.06 X C. f. leaf
21.			Digoxin	0.088 X C. f. leaf
22.	*D. ferruginea*		Digitoxin	0.003 X C. f. leaf
23.			Digoxin	0.014 X C. f. leaf

Contd…..

Table B 1.8 Contd.

No.	Plant Source	Class of Secondary Product	Secondary Product	Yield (x times) C. f. With Intact Plant
24.	*D. ambigua*	Steroids	Digitoxin	0.012 X C. f. leaf
25.			Digoxin	0.066 X C. f. leaf
26.	*Dioscorea composita*		Diosgenin	0.7 X C. f. tuber
27.	*Yucca schidigera*		Sarsaapogenin, Smilagenin	0.3 X C. f. mature rhizome
28.			Markogenin, Samogenin	0.66 X C. f. mature rhizome
29.			Gitogenin, Negitogenin	0.54 X C. f. mature rhizome
30.	*Chrysanthemum cinerariaefolium*	Isoprenoids	Pyrethrine, Cinerin, Jasmolin	-
31.	*Pelargonium fragrans*		Pinene, Sabinine, Farnesene, Carvone, Cadinine	0.5 X C. f. leaf
32.	*P. graveolens*		Geraniol, Citronellol, Citronellyl formate	-
33.	*P. tomentosum*		Menthone, Isomenthone	0.5 X C. f. leaf
34.	*Solanum nigrum*	Steroidal Alkaloids	Solasodine	0.35 X C. f. leaf
35.	*S. laciniatum*			10-20 X C. f. *In vitro* shoots of other spp.
36.	*Withania somnifera*		Withanolides	-
37.	*Citrus paradisi*	Phenyl-propanoids	Naringin,	0.18 X C. f. seedling leaf
38.	*Chrysosplenium americanum*		O-methylated flavonol glucosides	0.1 x C. f. first leaf
39.	*Foeniculum vulgare*		Anethole	-

Contd…..

Table B 1.8 Contd.

No.	Plant Source	Class of Secondary Product	Secondary Product	Yield (x times) C. f. With Intact Plant
40.	*Pimpinella anisum*	Phenyl-propanoids	Anethole	0.056 X C. f. shoot
41.			Epoxy-pseudoisoeugenol-(2-methylbutyrate)	0.54 X C. f. shoot
42.			Pseudoisoeugenol-(2-methylbutyrate)	0.14 X C. f. shoot
43.	*Taraxacum officinale*	Triterpenes	Triterpene acids, Triterpenols	**Different profiles of secondary products *in vitro* as compared to *in vivo* tissues.**

B. Hairy Root Cultures

Ever since the discoveries of Mendel, geneticists have been interested in the prospect of directed genetic change. Conventional plant breeding was the only source till the early part of 20th century to achieve this aim. But after the discovery of Smith and Townsend (1907), that a Gram negative soil bacterium, *Agrobacterium tumefaciens*, causes a tumorous plant disease known as **'crown gall'**, the options have widened. Later on, *Agrobacterium rhizogenes* was shown to cause **'hairy root phenotype'** in infected plants (White et al., 1982). These changes were mediated through transfer and subsequent integration into the host cell genome of a fragment of DNA known as **T-DNA**, from the bacteria. The expression of genes on this fragment in the plant cell environment led to the development of typical transformed phenotypes (Weising and Kahl, 1996 and references therein; Sheng and Citovsky, 1996).

Agrobacterium rhizogenes and Plant Interaction

Agrobacterium spp. are non-sporing, aerobic, Gram negative rods (0.6-1.0 µM x 1.5-3.0 µM) belonging to family Rhizobiaceae. They may occur as single cells or in pairs, are motile by 1-6 peritrichous flagella and secrete copious extracellular polysaccharide slime (Holt et al., 1994).

Based on their phytopathogenic characters (Gelvin et al., 1988-1990), agrobacteria are grouped into three classes namely:

I. *Agrobacterium tumefaciens*, causing crown gall disease.
II. *Agrobacterium rhizogenes*, causing hairy root disease.
III. *Agrobacterium radiobacter*, avirulent spp.

Based on their growth patterns (Gelvin et al., 1988-1990), agrobacteria can be classified into two groups :

1) Biotype 1, where the strains can grow upto 37°C, can grow on typical *E. coli*-type media and can produce ketolactose from lactose. Many *A. tumefaciens* strains and few *A. rhizogenes* strains fall in this category.
2) Biotype 2, where the strains can grow only at 28-29°C and not at 37°C, cannot grow on typical *E. coli*-type media but require special media and cannot produce

ketolactose from lactose. These strains can utilize erythritol as a carbon source. Many *A. rhizogenes* strains fall in this category.

Formation of crown gall or hairy roots is a direct expression of genes in the T-DNA of the Ti (Tumor-inducing) or Ri (Root-inducing) plasmids (Size: 200 kb) that are transferred to the host plant cell from agrobacteria and become stably integrated into its genome. Hairy root causing Ri plasmids are classified according to the opines (Agropine-, Mannopine- and Cucumopine-type) synthesized by them. The T-DNA, despite its prokaryotic origin, has eukaryotic transcriptional and translational control sequences that can be recognized by the host cell machinery. The whole process of *Agrobacteruim*-mediated T-DNA transfer in plant cells is depicted in **Fig. 1.1**. The figure summarizes a number of interactions involved in T-DNA transfer to plant cells. The distinct events are numbered consecutively as described in detail by Weising and Kahl (1996) and Sheng and Citovsky (1996).

The natural host range of *A. rhizogenes* includes most of the Dicots and some Gymnosperms. Monocots are generally not susceptible for *Agrobacterium rhizogenes* mediated transformation. However the constraint regards Monocots has been overcome *in vitro* for many economically important Monocots (Godwin et al., 1992). The main block for transformation in Monocots apparently occurs at the very first step of induction of *vir* genes by phenols that are absent at the wound site in Monocots. Amongst the Dicots, plants belonging to the family Solanaceae are highly susceptible for infections by all the strains of *Agrobacterium*.

Hairy roots are characterized by their rapid, highly branching growth on hormone-free media, plagiotropic root development, morphological and chromosomal stability, productivity of secondary metabolites and spontaneous and frequent regeneration into entire plants (Christey, 1997; Tanaka, 1997). Because of these reasons they are favored over suspension cell cultures for secondary metabolite production, as shown in **Table B 1.9**.

Fig. 1.1 A simplified Scheme Depicting *Agrobacterium*-mediated T-DNA Transfer in Plant Cells (Weising and Kahl, 1996; Sheng and Citovsky, 1996) (Not to scale).

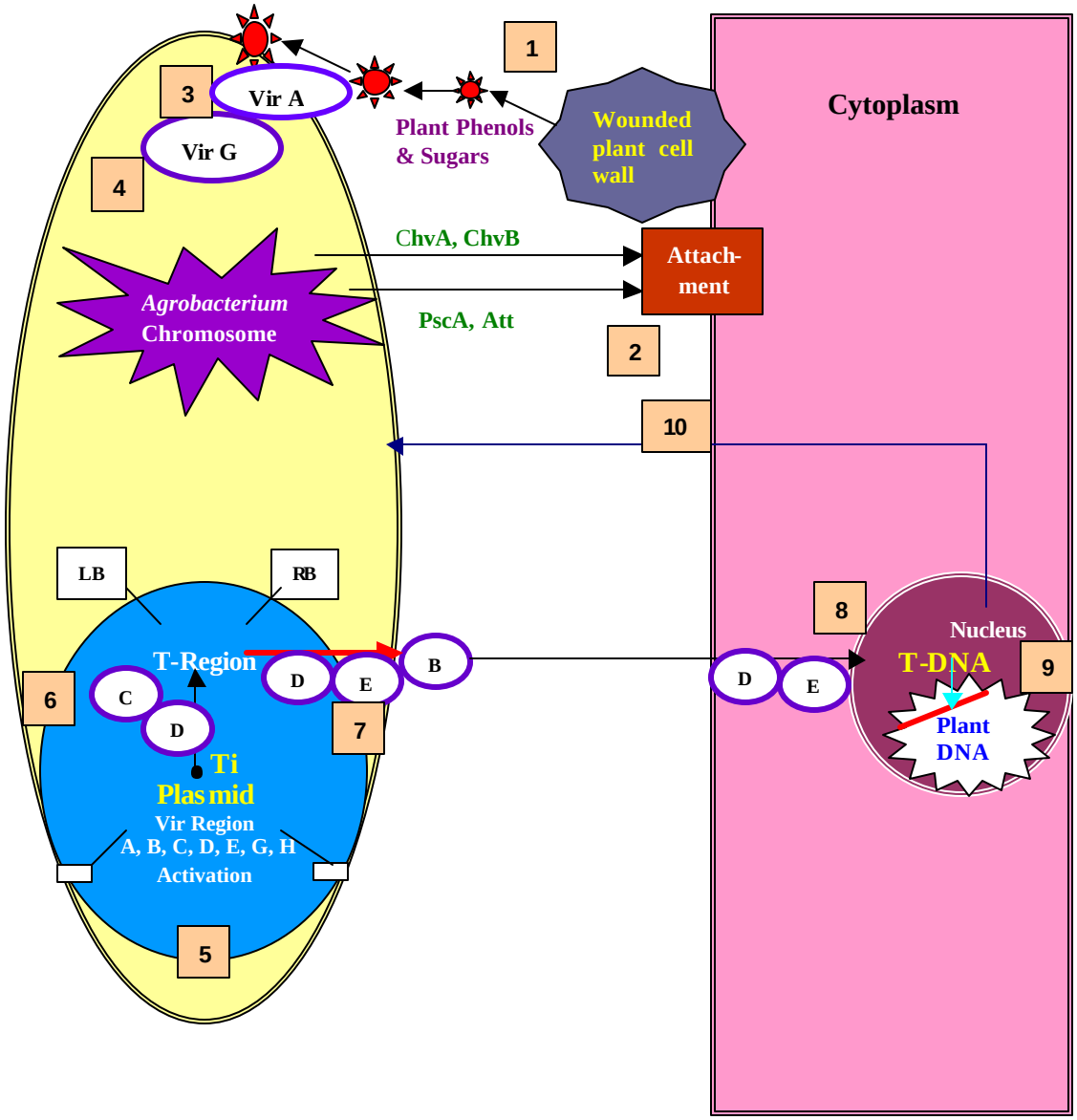


Table B 1.9 Various Advantages of Hairy Root Culture System over that of Cell Culture (Bhagyalakshmi and Ravishankar, 1998a).

No.	Parameter	Hairy Roots	Cell Suspension Cultures
1.	Aeration	High, 10-20 mM^{-1}	Low, 10 mM^{-1}
2.	Biomass doubling time	2-7 days.	0.7-14 days.
3.	Size	1-10 cm.	40-200 µM.
4.	Media	Simple, no vitamins and hormones.	Complex, requiring vitamins and hormones.
5.	Inoculum Density	5-10% V/V	5-10% V/V
6.	Morphology	Organized structure but highly tangled biomass with fragile cells.	Mixture of single and aggregated cells with fragile cell wall, highly vacuolated cells with less cytoplasm.
7.	Genetic Stability	Stable-euploid to polyploid, homogeneous.	Unstable-aneuploids, heterogeneous.
8.	Product Accumulation	Spectrum and their levels mostly as in parents but novel products also obtained.	Mostly an altered spectrum with low levels than the parent.
9.	Product Release	More often released.	Often released.
10.	Maximum Biomass Density Achieved	30 g. $dw.l^{-1}$	200 g. $dw.l^{-1}$
11.	Stability at High Density	Easily maintained.	O_2 requirement difficult to fulfil. Leads to cell damage.
12.	Inoculum Size	Independent.	Size-dependent.
13.	Growth Initiation	Minimum lag phase.	Often longer lag phase. Conditioning of medium required.
14.	Shear Sensitivity	Highly sensitive.	Sensitive but resistant lines can be selected.
15.	Continuous Operation	Easy: self filters available.	Problematic: requires support.
16.	Biomass Handling	Not easy, as non-pumpable.	Easy, as pumpable.

Hairy root cultures reported from over 30 plant families are known to produce a vast range of phytochemicals as seen from **Table B 1.10**.

Table B 1.10 Diverse Phytochemicals Synthesized by Hairy Roots. (Flores and Medina-Bolivar, 1995; Bhagyalakshmi and Ravishankar, 1998a; Mukundan et al., 1998).

No.	Family	Plant Species	Class of Compound
1.	Acanthaceae	*Salvia miltiorrhiza*	Diterpenoids
2.	Apocyanaceae	*Catharanthus roseus*	Indole Alkaloids
3.		*Catharanthus tricophyllus*	17 Indole Alkaloids
4.		*Rauwolfia serpentina*	Reserpine
5.		*Amsonia elliptica*	Indole alkaloids
6.	Araliaceae	*Panax ginseng*	Saponins, Steroids, Polyacetylenes
7.	Asteraceae	*Bidens* spp.	Polyacetylenes
8.		*Tagetes patula*	Thiophenes, Polyacetylenes
9.		*Echinacea purpurea*	Alkamids
10.		*Chaenactis douglasii*	Thiarubrins
11.		*Ambrosia* spp.	Thiophenes, Polyacetylenes
12.		*Rudbeckia* spp.	Thiophenes, Polyacetylenes
13.	Brassicaceae	*Armoracia lapathifolia*	Peroxidase, Isoperoxidase
14.	Boraginaceae	*Lithospermum erythrorhizon*	Naphthoquinones
15.	Chenopodiaceae	*Beta vulgaris*	Betalains
16.	Convolvulaceae	*Calystegia sepium*	Cuscohygrine
17.	Cucurbitaceae	*Trichosanthes kirilowii*	Chitinases
18.	Gentianaceae	*Swertia japonica*	Xanthones
19.	Geraniaceae	*Geranium thunbergii*	Tannins
20.	Rubiaceae	*Cinchona ledgeriana*	Quinonoline and Indole alkaloids
21.	Rutaceae	*Ruta graveolens*	Various phytochemicals

Contd…..

Table B 1.10 Contd.

No.	Family	Plant Species	Class of Compound
22.	Solanaceae	*Atropa belladona*	Tropane alkaloids, Calystegins
23.		*Anisodus luridus*	Tropane alkaloids
24.		*Brugmansia candida*	Tropane alkaloids
25.		*Datura stramonium*	Tropane alkaloids
26.		*Duboisia leichhardtii*	Tropane and Pyrrolidine alkaloids
27.		*Hyoscyamus niger, H. albus*	Tropane alkaloids
28.		*H. muticus*	Tropane alkaloids, Sesquiterpenes
29.		*Nicotiana tabacum*	Pyrrolidine alkaloids
30.		*Solanum khasianum*	Steroids : Solasodine
31.		*S. laciniatum*	Steroidal alkaloids
32.		*Scopolia* spp.	Tropane alkaloids
33.		*Withania somnifera*	Tropane alkaloids, Withasteroids
34.	Valeriaceae	*Valeria officinalis*	Valeoptriates
35.	Verbenaceae	*Lippia dulcis*	Sesquiterpenes

Generally hairy roots produce the same qualitative and quantitative spectrum of chemicals produced by the parent plant. But recently many culture systems have demonstrated the production of novel compounds in hairy roots which are not found in intact plants (**Table B 1.11**). In other systems, secondary metabolites accumulating at only low quantities *in vivo* are synthesized in significant amounts by hairy roots (Doran, 1994; Bhagyalakshmi et al., 1998b). These results can have many possible reasons as suggested by Doran (1994) and Robins (1998):

a) As hairy roots are not connected with any other plant organs, metabolites produced cannot be transported to other plant parts for modification, degradation or storage.
b) Many of the so-called novel compounds in hairy roots may be present in minor, undetectable quantities in roots *in planta* and these get accumulated in higher levels in the hairy roots due to altered environment.
c) *In vivo* sites of synthesis for many secondary compounds are unknown and many leaf-based compounds may be synthesized in roots *in vivo* and so hairy roots can be manipulated for their production.

Table B 1.11 Hairy Root Cultures Producing Novel Secondary Metabolites (Not Present in Parent Plants) (Kukreja et al., 1997).

Plant Spp.	***Agrobacterium rhizogenes* Strain**	**Novel Secondary Metabolite**
Astragalus membranaceous	ATCC 15834	Agroastragaloside I, saponins and polysaccharides
Fagopyrum esculentum	ATCC 15834	Flavonoids/catechins, procyanidins
Lobelia inflata	ATCC 15834	Polyacetylene/Robetyolinin
Rauwolfia serpentina	ATCC A4	12-hydroxyajmaline
Swertia japonica	ATCC 15834	8-o-primeverosyl bellidifolin

From the foregone Introduction, it is obvious that there is an urgent need to catalogue biodiversity, to identify and to characterize the chemistry of medicinal plants, as they are the storehouses of **"futuristic medicines"**. To continue such efforts which have been made by earlier researchers in obtaining significant results in the *in vitro* establishment and studies of a number of medicinal plants, we in the present thesis attempted to work with two plants namely, ***Taxus baccata* ssp. *wallichiana* Zucc. Pilg. (Himalayan Yew)** and ***Withania somnifera* (L.) Dun. (Ashwagandha)** that are known to produce antitumor secondary metabolites. The present thesis deals with **morphogenesis, micropropagation and secondary metabolite studies of these two plants *in vitro*.** Part I of the present thesis deals with *Taxus baccata* ssp. *wallichiana* Zucc. Pilg. (Himalayan Yew) while Part II deals with *Withania somnifera* (L.) Dun. (Ashwagandha). The overall objectives and the justification for undertaking the study with these two plants, in this work presented as a thesis here, are discussed in detail in respective chapters in Part I and Part II.

CHAPTER 2

Materials and Methods (General).

During the course of work presented in this thesis, different techniques related to plant tissue and cell culture, analytical TLC and HPLC, microbial culture (for growing and maintaining the contaminating bacteria and fungi isolated from *in vitro* cultures and also wild strains of *Agrobacterium rhizogenes* used in genetic transformation experiments), biochemical characterization and long and short term maintenance of the microbes were used.

The present chapter describes the general and common methods of plant tissue and cell culture used for both *Taxus* and *Withania* spp. Specific methodologies and techniques related to plant tissue and cell cultures, analysis of production of secondary metabolites *in vitro* as well as microbial culture, characterization and identification are described for both *Taxus* and *Withania* spp. in their respective chapters.

2.1 Glassware

Test tubes (2.5 x 15 cm), petri dishes (55 mm and 85 mm diameter), Erlenmeyer flasks and beakers (100, 250, 500, 1000 and 2000 ml capacity), pipettes (1, 2, 5 and 10 ml capacity) and measuring cylinders of all the capacities (10 ml-2000 ml) were purchased from "Borosil" India. In addition, glass, screw capped bottles (6 x 15 cm) purchased from "Ballarpur Industries", India were used for all tissue culture related experiments.

For the analytical work, Erlenmeyer flasks (250 ml, 500 ml and 1000 ml capacity), graduated and Pasteur pipettes, funnels, measuring cylinders, glass beakers, glass capillaries, glass sample vials were from "Borosil" India. Round bottom flasks of varying capacities were purchased from "Duran", Germany. 50 µl constant volume glass microsyringes needed for HPLC injections were purchased from 'Exmire' ("Ito Corp.", Japan).

2.1.1 Preparation of glassware

Glassware was cleaned by boiling initially in a saturated solution of sodium bicarbonate for one hour and subsequent washing in tap water. It was then immersed in 30% nitric acid solution for 30 min followed by a thorough wash with tap water. The glassware thus washed was then rinsed twice with glass distilled water and allowed to dry in an oven at 200^{o}C for two hours.

Test tubes and flasks needed for tissue culture work were plugged with absorbent cotton ("Seasons Healthcare Ltd.", Andhra Pradesh, India). Such plugged flasks, screw capped bottles and petri dishes were wrapped in brown paper prior to sterilization. Autoclaving was carried out at 121^{o}C and at a constant pressure of 1.1 kg.cm^{-2} for 1 h.

2.2 Plasticware

Sterile disposable plastic dishes (55 mm and 85 mm diameter) and disposable filter sterilization units having filters of pore size 22 µm were obtained from "Laxbro" (India). Microtips (20-1000µL) and Eppendorf tubes (1-2 ml) were obtained from "Sigma Chemical Co." (USA) as well as from "Tarsons" (India). The syringes and disposable small filter units used for filtering the callus sample extracts before HPLC, were purchased from "Millipore Corporation", (USA).

2.3 Chemicals

The chemicals used during the course of the study were of analytical grade. Inorganic salts were obtained from "Qualigens" and "S. D. fine chemicals", (India). All vitamins and growth regulators were obtained from "Sigma Chemical Co.", (USA). Agar-agar and sucrose were obtained from "Qualigens" and "S. D. fine chemicals", (India). Phytagel was purchased from "Sigma Chemical Co.", (USA). Casein acid hydrolysate, peptone, yeast extract and beef extract were purchased from "HiMedia Laboratories", (India).

Coconut milk (liquid endosperm) was obtained from green tender coconuts, brought from a local market. It was first filtered through rough filter paper and collected in 500 ml flasks (300 ml/flask). It was then autoclaved at 121^{o}C and at a pressure of 1.1

kg.cm^{-2} for 20 min, and then refiltered through Whatman No. 1 filter paper. The filtrate was stored at -20^{o}C as 300 ml aliquots in a freezer until required.

All the solvents used for the analytical work in HPLC and TLC were of "Lichrosolv" grade from "Merck", (India). Silica gels of various properties were purchased either loose or in the form of pre-coated plates from "Merck", (India).

2.4 Preparation of Plant Tissue Culture Media

Glass distilled water was used for the preparation of culture media. After addition of all constituents of media, the pH was adjusted to 5.8 using 0.1 N KOH or 0.1 N HCl. When activated charcoal ("Sarabhai Chemicals", India) was added to the medium, pH was adjusted to 6.0 before autoclaving the medium. Gelling agent (agar-agar or phytagel) was added as per requirement and the medium was steamed to melt the gelling agent. It was then dispensed into test tubes (20 ml per tube) or Erlenmeyer flasks (100 ml per 250 ml flask) or screw capped bottles (50 ml per bottle) and was autoclaved at 121^{o}C at a pressure of 1.1 kg.cm^{-2} for 20 min. When no gelling agent was added, each tube contained liquid medium with a filter paper bridge of Whatman No. 1 filter paper. Heat labile constituents like antibiotics were filter-sterilized by passing through a Millipore membrane (0.22 µm pore size) ("Millipore Corporation", USA) and added aseptically to the autoclaved medium just before gelling. All the plant growth regulators used during the course of the present work were added before autoclaving the medium. As per the requirements, the medium was also poured in sterile plastic dishes (12 ml per 55 mm dish, 20 ml per 85 mm dish) in a laminar flow hood.

The composition of various media used for culturing of explants of both the plant spp. (*Taxus* spp. and *Withania* spp.) are given in **Table 2.1** and a comparison of the concentrations of cations and anions in each of the media are indicated in **Table 2.2.**

Table 2.1: Composition of Inorganic and Organic Components ($mg.l^{-1}$) of Seven Plant Tissue Culture Basal Media* Used for Culturing Various Explants of *Taxus* spp. and *Withania* spp.**

Major Salts	WPM[a]	DCR[b]	LM[c]	MLM*[d]	MS[e]	B5[f]	SH[g]
NH_4NO_3	400	-	1650	800.97	1650	-	-
KNO_3	-	340	1900	1010.64	1900	2528	2500
$MgSO_4, 7H_2O$	370	370	1850	1850	370	246	400
$(NH_4)_2SO_4$	-	-	-	-	-	134	-
KCl	-	-	-	-	-	-	-
KH_2PO_4	170	170	340	340	170	-	-
NaH_2PO_4	-	-	-	-	-	150	-
Na_2SO_4	-	-	-	-	-	-	-
$CaCl_2, 2H_2O$	221	85	22	22	440	150	200
$Ca(NO_3)_2, 4H_2O$	556	556	-	-	-	-	-
K_2SO_4	990	-	-	-			
$NH_4H_2PO_4$	-	-	-	-	-	-	300
Na_2-EDTA	37.3	37.3	37.3	37.3	37.3	37.2	20
$FeSO_4, 7H_2O$	27.8	27.8	27.8	27.8	27.8	27.8	15
$Fe_2(SO_4)_3$	-		-	-	-	-	-

Contd…..

Table 2.1 Contd.

Minor Salts	WPM[a]	DCR[b]	LM[c]	MLM*[d]	MS[e]	B5[f]	SH[g]
H_3BO_3	6.2	6.2	31.0	31.0	6.2	3.0	5.0
$CoCl_2$, $6H_2O$	-	0.025	0.125	0.13	0.025	0.025	0.1
$CuSO_4$, $5H_2O$	0.25	0.25	0.5	0.5	0.025	0.025	0.2
$MnSO_4$, $4H_2O$	-	22.3	27.72	27.72	22.3	-	-
$MnSO_4$, H_2O	22.3				-	10.0	10.0
$ZnSO_4$, $7H_2O$	8.6	8.6	43.0	43.0	8.6	2.0	1.0
KI	-	0.83	4.15	4.15	0.83	0.75	1.0
Na_2MoO_4	0.25	0.25	1.25	1.25	0.25	0.25	0.1
$NiCl_2$	0.024	-	-	-	-	-	-
MoO_3	-		-	-	-	-	-
Organics							
Myo-inositol	100	200	100	100	100	100	1000
Nicotinic acid	0.5	0.5	0.5	0.5	0.5	1.0	5.0
Pyridoxin.HCl	0.5*	0.5	0.5**	0.5	0.5	1.0	0.5
Thiamine.HCl	1.0*	1.0	5.0**	5.0	0.1	10.0	5.0
Glutamine	-	-	-	1461	-	-	-
Glycine	2.0*	2.0	3.0**	3.0	2.0	-	-

[a] Lloyd & McCown's Woody Plant Medium (1981); [b] Gupta & Durzan's DCR Medium (1985); [c] Litvay's Medium (1981); [d] Modified Litvay's medium MLM by AAK (1997); [e] Murashige & Skoog (1962); [f] Gamborg et al. B5 Medium (1968); [g] Schenk & Hildebrandt (1972).

* : Modified in this thesis.
** : Vitamins according to MLM.
*** : For WPM and B5 media, some of the salt concentrations as suggested by Owen and Miller (1992) were used in some of the experiments.

Table 2.2 A Comparison of the Inorganic Nutrient Ion Concentration of Seven Plant Tissue Culture Basal Media Used for Culturing Various Explants of *Taxus* spp. and *Withania* spp. (Adapted from McCown and Sellmer, 1987).**

Macroelements = mM, Microelements = μM.

Inorganic Ions	WPM	DCR	LM	MLM*	MS	B5	SH
NH_4^+	4.94	5.0	20.6	10	20.61	2.02	2.6
K^+	12.61	4.62	22.52	10	20.04	24.7	24.7
Ca^{++}	3.0	2.69	0.15	0.15	2.99	1.02	1.4
Mg^{++}	1.5	1.5	7.5	7.5	1.5	1.01	1.6
Mn^{++}	0.132	0.132	0.124	0.132	0.132	0.059	0.059
Zn^{++}	0.03	0.03	0.15	0.03	0.029	0.007	0.014
Na^+	0.224	0.102	0.01	0.224	0.224	1.1	0.1
Fe^{++}	0.1	0.1	0.05	0.05	0.1	0.05	0.027
NO^{3-}	9.64	10.48	39.4	20	39.4	24.7	24.7
SO_4^{2-}	7.44	1.76	7.5	7.5	1.76	2.04	1.7
PO_4^{3-}	1.25	1.25	2.5	2.5	1.25	1.1	2.6
BO_3^{3-}	0.1	0.1	0.501	0.1	0.1	0.049	0.082
Cl^-	1.31	1.16	0.3	1.31	6.0	2.04	2.7
Fe-EDTA^{3-}	0.11	0.11	0.11	0.11	0.11	0.11	
Co^{++}	-	0.105	0.6	-	0.105	0.083	0.332
Cu^{++}	0.1	0.1	2.0	0.1	0.1	0.1	0.8
MoO_4^{2-}	1.03	1.61	5.17	1.03	1.03	1.03	0.412
I^-	-	5.0	25	-	5.0	4.52	6.0116
Ni^{++}	-	0.193	-	-	-	-	-
Total Ionic Strength of Media.	**43.516**	**36.042**	**144.185**	**60.736**	**100.480**	**65.738**	**69.838**

* : Modified in this thesis.

** : For WPM and B5 media, some of the salt concentrations as suggested by Owen and Miller (1992) were used in some of the experiments.

2.5 Growth and Maintenance of Plant Tissue and Cell Cultures

The stationary tissue cultures (callus and shoot cultures) belonging to both the plants on solid media were subcultured at regular intervals of 3-4 weeks. Observations were taken at the same time. The cell suspension cultures of *Taxus baccata* ssp. *wallichiana* Zucc. Pilg., were subcultured after every 4 days into the fresh liquid media (10% v/v) and the relevant observations of Packed Cell Volume (PCV), fresh and dry weights and the secondary metabolite contents were determined at regular intervals of 0 days, 9 days and 18 days. Liquid cultures of seedling and hairy roots of *Withania somnifera* (L.) Dun. were subcultured after every 6 days. Various details of such methodologies are described in relevant chapters of this thesis.

2.6 Statistical Analysis

"Randomized Block" was the experimental design used in this study. Depending upon the size and the availability of the explants, each experiment consisted of either 5, 10 or 50 replicates (mentioned in each of the chapters more specifically) and each experiment was repeated at least three times. In addition, the experiments were repeated over successive years. Standard Deviations (S. D.) and Standard Errors of Means (S. E.) were calculated in each experiment. Analysis of Variance (ANOVA) was carried out and the post-hoc significance of differences was calculated based on Student's t-test (Wardlaw, 1985).

CHAPTER 3

Control of Contamination and Phenolic Browning *in vitro*.

< **Identification and Control of Bacterial and Fungal Genera Associated with *in vitro* Cultures of *Taxus baccata* ssp. *wallichiana* Zucc. Pilg. and *Withania somnifera* (L.) Dun. as well as Control of Phenolic Browning.**

3.1. Introduction:

Plant tissue culture techniques are extensively used for mass production of elite plants as well as to study the basic aspects of primary and secondary metabolism, morphogenesis and genetic engineering etc. (George and Sherrington, 1984). But even after three decades of research and development in plant tissue culture, microbial contamination by yeasts, fungi, bacteria, viruses, mites and thrips is still the major problem that has hampered the establishment of truly aseptic plants and their successful micropropagation (Herman, 1990; Leifert et al., 1994). The influence of bacteria on shoot growth can range from total inhibition to no apparent effect (McCown, 1986). The contaminating bacteria and fungi may be endophytic or epiphytic, may be pathogenic or saprophytic (Debergh and Maene, 1981, 1984).

Another type of hazard for plant tissue and cell cultures is caused by 'latent' bacteria and viruses that do not produce any symptoms on the plant or any visible growth on the medium for long periods of time *in vitro*. They reduce the multiplication rates and productivity of tissue cultures and may become 'virulent' at a later stage due to changes in incubation temperatures, growth medium composition and removal of antibiotics from the medium (Leifert and Waites, 1994).

Increasing attention is being paid to ascertain sources of contamination, to develop procedures for their elimination by avoidance, rigorous manipulation of the nutritional and environmental conditions and by specific antibiotic treatments (DeFossard and DeFossard, 1988; Debergh and Read, 1991).

The earliest plant tissues cultured were cambii from woody Angiosperms and Gymnosperms (Gautheret, 1934). However the rate of progress in this field was relatively slow during the last 4 decades owing to a large number of bacterial and fungal contaminants associated with mature explants of Gymnosperms, due to problems of maturation (Gupta and Durzan, 1985), and also due to autotoxic phenolic exudations produced by them (Young et al., 1984).

3.1.1 Nature and occurrence of contaminants

The contaminants could be of endophytic or epiphytic origin (Debergh and Maene, 1984; Hennerty et al., 1988). An "endophyte" is defined as a fungus or a bacterium that causes inapparent, asymptomatic but parasitic infections within the tissues of the plant. These may be 'obligate endophytes' or 'facultative endophytes'. The latter are subdivided into 'constitutive' and 'inducible' types. Inducible endophytes are most frequently encountered during *in vitro* culture, since in nature, they are epiphytes of the parent plant (Gunson and Spencer-Phillips, 1994). An "epiphyte" is defined as a fungus or a bacterium associated with external plant surfaces that survive surface sterilization procedures (Leifert et al., 1989; Wilson and Power, 1989; Gunson and Spencer-Phillips, 1994). Other types of microbes could be air-borne and/or human associated (Leifert et al., 1989) and can be introduced via poor aseptic techniques, insufficient sterilization of instruments (Boxus and Terzi, 1988), failure of equipment (Trick and Lingens, 1985; Giles and Morgan, 1987) and laboratory environment with a high microbial load (Cassels, 1991). These various types of contaminants are summarized in **Table 3.1**.

Table 3.1 Contaminants from Various Sources (Adapted from: Leifert and Woodward, 1998).

Contaminant	Likely Source of Contamination	Comments
Bacteria		
Gram negative rods.	Inefficient disinfection of explants.	Often *Pseudomonas* spp. and Enterobacteriaceae.
Gram positive rods.	Inefficient laboratory procedures.	Often *Bacillus* and *Clostridium* spp.
Gram positive cocci (e.g. *Staphylococcus epidermidis*).	Poor aseptic techniques in subculturing of plants and pouring of media.	*Staphylococcus* spp. are considered as obligate human inhabitants.
Bacillus spp.	Inefficient sterilization of media.	Often *Bacillus subtilis* and *B. pumilus*.
	Inefficient sterilization of instruments used for subculture.	*Bacillus circulans* can survive in 70% alcohol.
Fungi/Yeasts		
General increase	Mite or thrip infestation of growth-rooms.	Especially when plant-specific growth rooms are affected.
Penicillium spp., *Candida* spp.	During pouring of media.	Especially when plant-specific growth rooms are affected.
Fusarium poe	Growth room infestation with *Sideroptes graminis* mites.	Fungus with white mycelium and a pink base.
Grey, black and green moulds (*Botrytis*, *Aspergillus*, *Alternaria*, *Penicillium* spp.)	High laboratory air contamination, Faulty laminar airflow cabinets.	Common air contaminants within buildings.
Pink yeasts (*Rhodotorula* spp.)		
Black moulds	Poor hygiene in growth rooms, plant and media cold stores.	Moulds are common in damp areas of the buildings. Spores are air-transmitted.
Cladosporium spp.	Insufficient protection of laboratory against outside air.	*Cladosporium* spores are common in outside air.

The contamination problem is acute especially with explants of mature tissue origin. It is a well-known fact that the incidence of contamination is closely linked with the state of the mother plant, which is denoted as "Stage 0" (Debergh and Read, 1991). When mother plants are of a great age and growing under natural environmental conditions, the incidences of contamination increase from "Stage 1", i.e. surface sterilization, onwards (Debergh and Read, 1991).

In spite of the controlled laboratory conditions, the external environmental conditions also affect the contamination rates in tropical countries since the parent plants support a rich microflora with them at "Stage 0" itself (Enjarlic et al., 1988; Watt et al., 1996). In addition, sudden seasonal outbreaks (just before the monsoon) of bacterial and fungal contamination are observed involving hitherto sterile cultures of a number of plant species. Such sudden outbreaks of fungal contamination are often associated with mites and thrips, the arthropods inhabiting culture rooms and acting as vectors for fungal spores (Debergh and Maene, 1984; Leifert et al., 1991a, 1994; Danby et al., 1994). The origin of sudden outbreaks of bacteria is not known. It has been suggested that PPFMs (pink pigmented facultative methylotrophic bacteria) like *Methylobacterium* spp. are the responsible agents. These bacteria are associated with 70 plant species across all the families *in vivo* (Holland and Polacco, 1994 and references therein).

3.1.2 Harmful effects of contaminants

In most laboratories, losses due to contamination average between 3-15% of plants at every subculture (Leifert et al., 1994), rendering commercial micropropagation less economical. It also leads to severe production losses, low progeny performance and rejections of entire shipments of plants due to quarantine regulations (Cassells, 1991). In scientific experimental setup, to offset the effects of contamination, sample size needs to be very large and same experiments have to be repeated many times. In addition, the continued presence of microbial contaminants alters the morphological nature and physiological response of cultures and makes it difficult to interpret the results obtained. There are many contaminating bacteria which cause diseases only *in vitro*, and on all the varieties of plants (**"Vitropaths"**, Herman, 1990). Considering the high frequency of mutations and variations in bacteria, such organisms can become pathogenic and cause epidemics *ex vitro*. They may also block the vascular tissues.

Latent bacterial contamination during the proliferation phase adversely affects rooting due to changes in growth regulators and water potential of the media (Leifert et al., 1994) and also kills young trees after weaning (Boxus and Terzi, 1988). Many *Bacillus* spp. increase pH of the medium, inhibiting proton pumps involved in plant nutrient uptake and cause precipitation of many nutrients from the medium (Leifert et al., 1994). Cultures of apricot with latent bacterial contaminants showed high CO_2 and low O_2 concentration in the vessel headspace, thereby decreasing the proliferation and photosynthetic rates (Marino et al., 1996).

3.1.3 Beneficial effects of contaminants

Bacterial and fungal contaminants are also known to exhibit some beneficial effects *in vitro*. Nowak (1996) has coined the terms **"Bacterization"** and **"Biotization"** referring respectively to the addition of bacteria or other microbes to cultures or to *in vitro* cultured plants to get an enhanced biological effect. He isolated a strain of *Pseudomonas* from surface-disinfected onion roots which stimulated growth of potatoes *in vitro*, induced root branching, root hair formation, stem elongation, lignin deposition, better acclimatization and better resistance to fungal pathogens *ex vitro*. Same results were also obtained for a variety of other plants.

A *Pseudomonas* spp. isolated during *in vitro* culture of Oregano was reported to control vitrification and to improve acclimatization *ex vitro* (Shetty et al., 1995). Two strains of *Pseudomonas maltofila* increased the regeneration potential of recalcitrant calli of Soybean under co-cultivation (Herman, 1995). Culture extracts of *Trichoderma viride* (Strain A) and *Bacillus subtilis* (Strain B) showed antifungal activity while extracts from *Pseudomonas fluorescens* (Strain X) had the highest antibacterial activity (Hussain et al., 1994). When these were added to cultures of tobacco, they reduced the growth of accidental contaminants. A selected strain of a bacterial endophyte, *Bacillus subtilis* from a healthy Chestnut tree, showed broad-spectrum anti-fungal activity against a number of phytopathogenic fungi like *Cryphonectria parasitica*, *Alternaria radicina* etc. (Wilhelm et al., personal communication). A new strain of *Bacillus circulans* was found to promote somatic embryogenesis in *Pelargonium* when combined with TDZ (Murthy et al., 1999).

A number of bacterial endophytes isolated from *Taxus* spp. produce **Taxol** and related **Taxanes** (Page et al., 1999). It is believed that due to close associations of such endophytes with hosts, some genetic exchange takes place and the endophytes develop

the ability to synthesize particular metabolites. Similarly a number of fungal genera isolated from *Taxus* spp. produce **Taxol** and related **Taxanes** (Stierle et al., 1995; Strobel et al., 1996).

In another example (Czembor and Strobel, 1997), indigenous isolates of *Fusarium avenaceum* were shown to inhibit seed germination of spotted knapweed (*Centaurea maculosa*), without adversely affecting seed germination of wheat or oat. Thus this can be an effective bio-control measure. Phytotoxic metabolites from culture filtrates of *Alternaria solani* stimulated adventitious shoot induction in potato tubers when co-cultivated. Jasmonic acid and two other potato tuber-inducing principles were isolated from culture filtrates of fungus *Lasiodiplodia theobromae* which can be beneficially used for *in vitro* cultures of potato (Herman, 1995).

A recent hypothesis suggests that microbes may actually be essential to all cytokinin-autotrophic plant tissue cultures as well as to all plants *in vivo* as cytokinins are synthesized by these microbes like *Methylobacterium* spp. (Holland, 1997).

3.1.4 Control of contaminants

The use of anti-microbial agents (anti-bacterial as well as anti-fungal) to control contamination is the preferred method (Thurston et al., 1979; Falkiner, 1988; Wilson and Power, 1989; Kneifel and Leonhardt, 1992; Watt et al., 1996). However their indiscriminate use may lead to phytotoxicity problems (Phillips et al., 1981; Pollock et al., 1983) and development of resistant strains (Leifert et al., 1991b). Hence characterization of contaminating microbes (bacteria and fungi) to generic and species level can yield important information about the source of contamination, the relative importance of different sources, pathogenicity, if any, of the isolated genera (Danby et al., 1994) and more specifically the development of versatile antibacterial and antifungal therapies (Falkiner, 1988; Leifert et al., 1989).

3.1.5 Phenolic browning/oxidation *in vitro*

The presence of phenolic compounds causing death of explants has been another important problem of tissue cultures of woody perennials (Compton and Preece, 1986) in addition to various bacterial and fungal contaminants as discussed above. Some of these exudates appear as a reaction to injury and/or infection. In tissue culture they appear after tissue excision and are many times aggravated by growth media constituents (Seneviratne and Wijesekara, 1996). Tissue blackening occurs due to action of copper-containing oxidase enzymes: **polyphenoloxidases** like tyrosinases, which are released or synthesized in oxidative conditions after tissue wounding and they oxidize o-diphenols released due to cellular wounding to o-quinones (Scalbert et al., 1988; Marks and Simpson, 1990 and references therein). The onset of tissue browning has been found to be associated with changes in protein pattern, amino acid content, ethylene production and the occurrence of saccharose and accumulation of starch (Lindfors et al., 1990). These changes eventually lead to growth inhibition or death of explants. Other types of phenolic exudates appear at the end of incubation period and are apparently products of dying cells (Seneviratne and Wijesekara, 1996). The phenolic exudation is aided by light and is autocatalytic.

A number of methodologies have been employed to overcome the phenolic oxidation. Some of them are as follows (Pierik, 1987):

- Addition of activated charcoal (0.2-3.0% w/v) to the medium.
- Addition of polymeric polyvinylpyrrolidone (PVP) or polyvinylpolypyrrolidone (PVPP) to the medium. These absorb phenols through hydrogen bonding.
- Additions of anti-oxidants/reducing agents like citric and ascorbic acids, thiourea glutathione and L-cysteine in the medium or before surface sterilization. These reduce the redox potential of explants and stop the oxidation reactions (Marks and Simpson, 1990).
- Addition of diethyl-dithiocarbonate (DIECA) ($2g.l^{-1}$) in the rinses after surface sterilization and as droplets at the time of micro grafting.
- Addition of amino acids like glutamine, arginine and asparagine to the media.
- Frequent subcultures onto fresh media.
- Use of liquid medium for easier and quicker dilution of toxic products.
- Reduction of wounded tissues to decrease exudation.
- Reduction of salt concentration in the growth media.

- Omission of plant growth regulators as they aid in medium darkening.
- Soaking of explants in water before culturing to reduce browning.
- Incubation of shoot bases in darkness during culture period.
- Immediate inoculation of explants after removing them from the parent trees.
- Sealing ends of explants with paraffin wax.

During the course of the present work, the problem of phenolic oxidation was particularly encountered on culturing stem bits and needle explants of *Taxus* spp. since they were derived from mature trees (80-120 year old). Due to oxidation of externally released polyphenols, the explants (stem bits, needles) as well as the nutrient medium became brown and the explants did not respond *in vitro* and a strategy to control phenolic oxidation had therefore become a necessity if not warranted.

The problem of phenolic oxidation was relatively less in cultures of *Withania* spp. because it is a herbaceous plant and the explants were derived from *in vitro* grown seedlings and were juvenile.

Bacterial and fungal contamination of cultures, especially initiated with mature explants of *Taxus* spp. for establishment of callus (**Chapter 4** of this thesis) and shoot cultures (**Chapter 5** of this thesis) under aseptic conditions, was yet another major problem and in the initial experiments all the cultures (100%) were contaminated. In case of cultures of *Withania* spp., the internodal explants (**Chapter 6** of this thesis) showed presence of typical yellow bacteria hindering the regeneration response. These bacteria were however absent in all the other cultures raised with explants such as nodes, leaves, hypocotyls, embryos and seeds. To our knowledge, there are neither detailed reports describing the contamination in tissue cultures of these two plants nor methods to control contamination observed especially for *in vitro* cultures of *Taxus* explants in literature. Probably contamination was not a problem in these studies reported earlier for these two species or serious attempts have not been made to control the observed contamination. However, some authors like Gibson et al. (1993) have mentioned presence of contaminants in tissue cultures of *Taxus brevifolia* and have used broad-spectrum anti-fungal and anti-bacterial agents to control them. However neither the methods adapted to control contamination nor the percentages of contamination were reported. On the other hand, a large number of endophytic bacteria and fungi have been isolated from trees of

Taxus spp. growing under natural habitats and many have been shown to produce the **Taxane** class of anti-cancer agents independent of the parent plants (Stierle et al., 1995; Strobel et al., 1996). However, the association of these fungi and bacteria and their effect on *in vitro* cultures is not known. On culturing of various explants, especially of *Taxus* spp. in various nutrient media in our studies, all the cultures got contaminated and use of broad spectrum antifungal and antibacterial agents to control them, as reported by Gibson et al. (1993), did not yield significant positive response and results. Therefore an attempt has been made to systematically identify and characterize the bacterial and fungal associates and contaminants of *Taxus* spp. and *Withania* spp. and to develop a contamination control strategy in order to obtain sterile cultures of *Taxus* spp. and *Withania* spp. Furthermore, since the explants of *Taxus* spp. in particular, were derived from mature trees, they exuded phenolics hampering the *in vitro* response. Hence it had become necessary to formulate a strategy to overcome not only the problem of phenolic oxidation but also the high degree of fungal and bacterial contamination associated with cultures of *Taxus* spp. and *Withania* spp. under *in vitro* conditions.

The objectives of the work in the present chapter have therefore been:

1. To elucidate the nature of the contaminants associated with *in vitro* cultures of *Taxus* spp. and *Withania* spp., which are varied in their habits and are collected from diverse habitats.
2. To identify the contaminants upto the generic and species level.
3. To devise a suitable and very specific control strategy to overcome both bacterial and fungal contaminants encountered in both the species based on the know-how generated.
4. To develop a common protocol applicable not only to control phenolic browning but also contamination associated with cultures of *Taxus* spp. and *Withania* spp. so as,
5. To establish sterile and healthy cultures in both the species for further *in vitro* studies as an acid test for the effectiveness of the developed protocol for control of contamination and phenolic browning.

While the bacterial contaminants and associates observed commonly in *Taxus* and *Withania* spp. have been described under **Subsection 1** of the present chapter, **Subsection 2** of this chapter deals with the fungal contaminants and phenolic oxidation/browning.

3.2. Materials and Methods

3.2.1 Collection of plant material

While the twigs of *Taxus* spp. were collected from different locations in the Himalayas as described in detail in **Chapter 4, Section 4.2.1**, the seeds of the cultivated variety WS-20 of *Withania* spp. were collected as described in detail in **Chapter 6, Section 6.2.1**.

3.2.2 Surface sterilization and culture of explants

3.2.2.1 *Taxus baccata* ssp. *wallichiana* Zucc. Pilg.

Stem bits with axillary buds and trimmed needles (3-5 cm long), stem bits without axillary buds, needles, and seeds were first given a liquid soap treatment with 1% (v/v) Teepol ("Bennett & Colman", India) for five minutes with constant agitation. After washing off the soap solution, one minute long treatment with 95% (v/v) ethyl alcohol followed by ten minute duration treatment with 0.1% (w/v) mercuric chloride ($HgCl_2$) under vacuum (equivalent to 20" Hg) was given. $HgCl_2$ was washed off with six washes of sterile distilled water in a laminar airflow cabinet. This methodology of surface sterilization of explants shall be hereinafter referred to as the **"Routine Surface Sterilization Protocol"**. The surface sterilized explants were then inoculated on nutrient media and incubated under conditions as described in **Section 3.2.3.1**. The percentage of contamination either due to bacteria or fungi or by both was scored.

3.2.2.2 *Withania somnifera* (L.) Dun.

Seeds of *Withania* spp. used for raising *in vitro* seedlings were rinsed in a mixture of 1% (v/v) Teepol and 70% (v/v) ethyl alcohol for 15 seconds followed by treatment with 3% sodium hypochlorite (v/v) for five minutes. Glass distilled water was used for all the washing steps. The seeds were then washed with six washes of sterile distilled water in a laminar airflow cabinet. This methodology of surface sterilization of explants shall be hereinafter referred to as the **"Routine Surface Sterilization Protocol"**. These seeds were used for raising seedlings. The internode explants were derived from such two-month old *in vitro* grown seedlings as described in **Chapter 6, Sections 6.2.2 and 6.2.3**. Explants were inoculated on nutrient media and incubated under conditions as described

in **Section 3.2.3.2**. The percentage of contamination either due to bacteria or fungi or by both was scored.

3.2.3 Media and culture conditions

3.2.3.1 *Taxus baccata* ssp. *wallichiana* Zucc. Pilg.

The stem and needle explants of *Taxus* spp. were inoculated in plastic dishes (55 mm diameter) containing full strength woody plant medium (WPM) (Lloyd and McCown, 1981) with 2% sucrose, 0.3% phytagel supplemented individually with 2, 4, 6, 8 or 10 $mg.l^{-1}$ 2,4-D for callus induction. All the details of media composition and media preparation were as described earlier in **Chapter 2, Section 2.4**. The cultures were incubated in total darkness at 25 $\pm$ 2°C. In addition, stem explants with axillary buds were inoculated in culture tubes containing WPM with its major salts reduced to half strength (1/2 WPM) without plant growth regulators but with 2% sucrose, 0.8% agar for enhanced axillary budding. All the details of media composition and media preparation were as described earlier in **Chapter 2, Section 2.4**. The cultures were incubated under 16 hour photoperiod provided by cool white fluorescent tubes of light intensity 4.8-19 $\mu E.m^{-2}s^{-1}$ at 25 $\pm$ 2°C. The embryos and endosperm halves dissected out from seeds were cultured in plastic dishes (55 mm diameter) on full strength WPM medium without plant growth regulators and with 2% sucrose, 0.3% phytagel. The cultures were incubated in total darkness at 25 $\pm$ 2°C.

3.2.3.2 *Withania somnifera* (L.) Dun.

All the internode explants of *Withania* spp. were inoculated in culture tubes containing full strength MS medium (Murashige and Skoog, 1962) supplemented with 2% sucrose, 0.8% agar and 2, 4, 6, 8 and 10 $mg.l^{-1}$ 2,4-D independently for callus induction. All the details of media composition and media preparation were as described earlier in **Chapter 2, Section 2.4**. The cultures were incubated under 24 hour photoperiod provided by cool white fluorescent tubes of light intensity 38 $\mu E.m^{-2}s^{-1}$ at 25 $\pm$ 2°C.

3.2.4 Isolation of bacterial contaminants

The bacteria appeared either from the cut end of the needles and stem bits of *Taxus* spp. during the first week of culture or after one month from the developing calli while the contaminating bacteria appeared within 15 days from the internodes of *Withania* spp. causing them to become spongy and unresponsive for regeneration. The isolated bacteria were grown and stored as follows.

3.2.5 Growth, storage, identification and control of contaminating bacteria

3.2.5.1 Growth, purification and storage

The contaminant bacteria were removed with a sterile wire loop from cultures of *Taxus* spp. and *Withania* spp. and grown either in 5 ml of nutrient broth (NB) prepared according to the manufacturer's instructions ("Difco Laboratories", USA) or on plates of nutrient agar (NA) [NB fortified with 2% bacto-agar ("Difco Laboratories", USA)]. Glass distilled water was used in media preparations and pH was adjusted to 7.0 before autoclaving, using 0.1 N KOH or 0.1 N HCl. Autoclaving was done at 121^{o}C at a pressure of 1.1 kg.cm^{-2} for 20 min.

Bacteria in NB were grown in dark for 24-48 hrs at 200 rpm in an incubator shaker ("Steelmate", India) at 26 $\pm$ 2^{o}C. The bacterial suspensions were then streaked onto sterile plates of nutrient agar (NA) to isolate single colonies and to check for the purity of the various isolates. Wherever the colonies were morphologically dissimilar, purification was carried out with the serial dilution method of Bradbury (Bradbury, 1988). Short-term storage of pure bacterial cultures was achieved by maintaining them in the form of single colonies on plates of NA kept at 4^{o}C. Long term storage was achieved by making glycerol stocks of each of the pure strains and storing them at a temperature of –70^{o}C (Maniatis et al., 1982).

The isolated, pure bacteria were used for their identification through morphological and biochemical characters.

3.2.5.2 Identification through morphological and biochemical characters

Bacterial cultures initiated from single colonies and in the log phase of growth (Usually grown for 24-48 hours at 26 $\pm$ 2ºC, in dark and at 200 rpm on a rotary shaker/incubator) (O. D. = 1 at wavelength of 600-650 nm) (Senior et al., 1995) were used for all the experiments of morphological and biochemical characterization.

For morphological characterization, cultures in log phase of growth were streaked and grown for 24 hrs on NA plates (at 26 $\pm$ 2ºC, in dark, in an incubator) and were visually examined for colony characters (size, shape, margin, elevation, colour and opacity).

A number of biochemical tests were performed (See **Tables 3.4 and 3.5**) with bacterial cultures in the log phase of their growth, for their identification, as described in Bradbury (1988), the Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and Principles of Biochemical Tests in Diagnostic Microbiology (Blazewic and Ederer, 1975). Since all these tests are routinely carried out in Microbiological practice for identification of various microflora, their details are not given here. The results obtained through morphological and biochemical tests of characterization were tabulated as **'Observed Values'** (**Tables 3.4 and 3.5**). These **'Observed Values'** were computerized and used in the identification of bacterial contaminants through a software program specially developed for this purpose.

3.2.5.3 Identification of the bacterial isolates based on a software program

The theoretical values as given in tables 4.1, 5.1 and 5.2 from Bergey's Manual (Holt et al., 1994), were computerized and regarded as the **'Master Values'**. The **'Observed Values'** were also computerized and a program in Visual Basic was prepared to count the number of matches (**'Similarity Indices'**) between the **Observed** and the **Master values**. The results obtained were processed for calculating the percentages and for determining the maximum percentages for each strain. The strains from the respective tables and with the highest two percentages of matches were considered to be the identities of the contaminant bacteria. After specific identification, antibiogramme assays were carried out to identify the effective antibiotics and their bactericidal concentrations for each of the identified bacteria.

3.2.5.4 Antibiogramme assay

Susceptibility of the bacterial isolates to different antibiotics was assayed by using commercially available filter discs, Biodisc-12 ("Pathoteq Laboratories", India), impregnated with known concentrations of twelve different antibiotics (See **Table 3.2**), by the top agar method (Blazewic and Ederer, 1975). The efficiency of each individual antibiotic agent against each isolate was deduced by measuring the diameter of the clearing zone around each filter disc after incubation of the cultures at 26 $\pm$ 2ºC for 24 hours in the dark, in order to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the effective antibiotic.

Table 3.2. Antibiotics and Their Concentrations Employed in Biodisc-12 ("Pathoteq Laboratories", India).

No.	Antibiotic Agent	Symbol	Type of Antibiotic	Concen-tration (mg)
1.	Ampicillin	AS	β-lactam group, a derivative of penicillin. Specifically inhibits bacterial cell wall synthesis.	20
2.	Co-Trimoxazole	BA	-	25
3.	Cefotaxime	CF	β-lactam group (Cephalosporins). Specifically inhibits bacterial cell wall synthesis.	30
4.	Piperacillin	PC	β-lactam group. (Semisynthetic acylamino penicillin). Specifically inhibits bacterial cell wall synthesis.	100
5.	Chloramphe-nicol	CH	Broad spectrum, bacteristatic. Inhibits polypeptide synthesis.	30
6.	Ciprofloxacin	CP	Quinolone group. Inhibits DNA gyrase during replication.	5
7.	Ceftizoxime	CI	β-lactam group (Cephalosporins). Specifically inhibits bacterial cell wall synthesis.	30
8.	Tetracycline	TE	Quinone group. Inhibits polypeptide synthesis, cell wall synthesis and bacterial respiratory systems.	30
9.	Ofloxacin	OF	Quinolone group. Inhibits DNA gyrase during replication.	5
10.	Gentamicin	GM	Aminoglycoside group. Inhibits polypeptide synthesis.	10
11.	Amikacin	AK	Aminoglycoside group. (Semisynthetically derived from Kanamycin-A). Inhibits polypeptide synthesis.	30
12.	Pefloxacin	PF	Quinolone group. Inhibits DNA gyrase during replication.	10

3.2.5.5 Determination of MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) by liquid assay

Liquid bioassays were carried out for each of the seven effective antibiotics identified by the Bio-discs on solid media, to determine the efficacy of each individual antibiotic at three different concentrations. For each bacterial isolate, three tubes of NB were prepared for each antibiotic agent. First tube contained NB supplemented with a sterile solution of an antibiotic at the concentration used in the biodisc. In the second tube, a sterile solution of the same antibiotic one unit concentration below the concentration used in Biodisc-12 was added to NB. In the third tube a sterile solution of the antibiotic one unit concentration above the concentration used in the Biodisc-12 was aseptically added to NB (**Table 3.9**). The presence or absence of growth of each bacterial isolate in these amended media was determined visually by the turbidity of the medium after growth for 24 - 48 hours at 26 $\pm$ 2ºC at 200 rpm on an incubator-shaker in the dark.

Bacteria cultured in the amended media were streaked on plates of plain NA to confirm the results of this liquid bioassay. Wherever the growth of the bacterium was observed on these plates, the original antibiotic concentration was designated as Minimal Inhibitory Concentration (MIC) and wherever the bacteria were unable to resume their growth, that antibiotic concentration was designated as Minimal Bactericidal Concentration (MBC). Wherever the growth of a bacterium was not hampered by an antibiotic at all, it was considered to be resistant to that particular antibiotic.

The type of antibiotic and its effective concentration derived, based on the above methodology to identify bacterial contaminants, was used along with antifungal agents in a strategy of pre-treatment as discussed under **Section 3.3.4**.

Ξ The identification of useful antifungal agents and their effective concentrations against the identified fungal contaminants observed in cultures of *Taxus* spp. and *Withania* spp. are described in the following sections.

3.2.6 Isolation and storage of fungal contaminants

3.2.6.1 *Taxus baccata* ssp. *wallichiana* Zucc. Pilg.

With the **"Routine Surface Sterilization Protocol"** (C. f. **Section 3.2.2**), almost all the cultures of *Taxus* spp. became contaminated with fungi during the first two weeks (**Fig. 3.1 C, D)**. The isolated fungal contaminants were purified by the standard hyphal tip and serial dilution methods (Alexopoulos and Benecke, 1962; Ainsworth and Sussman, 1966-1968) and grown on slants of potato dextrose agar (PDA) medium prepared according to the manufacturer's instructions ("Difco Laboratories", USA). Pure fungal cultures were maintained on slants of PDA kept at 4°C (Alexopoulos and Beneke, 1962).

3.2.6.2 *Withania somnifera* (L.) Dun.

The fungal contaminants observed in internode explants of *Withania* spp. were purified, grown and stored as described above in **Section 3.2.6.1**.

The isolated fungi from both the plants, in pure form, were further used for their identification.

3.2.7 Identification of fungal contaminants

The identification was carried out based on macroscopic and microscopic morphological characters of vegetative and reproductive structures (Ainsworth and Sussman, 1966-1968). Once definitive identification of fungal genera was known, various antifungal agents could be tried to control fungal contamination in cultures of *Taxus* spp. and *Withania* spp.

3.2.8 Use of antimicrobial agents to control bacterial and fungal contaminants

A number of anti-microbial agents were dissolved in glass distilled water and used at following concentrations: Bavistin ("BASF", India) at 1% (w/v), Aureofungin ("Hindustan Antibiotics Ltd.", India) at 0.5% (w/v), 8- Hydroxyquinoline, Hemisulphate salt (8-HQS) ("Sigma Chemical Co.", USA) at 0.1% and 0.25% (w/v), Augmentin ("Time Carp Pharma Labs Pvt. Ltd.", India) at 500 $mg.l^{-1}$, Streptopenicillin ("Hindustan Antibiotics Ltd.", India) at 500 $mg.l^{-1}$, Ofloxacin (Ofloxacin infusion, Tarivid® i. v., "Hoechst Marion Roussel Ltd.", India) at 25 mgl^{-1}, Pefloxacin (Pefloxacin infusion, Pelox*-400, "Wockhardt Ltd.", India) at 25 mgl^{-1} and Ciprofloxacin (Ciprofloxacin injection IP, Ciplox, "Cipla Ltd.", India) at 25 mgl^{-1}.

These anti-microbial agents individually or in combination were not incorporated in the tissue culture media but were used in the **"Modified Surface Sterilization"** procedure which was developed as a strategy to contain the bacterial and fungal contamination as well as phenolic browning based on the results obtained in this chapter. For details see **Section 3.3.4**.

The percentage of fungal contamination was scored for **"Routine Surface Sterilized"** explants without pre-treatment and compared against the **"Modified Surface Sterilization"** procedure involving a pre-treatment strategy (**Flow Chart 3.3**) containing antifungal agents individually and in combination with antibacterial agents as well as antioxidants. For details refer to **Sections 3.3.4, 3.3.5 and 3.3.6**.

3.2.9 Control of phenolic browning/oxidation

A number of strategies were used to control phenolic browning. The most basic strategy was the use of anti-oxidation agents such as Ascorbic acid and Citric acid ("HiMedia Laboratories", India) and soluble PVP, Mol. Wt. 3,60,000 ("Sigma Chemical Co.", USA) at 2% (w/v) strength in the pre-treatment solution (For details refer to **Sections 3.3.5 and 3.3.6**).The pre-treated explants were used either for axillary sprouting or for callus initiation experiments. The other methods used to control phenolic browning included use of 1/2 WPM (Strength of major salts reduced to 1/2) without plant growth regulators in liquid form (absence of gelling agents) or in solid form (presence of gelling agents) for inoculating the stem bit explants intended for enhanced axillary budding.

Further the media were modified in some cases by addition of 0.5% (w/v) activated charcoal (AC). The control of phenolic browning in cultures is described in detail in **Section 3.3.5**.

3.2.10 Statistical analysis

All the morphological and biochemical tests were carried out in triplicate for each of the bacterial and fungal isolates and the sets of experiments were repeated twice. The experiments on control measures for the contaminants were carried out in triplicate over three successive years and the Standard Errors of the Means (SE) and/or Analysis of Variance (ANOVA) were calculated wherever applicable. Post-hoc comparisons were performed with t-test (Wardlaw, 1985).

Each treatment in each of the experiments carried out to control phenolic browning/oxidation consisted of 50 replicates and each experiment was repeated thrice All the experiments were repeated over three successive years. Standard Errors of the Means (SE) and Analysis of Variance (ANOVA) were calculated wherever applicable. Post-hoc comparisons were performed with t-test (Wardlaw, 1985).

3.3 Results and Discussion

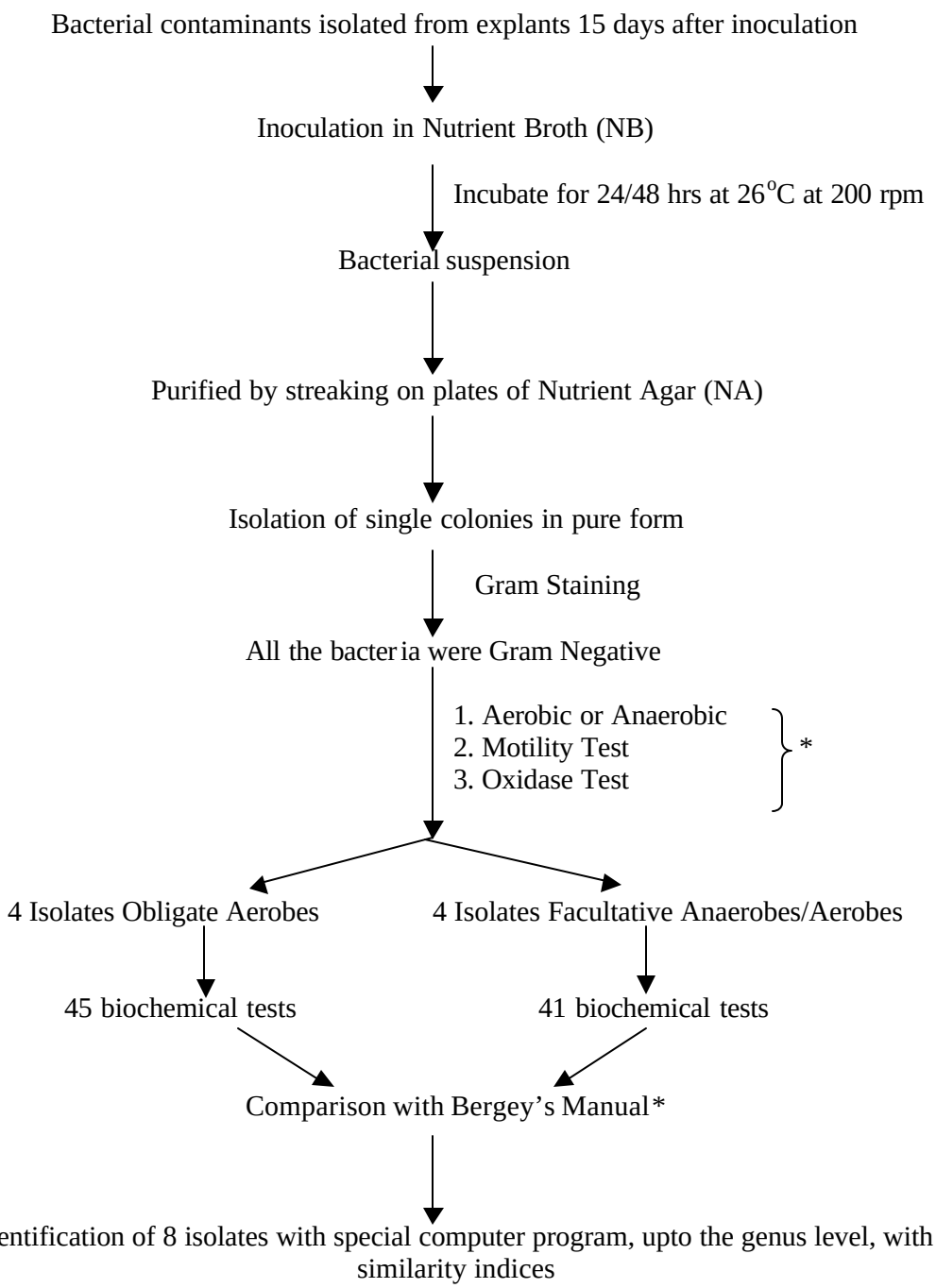
When the explants of *Taxus* spp. and *Withania* spp. were inoculated after using the **"Routine Surface Sterilization Protocol"** , high degree of either bacterial or fungal or bacterial as well as fungal contamination was observed.

Subsection 1: Bacterial Contamination

Plant tissue cultures are known to host a large number of microbes due to the favorable conditions present in the plant tissue culture media (high amount of sugars and organic compounds) and the microenvironment. In the earlier years, most of the efforts directed at overcoming these contaminants involved a treatment of the infected explants with a variety of antibiotics without identifying the contaminants (Young et al., 1984; Falkiner, 1988; Kneifel and Leonhardt, 1992). A number of researchers in the recent years have concentrated on identifying these contaminants up to the genus and species level for the development of very specific strategies to overcome these contaminants (Leifert et al., 1989; Leifert et al., 1991a, b; Buckley et al., 1995; Reed et al., 1995; Fellner et al., 1996) and also to inhibit the indiscriminate use of effective antibiotics, which may give rise to new and more virulent resistant strains. In addition, a large number of antibiotics are known to be highly phytotoxic to plant tissue cultures (Thurston et al., 1979; Pollock et al., 1983).

The methodology followed for identification of the contaminant bacteria in this thesis is depicted concisely in **Flow Chart 3.1**.

Flow Chart 3.1. Identification of Contaminant Bacteria upto the Genus Level



* Methodology for tests is as per Bergey's Manual of Determinative Bacteriology (Holt et al., 1994)

3.3.1 Isolation, characterization and identification of bacteria

After isolating the contaminant bacteria in pure, single colony forms on NA, a total of 8 isolates could be distinguished (See **Table 3.3**). Four isolates, namely T-W, T-WH3, T-CL1, T-EMB-W, were always associated with tissue cultures of *Taxus* spp. while two isolates, namely, W-Y(O) and W-Y(T), were always associated with tissue cultures of *Withania* spp. Two isolates, namely P-PNK AND P-PNK(S), were common to both the cultured plants and were of an 'occasional' or 'opportunistic' nature (**Fig. 3.1 A, B**). In case of both the plants, except the 'occasional' contaminants (P-PNK and P-PNK(S)), all the other bacteria (T-W, T-WH3, T-CL1, T-EMB-W, W-Y(O) and W-Y(T)) were strictly endophytic/closely associated with the particular plant. This was proven by the fact that despite surface sterilization of the explants with $HgCl_2$, the bacteria continued to appear and their frequencies were constant over two years. Also each of these two plants exhibited very specific types of bacteria and these bacteria never appeared in cultures of other plants. Hence these bacteria are referred to as **"associates"**. **Table 3.3** depicts the frequency of occurrence of these contaminant bacteria.

Table 3.3 Percentages of Contaminant Bacteria Isolated from Tissue Cultures of *Taxus baccata* ssp. *wallichiana* and *Withania somnifera* Over a Period of Two Years.

No.	Bacterial Isolates	Incidence of Associated Bacteria (%)	
		***Taxus* spp.**	***Withania* spp.**
1.	T-W	10.0-20.0	0.0
2.	T-WH3	10.0-20.0	0.0
3.	T-CL1	7.0	0.0
4.	T-EMB-W	10.0	0.0
5.	W-Y(T)	0.0	30.0
6.	W-Y(O)	0.0	30.0
7.	P-PNK	70.0	70.0
8.	P-PNK(S)	80.0-90.0	80.0-90.0

Fig. 3.1

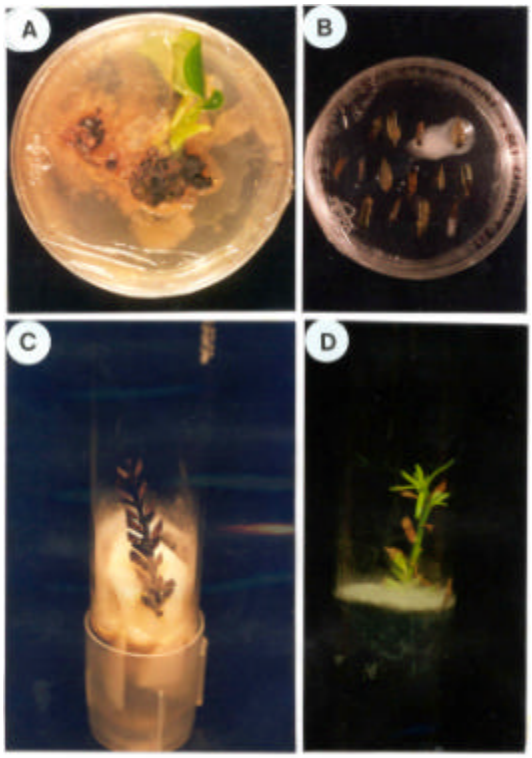
Infected cultures of *Taxus* and *Withania* spp.

A. P-PNK, the occasional contaminant infecting *in vitro* cultures of *Withania* spp.

B. T-CL1, the white contaminant bacterium of *Taxus* spp. callus cultures.

C. *Taxus* spp. stem explant infected with fungus and turning brown due to phenolics.

D. *Taxus* spp. culture infected with the non-sporulating opportunistic fungus.



It is clear from **Table 3.3** that out of the eight bacteria isolated; P-PNK and P-PNK(S) were common to both the plants and were found to be opportunistic and occasional. They were pink and intense pink pigmented contaminants respectively, appearing in distinct outbreaks that were unrelated to the monsoon season. In between these sudden outbreaks, all the cultures remained sterile. The intense pink bacteria (P-PNK(S)) were more predominant in the cultures and because of their fast growth rate, the cultures were killed within two days.

Four isolates (T-W, T-WH3, T-CL1 and T-EMB-W) were always associated with tissue cultures of *Taxus baccata* ssp. *wallichiana*. All of them were white in colour. One isolate, T-EMB-W was associated with embryos of this plant and was slow growing. But once this contaminant appeared in the cultures (10%), further growth of the embryos was arrested. The isolate T-CL1 was present in callus cultures (7%) while T-W and T-WH3 were associated with newly inoculated needles (10-20%) and appeared within one week of inoculation. As compared to the pigmented opportunistic contaminants, these bacteria were slow growing in plant tissue culture media but eventually succeeded in killing the explant or the callus.

Two types of yellow bacteria, W-Y(O) and W-Y(T) were isolated (30%) only from the cultured internodes of *Withania somnifera*. They oozed out from the explant, making it spongy and unresponsive. They were fast growing and killed the explants within three days after their appearance.

Following the isolation of contaminant bacteria in pure, single colony form, morphological and biochemical characterizations were performed which are described in following subsections.

3.3.1.1 Morphological characterization

All the bacterial types had round and smooth walled colonies and only the yellow colored colonies exhibited fluorescence. The white and pink colored colonies did not have any fluorescence.

All the contaminants were Gram stained using the double stain method and were found to be either Gram-negative rods or coccobacilli (**Figs. 3.2** and **3.3**). A distinct cycle

Fig. 3.2

Figs. A. – D. Gram staining of 'associated' contaminant bacteria from *in vitro* cultures of *Taxus* spp. (Magnification: 100x).

A. T-W, with long, rod shaped cells.

B. T-CL1, with both rods and cocci.

C. T-EMB-W, with slender rod shaped cells.

D. T-WH3, with short, rod shaped cells.

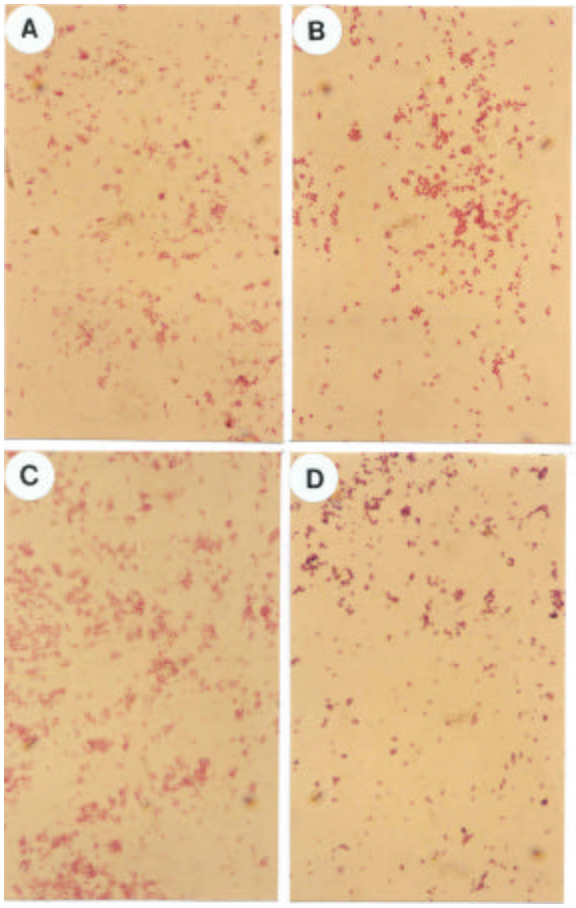


Fig. 3.3

Figs. A. – B. Gram staining of 'associated' contaminant bacteria from *in vitro* cultures of *Withania* spp. (Magnification: 100x).

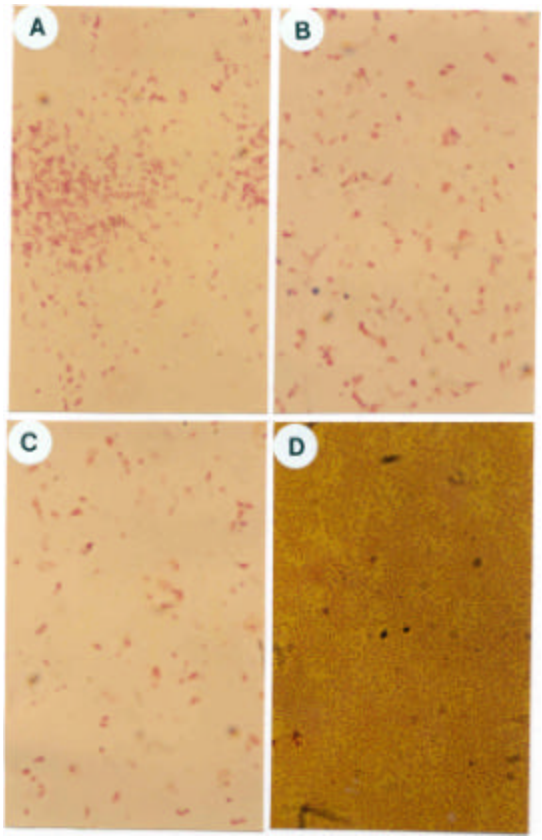
A. W-Y(T), with long, rod shaped cells.

B. W-Y(O), with intermediate sized, rod shaped cells.

Figs. C. – D. Gram staining of 'occasional'/'opportunistic' contaminant bacteria from *in vitro* cultures of *Taxus* spp. and *Withania* spp. (Magnification: 100x).

C. P-PNK, with squat, rod shaped cells.

D. P-PNK-S, short, rod shaped cells.



of rods and cocci was found in the isolate T-CL1. The younger cultures had a predominance of cocci while the older cultures showed maximum number of rods. None of the isolates were gram positive in nature.

3.3.1.2 Biochemical characterization

Among the biochemical tests, the test to determine the oxidative/fermentative mode of metabolism (Aerobic or anaerobic respiration) was initially carried out by stab inoculation and the oil overlay method, followed by the tests for the presence of motility and oxidase enzyme (Holt et al. 1994; Blazewic and Ederer, 1975), to determine whether the bacteria were aerobic or anaerobic in nature. None of the isolates were truly anaerobic in nature. Based on these results, the 8 isolates could be grouped into two classes.

Class 1: Obligate aerobes: Two isolates from *Taxus* spp. (T-WH3 and T-CL1), one isolate from *Withania* spp. (W-Y(O)) and the common contaminant P-PNK.

Class 2: Facultative aerobes/anaerobes: Two isolates from *Taxus* spp. (T-EMB-W and T-W), One isolate from *Withania* spp. (W-Y(T)) and the common contaminant P-PNK(S).

A large number of other biochemical tests were carried out on all the isolates and the results are represented in **Table 3.4** for obligate aerobes and **Table 3.5** for facultative aerobes. Results of some of the tests are represented in **Fig. 3.4**.

The results of morphological and biochemical tests were used to identify the bacterial isolates upto the genus level based on similarity indices with the well-documented results recorded in the Bergey's Manual under Tables 4.1, 5.1 and 5.2.

Table 3.4. Morphological and Biochemical Characterization of Obligate Aerobic Contaminant Bacteria Infecting Cultures of *Taxus baccata* ssp. *wallichiana* and *Withania somnifera*.

No.	Tests/Characters	Bacterial Isolates			
		P-PNK	T-WH3	T-CL1	W-Y(O)
1	Gram Reaction	-	-	-	-
2	Shape	Rods	Rods	Cycles of Rods and Cocci	Rods
3	Cyst or Cyst Like Forms Appear	-	-	-	-
4	Cells Arranged in Square Tablets	-	-	-	-
5	Motility	+	-	-	-
6	Cells in Gelatinous Matrices	-	-	-	-
7	Fluorescent Pigment	-	-	-	-
8	Colony Color	Pink	White	White	Yellow
9	Oxidase	-	+	-	-
10	Indole from Tryptophan	-	-	-	-
11	N2 Fixation	-	-	-	-
12	Denitrification to N2	-	-	-	-
13	Marine or Brine Bacteria	-	-	-	-
14	MR	+	-	+	-
15	VP	-	+	-	+
16	Citrate	-	+	+	-
17	Growth on one Carbon compounds	-	-	-	-
18	Potato Rot	-	+	+	+
19	Catalase	+	+	+	+
20	Starch	+	+	+	-
21	Levan	-	+	+	+
22	Spore Test	-	-	-	-
23	Nitrate Reductase	+	+	-	-
24	Urease	-	-	-	-

Contd…..

Table 3.4 Contd.

No.	Tests/Characters	Bacterial Isolates			
		P-PNK	T-WH3	T-CL1	W-Y(O)
25	Growth at 5°C*	-	-	-	-
26	Growth at 27°C	+	+	+	+
27	Growth at 37°C	+	+	+	+
28	Growth at 60°C**	-	-	-	-
29	Growth in 20 % NaCl	-	-	-	ND
30	Anaerobic Growth (stab method)	-	-	-	-
31	Anaerobic Growth (oil overlay method)	-	-	-	-
32	Growth in Ashby's Medium (Without N2)	V	-	-	-
33	Gas Production in Glucose Medium	-	-	-	-
34	Acid Production in Glucose Medium	-	+	-	+
35	Growth in Glucose Medium	-	+	+	+
36	Acid Production in Mannose Medium	-	-	+	ND
37	Growth in Mannose Medium	-	+	+	+
38	Acid production in Lactose Medium	+	-	+	-
39	Growth in Lactose Medium	+	+	+	+
40	Acid production in Rhamnose Medium	-	-	+	-
41	Growth in Rhamnose Medium	+	+	+	+
42	Acid production in Sucrose Medium	+	-	-	-
43	Growth in Sucrose Medium	+	+	+	+
44	Acid production in Mannitol Medium	-	-	-	-
45	Growth in Mannitol Medium	+	+	+	+

* : Growth occurs after transfer to 27 °C in the strains, which do not grow at 5°C.
** : Growth does not occur even after transfer to 27°C.
\+ : Positive test result.
\- : Negative test result.
ND : Not determined.
V : Variable results.

Table 3.5. Morphological and Biochemical characterization of Facultative Anaerobic/Aerobic Contaminant Bacteria Infecting Cultures of *Taxus baccata* ssp. *wallichiana* and *Withania somnifera*.

No.	Tests / Characters	Bacterial Isolates			
		P-PNK(S)	T-EMB-W	T-W	W-Y(T)
1	Gram Reaction	-	-	-	-
2	Cell Shape	Rods	Cocco-bacilli	Rods	Rods
3	Motility	+	+	+	-
4	Cysts or Cyst-Like Forms Present	-	-	-	-
5	Cells Arranged in Square Tablets	-	-	-	-
6	Oxidase	+	-	+	+
7	Fluorescent pigment	-	-	-	-
8	Colony Color	Intense Pink	White	White	Yellow
9	Indole from Tryptophan	-	-	-	-
10	MR	-	+	-	+
11	VP	+	+	-	-
12	Citrate	+	+	+	+
13	Growth on one Carbon compounds	-	+	-	-
14	Potato Rot	-	+	+	+
15	Catalase	+	+	+	+
16	Starch	+	+	+	-
17	Levan	+	+	+	-
18	Spore Test	-	-	-	-
19	Nitrate Reductase	+	+	-	+
20	Urease	ND	-	-	+
21	Growth at 5°C*	-	+	+	-
22	Growth at 27°C	+	+	+	+
23	Growth at 37°C	+	+	+	+
24	Growth at 60°C**	-	-	-	-

Contd…..

Table 3.5 Contd.

No.	Tests / Characters	Bacterial Isolates			
		P-PNK(S)	T-EMB-W	T-W	W-Y(T)
25	Growth in 20 % NaCl	-	-	-	-
26	Anaerobic Growth (Stab Method)	-	+	-	-
27	Anaerobic Growth (oil overlay method)	+	+	+	+
28	Growth in Ashby's Medium (No N_2)	-	-	-	-
29	Gas Production in Glucose medium	-	+	-	+
30	Acid Production in Glucose Medium	+	+	+	+
31	Growth in Glucose Medium	+	+	+	+
32	Acid Production in Mannose Medium	+	+	-	+
33	Growth in Mannose Medium	+	+	+	+
34	Acid Production in Lactose Medium	+	+	-	-
35	Growth in Lactose Medium	+	+	+	+
36	Acid production in Rhamnose Medium	+	+	-	+
37	Growth in Rhamnose Medium	+	+	+	+
38	Acid production in Sucrose Medium	+	-	-	+
39	Growth in Sucrose Medium	+	+	+	+
40	Acid production in Mannitol Medium	+	+	-	+
41	Growth in Mannitol Medium	+	+	+	+

* : Growth occurs after transfer to 27°C in the strains, which do not grow at 5°C.
** : Growth does not occur even after transfer to 27°C.
\+ : Positive test result.
\- : Negative test result.
ND : Not Determined.

Fig. 3.4

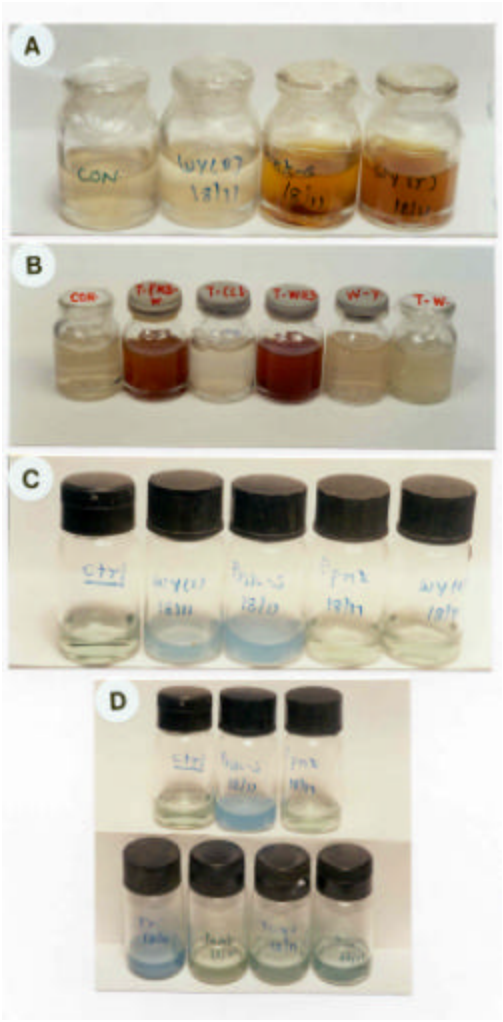
Figs. A – D A representation of biochemical tests performed for characterization of contaminant bacteria from *in vitro* cultures of *Taxus* spp. and *Withania* spp.

A. Presence (reddish brown coloration) or absence (absence of coloration) of nitrate reductase in contaminant bacteria from *Withania* spp.: W-Y(O), W-Y(T) and common contaminant: P-PNK(S). The leftmost bottle is control without bacteria.

B. Presence (reddish brown coloration) or absence (absence of coloration) of nitrate reductase in contaminant bacteria from *Taxus* spp.: T-EMB-W, T-CL1, T-WH3, T-W. The leftmost bottle is control without bacteria.

C. Presence or absence of utilization of citrate by contaminant bacteria from *Withania* spp.: W-Y(T) and W-Y(O) and two common contaminants: P-PNK and P-PNK(S). The leftmost bottle is control without bacteria Blue coloration indicates conversion of citrate in the medium by the bacterial enzymes.

D. Presence or absence of utilization of citrate by contaminant bacteria from *Taxus* spp.: T-W, T-EMB-W, T-WH3 and T-CL1 and two common contaminants: P-PNK and P-PNK(S). Blue to bluish-green coloration indicates conversion of citrate in the medium by bacterial enzymes. The leftmost bottle in the upper-hand corner is control without bacteria



3.3.1.3 Identification of bacteria based on a software program

The results obtained and recorded in **Tables 3.4 and 3.5** indicated that none of the isolates could be unambiguously assigned to a single genus of bacteria described in Bergey's Manual. Hence a software program developed in Visual Basic by Dr. A. P. Kulkarni was used as described in **Section 3.2.5.3** for identification of obligate and facultative aerobic bacteria based on similarity indices and the results obtained are represented in **Tables 3.6 and 3.7** respectively.

Table 3.6. Identification of Obligate Aerobic Contaminant Bacteria Infecting Cultures of *Taxus baccata* ssp. *wallichiana* and *Withania somnifera* Based on the Software Program Employing Similarity Indices Upto the Genus Level.

(Genera Showing Two Highest Percentages of Similarities According to Table 4.1 of Bergey's Manual are Indicated).

No.	Bacterial Isolates	Bacterial Genera from Table 4.1 of Bergey's Manual (% Similarity)		
1.	P - PNK	*Pseudomonas* (87.9)	*Aminobacter* (84.8)	
2.	T-WH3	*Aminobacter* (88.2)	*Pseudomonas* (88.2)	*Psychrobacter* (85.3)
3.	T-CL1	*Pseudomonas* (85.3)	*Paracoccus* (82.4)	
4.	W-Y(O)	*Pseudomonas* (88.2)	*Aminobacter* (85.3)	

All the obligate aerobic contaminants were assigned to the highly heterogeneous genera of *Pseudomonas* and *Aminobacter* with the highest percentages of similarity (**Table 3.6**). *Aminobacter* has recently been separated from the genus *Pseudomonas*. Since Psychrobacter is able to grow at 5°C but unable to grow at 37°C, T-WH3 could not belong to this genus. Since *Paracoccus* appears both as rods and cocci, T-CL1 has a good chance of belonging to this genus, based on the similarity percentages.

Table 3.7 Identification of Facultative Anaerobic/Aerobic Contaminant Bacteria Infecting Cultures of *Taxus baccata* ssp. *wallichiana* and *Withania somnifera* Based on the Software Program Employing Similarity Indices Upto the Genus Level.

(Genera Showing Two Highest Percentages of Similarities According to Tables 5.1 and 5.2 of Bergey's Manual Are Indicated).

No.	Bacterial Isolates	Bacterial Genera from Tables 5.1 & 5.2 of Bergey's Manual (% Similarity)				
1.	P-PNK(S)	*Erwinia perisicinus* (73.68)	*Serratia entomophila* (73.68)	*Enterobacter taylorae / Erwinia cancerogena* (68.42)	*Serratia liquefaciens* (68.42)	*Serratia marcescens* (68.42)
2.	T-EMB-W	*Enterobacter / Erwinia nimipressuralis* (89.47)	*Buttiauxella agrestis* (84.21)	*Enterobacter aerogens* (84.21)	*Enterobacter intermedius* (84.21)	
3.	T-W	*Leminorella richardii* (78.95)	*Erwinia amylovora* (68.42)	*Erwinia trachephila* (68.42)	*Leminorella gimontii* (68.42)	*Pragia fontium* (68.42)
4.	W-Y(T)	*Salmonella choleraesuis* ssp. *choleraesuis* (73.68)	*Salmonella choleraesuis* ssp. *houtenae* (73.68)	*Salmonella choleraesuis* ssp. *salamae* (73.68)	*Yokenella regensburgei (Koserella tabulsii)* (73.68)	*Buttiauxella agrestis* (68.42)
		Citrobacter freundii (68.42)	*Enterobacter gergoviae* (68.42)	*Klebsiella planticola* (68.42)	*Rahnella aquatilis* (68.42)	*Salmonella bongon* (68.42)
		Salmonella choleraesuis ssp. (68.42)	*Salmonella choleraesuis* ssp. (68.42)			

Because of the highly heterogeneous nature of the family Enterobacteriaceae, many matches were found for the facultative anaerobic/aerobic contaminants (**Table 3.7**). Maximum matches were found in the *Enterobacter-Erwinia* complex which is associated with both human and plant tissues. This is followed by the pigmented genus *Serratia* especially *S. entomophila*. This intense pink bacterium is spread by mites and hence must have appeared in various cultures in distinct outbreaks. Amongst the other probable genera, *Buttiauxella, Rahnella* and *Pragia* are fresh water bacteria while *Salmonella* is a

very big and heterogeneous assemblage associated with humans and animals. *Leminorella* is also a human associated form.

It is clear from these results that further definitive assignation of bacteria to the species level can only be done after either fatty acid or DNA sequence analysis. The various databases available over the world for bacteria are mainly concerned with medically important bacteria and not much information is available for plant related organisms. There is a need to build up such databases urgently.

As described by Leifert et al. (1994), the gram negative Pseudomonads infected more than 50% of stage 1 and stage 2 cultures while *Erwinia* spp. infected 10% of such cultures. This is because these genera are associated with aerial surfaces of plants under field conditions (Last and Deighton, 1965). Since none of the contaminants were gram positive, the possibility of their introduction from the laboratory air is excluded. Since none of the contaminants formed endospores, *Bacillus* spp. that can spread from inefficient autoclaving and insufficient sterilization of instruments used for inoculations and subcultures were absent.

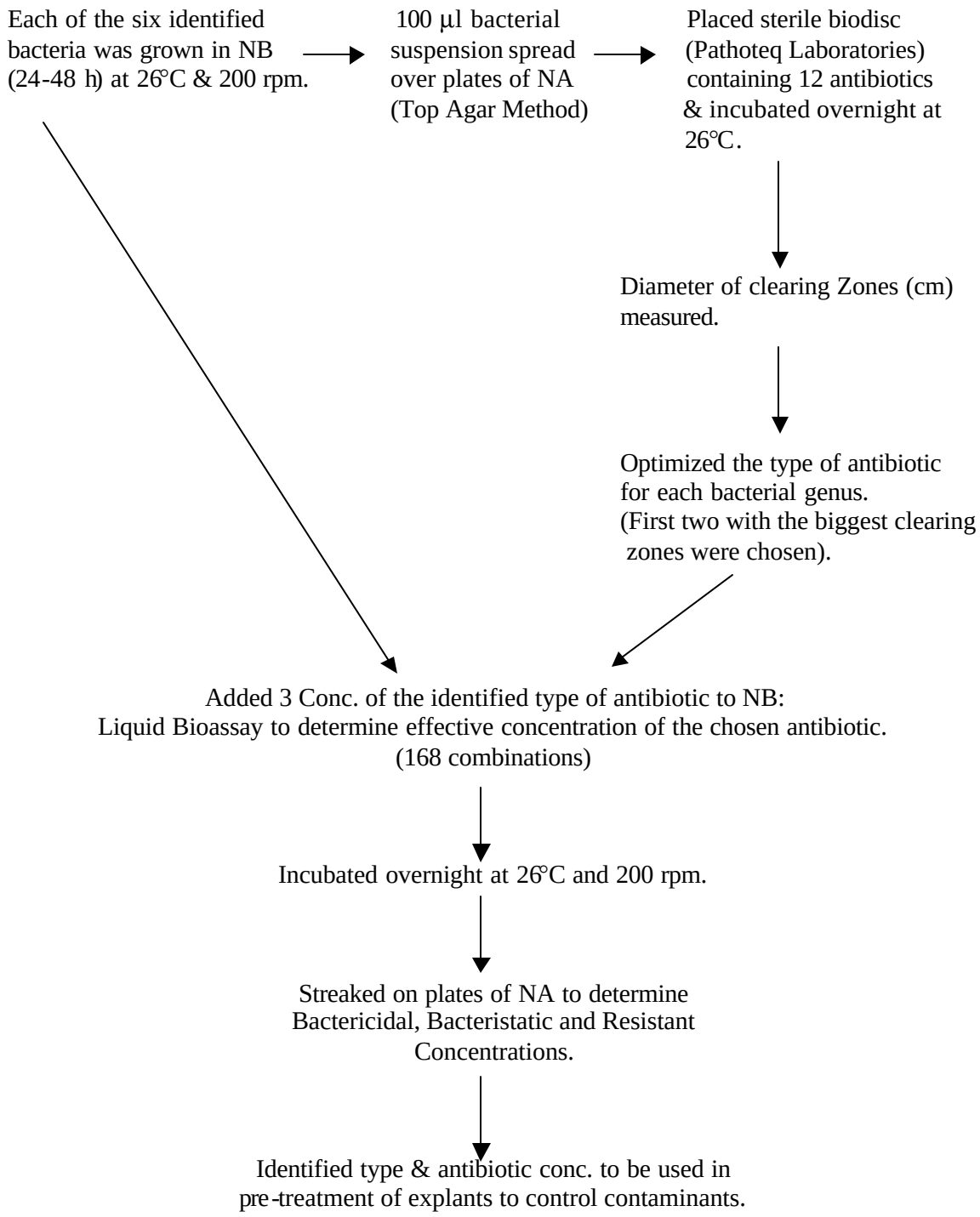
From this, we can reasonably conclude that most of the bacteria found *in vitro* were associated with plants either exogenously or endogenously, except those belonging to genera *Serratia* , *Salmonella* and *Leminorella* .

Effective control measures against contaminant bacteria using antibiogramme assays were carried out subsequently, after bacterial identification upto the generic level.

3.3.2 Control measures

Antibiogramme assays, both in solid and liquid media, were carried out (**Flow Chart 3.2**) and the results of, which are presented in **Tables 3.8 and 3.9** . These assays helped in the identification of effective antibiotics and their minimum concentration required to control the identified bacteria effectively. The results obtained are described herewith.

Flow Chart 3.2. Identification of Effective Antibiotics and Their Optimum Concentration for Control of Each of the Contaminant Bacteria



3.3.2.1 Antibiogramme assay in solid media

Table 3.8. Results of Antibiogramme Test Showing Effect of Different Antibiotics from Biodisc-12 on Contaminant Bacteria Isolated from *Taxus baccata* ssp. *wallichiana* and *Withania somnifera*.

Antibiotic Agent	*Diameter of Clearing/Inhibition Zone (cm) for Bacterial Strains*							
	P-PNK	**P-PNK(S)**	**T-W**	**T-WH3**	**T-CL1**	**T-EMB-W**	**W-Y(0)**	**W-Y(T)**
Ampicillin (AS)	-*	1.0	-	0.5	-	0.3	0.5	-
Co-Trimoxazole (BA)	-	1.2	0.4	-	-	-	-	**0.5**
Cefotaxime (CF)	-	**1.4**	0.4	0.4	-	**1.5**	-	0.1
Piperacillin (PC)	-	**1.4**	1.0	0.3	0.4	0.7	-	**0.5**
Chloramphenicol (CH)	-	0.4	0.3	0.9	0.6	0.3	0.2	**0.5**
Ciprofloxacin (CP)	-	**2.2**	**2.0**	0.5	0.9	-	**0.8**	0.2
Ceftizoxime (CL)	-	1.0	0.0	0.15	-	-	0.4	0.1
Tetracycline (TE)	0.9	0.2	1.2	**2.1**	0.5	1.0	0.6	0.5
Ofloxacin (OF)	-	1.3	0.7	1.1	0.6	0.2	-	**0.9**
Gentamicin (GM)	0.7	0.6	0.5	0.5	0.2	0.95	0.5	0.4
Amikacin (AK)	**1.0****	0.9	1.0	1.0	**1.0**	0.85	**0.7**	0.3
Pefloxacin (PF)	**1.0**	1.3	**1.3**	**1.7**	**1.2**	**1.1**	0.2	**0.9**

*: '-' represents absence of clearing/inhibition zone signifying that the particular antibiotic could not kill that particular bacterium.

: Values in **BOLD in a column represent the highest two values observed for zones of clearing/inhibition, of an antibiotic, for each bacterial isolate.

In the solid antibiogramme assay (**Fig. 3.5**), antibiotic BA was the least effective (activity against 3 isolates) with very small zones of inhibition. AS could control the growth of 4 isolates while CF and CL could control the growth of 5 isolates. PC, CP, OF inhibited growth of 6 isolates while CH could inhibit growth of 7 isolates. TE, GM, AK, PF were broad-spectrum antibiotics controlling growth of all the eight isolates (**Table 3.8**).

Among the bacteria, T-W and W-Y(T) were controlled by all the antibiotics tested except AS. T-CL1, T-WH3, T-EMB-W, W-Y(O) and P-PNK were resistant to BA. Growth of T-CL1 and W-Y(O) was not hampered by four antibiotics each. P-PNK was not sensitive to 8 antibiotics out of 12 antibiotics tested, whereas P-PNK(S) was susceptible to all the antibiotics.

3.3.2.2 Antibiogramme assay in liquid media

To evaluate the efficiency of a single antibiotic agent with respect to the effective concentration and to confirm the preliminary results of the antibiogramme assay, antibiotic sensitivity tests were carried out in NB. Since the results of antibiogramme assay in solid media are affected by the diffusibilty of the antibiotics, which are in turn dependent upon the structure and molecular weights of the antibiotics, sensitivity of the organism, concentration of the drug on the disc and the medium constituents, the experiment was repeated in liquid media. The concentrations used in the Biodisc-12 were further manipulated for this test. For the liquid assay only seven most effective antibiotics were used on the basis of the results of inhibition zone diameters obtained in the solid antibiogramme assay. These were AK, TE, GM, PF, PC, CP and OF. Each antibiotic was aseptically added to tubes of NB at three different concentrations (**Table 3.9**) and these tubes were then inoculated individually with a loopful of bacteria. In all 168 combinations were tried. In many cases, the antibiotic concentrations used in the Biodisc-12 were found to be bacteristatic and not bactericidal (**Table 3.9**).

Table 3.9. Results of Liquid Bioassay Determining Effect of Different Concentrations (mg) of Effective Antibiotics on Different Contaminant Bacteria Isolated from *Taxus baccata* ssp. *wallichiana* and *Withania somnifera*.

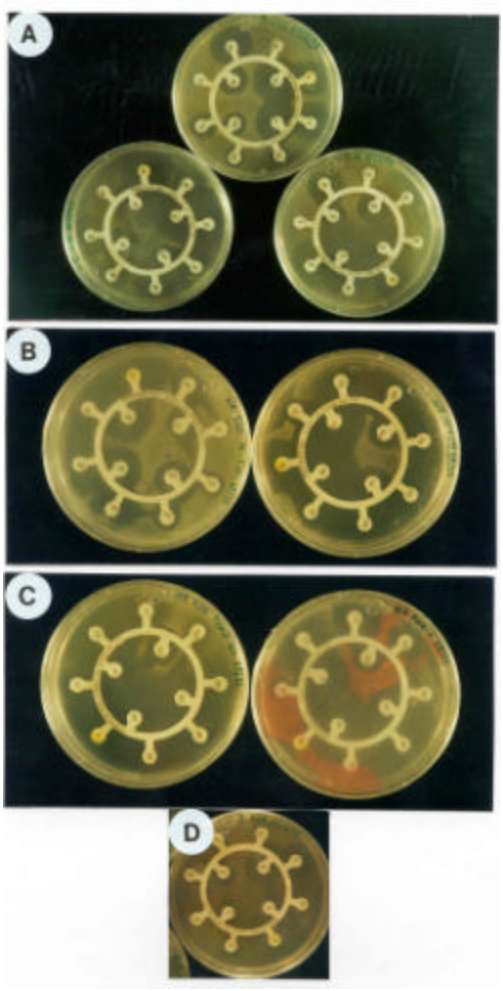
Antibiotic Agent	mg	Bacterial Isolates							
		P-PNK	P-PNK (S)	T-W	T-WH3	T-CL1	T-EMB-W	W-Y(0)	W-Y(T)
Amikacin (AK)	10	R	**C**	S	**C**	R	R	**C**	**C**
	30	R	C	S	C	R	S	C	C
	50	R	C	C	C	S	R	C	C
Tetra-cycline (TE)	10	R	R	S	**C**	R	S	**C**	**C**
	30	R	R	S	C	R	S	C	C
	50	R	R	S	C	R	S	C	C
Genta-micin (GM)	5	R	**C**	S	**C**	R	S	S	**C**
	10	R	C	S	C	R	S	S	C
	15	R	C	C	C	R	S	S	C
Pipera-cillin (PC)	50	R	R	R	R	R	R	S	R
	100	R	R	R	R	R	R	S	R
	200	R	R	R	S	R	R	**C**	**C**
Ciproflo-xacin (CP)	2.5	S	**C**	**C**	**C**	R	**C**	**C**	**C**
	5	S	C	C	C	R	C	C	C
	10	**C**	C	C	C	R	C	C	C
Ofloxacin (OF)	2.5	R	**C**	**C**	**C**	R	**C**	**C**	**C**
	5	R	C	C	C	R	C	C	C
	10	S	C	C	C	**S**	C	C	C
Pefloxacin (PF)	5	S	**C**	**C**	**C**	R	**C**	**C**	**C**
	10	S	C	C	C	R	C	C	C
	15	**C**	C	C	C	**S**	C	C	C

C: bactericidal, R: resistant, S: bacteristatic.

Fig. 3.5

Figs. A. – D. 'Biodisc Assay' for bacterial contaminants of *Taxus* spp. and *Withania* spp. (Identification of effective antibiotics for control measures).

A. For bacterial isolates: T-CL1, T-WH3 and T-W.
B. For bacterial isolates: W-Y(O) and W-Y(T).
C. For bacterial isolates: T-EMB-W, P-PNK(S)
D. For bacterial isolate: P-PNK.



From **Table 3.9** it is clear that the two common contaminants (P-PNK and P-PNK(S)) were controlled by the Quinolone group of antibiotics (CP, OF and PF). In case of contaminants from *Taxus* spp. and *Withania* spp., all of the isolates had at least one antibiotic giving a bactericidal response and hence could be used to control them. Only T-CL1 was found to be resistant to all the antibiotics in liquid bioassay. It gave a bacteristatic response for 10 µ*g* of ofloxacin and 15 µ*g* of pefloxacin. None of the antibiotics proved to be bactericidal at the given concentration. It is reasonable to believe that higher concentrations of the same antibiotics will prove to be bactericidal.

In general, it is thus clear that for all the 8 isolates, the Quinolone group of antibiotics comprising of CP, OF and PF are very effective in terms of their bactericidal activities at 2.5, 2.5 and 5 µg respectively and can be used in the strategy to be adopted to control various contaminant bacteria.

Using the standardized strategy of isolation of contaminant bacteria from *Tauxs* spp. and *Withania* spp. *in vitro* cultures, followed by their characterization, identification and antibiogramme assay, the minimum bactericidal concentration (MBC) of a specific antibiotic against each of the contaminant bacteria was determined. The determined MBC was used (**A**) for preventing the bacterial contamination in cultures of *Taxus* spp. and *Withania* spp. and (**B**) for combining with anti-fungal agents in a strategy to control fungal and bacterial contamination as described under **Section 3.3.4**.

Subsection 2: Fungal Contamination and Pheno lic Oxidation/Browning

Even though *Taxus* spp. plants *in vivo* are known to produce **Taxanes** with antifungal properties (Young et al., 1992; Wagner and Flores, 1994; Elmer et al., 1994), *in vitro* cultures showed many fungal contaminants (**Fig. 3.1 C, D**). Two types of contaminants were identified: **'Constant'** (endophytes or plant associates) and **'Occasional'** or **'Opportunistic'** (appearing suddenly, mostly dependant on environmental conditions like monsoon). Based on the identified fungal genera very specific anti-fungal strategies have been developed in order to circumvent the possibility of development of resistant strains. In addition, this may help in pinpointing the source of contamination and to check for the pathogenicity, if any, of the isolated genera (Danby et al., 1994). In case of *Taxus* spp., it would also help to determine whether any of the isolated genera are **Taxol/Taxane** producers.

3.3.3 Isolation and identification of fungal contaminants

3.3.3.1 Constant/endophytic contaminants

The plant tissue cultures especially of woody plants show a steady relatively unchanged rate of contamination that is caused by the 'constant' contaminants. These constant contaminants are associated with the plants and introduced through the explants (Stage 0) or due to insufficient surface sterilization as in many cases the sterilant can not reach all the crevices that are usually found on mature explants (Stage 1) (Leifert and Waites, 1994). The endophytic nature of several bacteria and fungi sharing different ecological associations with the plants supports this assumption (Knogge, 1996; Alfano and Collmer, 1996). In case of *Taxus* spp., there are various reports in literature enlisting different endophytic bacterial and fungal genera associated with this plant (Stierle et al., 1995; Strobel et al., 1996). Incidentally many of these genera have been identified in tissue cultures of the Himalayan *Taxus* spp. during the course of the present work, based on macroscopic and microscopic morphological characters of vegetative and reproductive structures and are listed below.

1. *Alternaria* spp. Nees (1816) Anamorphic Pleosporaceae, Dothidiales, Ascomycota (Hawksworth et al., 1995). Found in 20% of cultures (**Fig. 3.6 A**).

In natural environments, 50 species of the genus are widespread and include many plant pathogens. Remaining species are cosmopolitan saprobes.

2. *Phoma* spp. Sacc. (1880), Anamorphic Pleosporaceae, Dothidiales, Ascomycota (Hawksworth et al., 1995). Found in 60% of cultures. This is reported to be an endophyte of *Taxus* spp (Yang et al., 1994) with the ability to produce **Taxol**. (**Fig. 3.6 B**).

   In natural environments, it is widespread and is saprobic or necrotrophic on leaves and stems. It is also reported to be quite common *in vitro* across various genera (Danby et al., 1994).

3. *Pestalotiopsis* spp. Steyaert (1949), Anamorphic Amphisphaeriaceae, Xylariales, Ascomycota (Hawksworth et al., 1995). Found in 15% of cultures. This is reported to be an endophyte of *Taxus* spp (Strobel et al., 1996) with the ability to produce **Taxol**. (**Fig. 3.6 C**).

In natural environments, it is widespread and is saprobic or necrotrophic on leaves and stems. It is also a known parasite of bark and wood and hence is closely associated with the trees.

4. *Phyllosticta* spp. Pers (1818), Anamorphic Mycosphaerellaceae, Dothidiales, Ascomycota (Hawksworth et al., 1995). Found in 10% of cultures (**Fig. 3.6 D**).

   In natural environments, it is a key spp. on conifers, growing as biotrophic or necrotrophic or saprobic on the plants. Its teleomorph *Guignardia* is highly parasitic causing leaf spot and leaf blight diseases.

5. *Fusarium* spp. Link (1809), Anamorphic Hypocreaceae, Hypocreales, Ascomycota (Hawksworth et al., 1995). Found in 20% of cultures (**Fig. 3.6 E, F**).

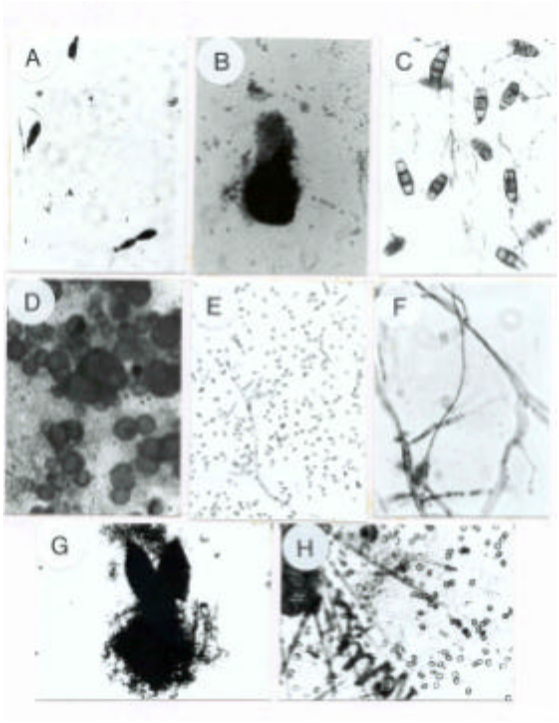
   In natural environments, the genus is widespread and includes saprobic, necrotrophic and parasitic spp. Its another anamorph *Acremonium* is reported to be endophytic in *Taxus* (Strobel et al. 1997). *Fusarium* is also reported to be quite common *in vitro* across various genera (Danby et al., 1994).

6. *Chaetomium* spp. Kunze (1817), Chaetomiaceae, Sordariales, Ascomycota (Hawksworth et al., 1995). Appeared in 10% of cultures and quickly sporulated forming dark brown ascospores (**Fig. 3.6 G, H**).

Fig. 3.6

Figs. A. – H. Contaminating fungi of *Taxus* spp. and *Withania* spp. Magnification given in the brackets.

A. *Alternaria* spp. Nees. (1816) (40x)
B. *Phoma* spp. Sacc. (1880) (40x)
C. Spore mass of *Pestalotiopsis* spp. Steyaert (1949) (100x)
D. *Phyllosticta* spp. Pers. (1818) (10x)
E. *Fusarium* spp. Link. (1809) (40x)
F. *Fusarium* spp. Link. (1809): Conidia on phyllides (100x)
G. *Chaetomium* spp. Kunze (1817) (10x)
H. *Chaetomium* spp. Kunze (1817) (40x)



In contrast to the various endophytic and constant fungal contaminants in *Taxus* spp., no constant/endophytic contaminants were found in cultures of *Withania* spp. *in vitro*.

3.3.3.2 Occasional/opportunistic contaminants

The 'Occasional' fungal contaminants, usually of very specific genera, are often associated with mites and thrips, the arthropods that inhabit the culture rooms and act as vectors of fungal contaminants (Debergh and Maene, 1981; Blake, 1988; Leifert et al, 1991a, 1994). Their occurrence is also corelated with the spore load in the environment that is very high during the monsoon season in the tropics. Following fungal genera were identified during the course of the present work. *Fusarium oxysporum* was the only fungus observed in cultures of *Withania* spp. *in vitro* at a frequency of 70-80% whereas the following fungi were found to appear in *Taxus* spp. cultures:

1. *Fusarium oxysporum* Anamorphic Hypocreaceae, Hypocreales, Ascomycota (Hawksworth et al., 1995). Most frequently observed in the monsoon season (May-August) and infected cultures at a frequency of 80-100% irrespective of species, developmental stage or explant. Leifert et al., (1994) have reported that sudden fungal contamination by an allied spp., *Fusarium poe* was often associated with mites which actually feed on this fungus.
2. *Chaetomium bostrichodes* Zopf., Chaetomiaceae, Sordariales, Ascomycota (Hawksworth et al., 1995). Found to infect 20-30% cultures in the monsoon season. Slow-growing nature of this fungus restricted the losses.
3. Occasionally, an unidentified black mycelium (never formed reproductive structures for definitive identification) caused extensive losses of the cultures to the tune of 70-80%.
4. *Neurospora* spp. Shear and Dodge (1927), Teleomorphic Sordariaceae/Neurosporaceae, Sordariales, Ascomycota (Hawksworth et al., 1995). Appeared irrespective of the genera, at a frequency ranging from 60-70%. This fungus is a widespread saprobe on rotting wood and soil.

The identification of fungal genera occurring in cultures of *Taxus* spp. and *Withania* spp. as 'constant' and 'occasional/opportunistic' contaminants has led us to devise a **"Contamination Control Strategy"** to control fungal contaminants with the

help of antifungal and antimicrobial agents and in combination with antibacterial agents, especially of the quinolone group, specific for the identified bacteria, to control both fungal as well as bacterial contamination by way of a pre-treatment of explants before their surface sterilization and subsequent culture in their respective optimized media. For details see **Section 3.3.4**.

3.3.4 Control of contamination – the strategy

3.3.4.1 *Taxus baccata* ssp. *wallichiana* Zucc. Pilg.

Diverse chemicals have been employed to combat both bacterial and fungal contamination. These include the use of routinely used surface sterilization agents like alcohol, commercial bleach, mercuric chloride, hydrogen peroxide (Grayson, 1982) as well as new agents like diethylpyrocarbonate (Macek et al, 1994), 8-hydroxy-quinolinol-sulfate (Laimer da Camara Machado et al, 1991) and sulfite pads (Shure et al., 1994). In addition, use of antifungal agents such as benomyl, chlorothalonil, Amphotericin-B (Watt et al, 1996) as well as benlate, captan, ethirimol and many others (Thurston et al., 1979) has also been reported. Similarly use of antibacterial agents in the medium to control various contaminating bacteria in tissue culture is well known (Falkiner, 1988; Leifert et al., 1991; Meyer et al., 1992). However, the combined use of both antifungal and antibacterial agents to control both bacteria and fungi is the preferred method (Thurston et al., 1979; Wilson and Power, 1989; Cassells, 1991; Watt et al., 1996). But their indiscriminate use may lead to phytotoxicity problems (Phillips et al., 1981; Pollock et al., 1983) and development of resistant strains (Leifert et al., 1991b). Hence characterization of contaminating microbes (bacteria and fungi) to generic and species level can lead to more specific development of versatile antibacterial and antifungal therapies (Falkiner, 1988; Leifert et al., 1989). As the bacterial and fungal contaminants occurring in cultures of *Taxus* spp. and *Withania* spp. have clearly been identified upto genus and species level in this chapter, a strategy to treat the explants before **"Routine Surface Sterilization"** and culture of explants, in a pre-treatment solution consisting of antibacterial and antifungal agents, has been envisaged so as to overcome the phytotoxicity problems and development of resistant strains of bacteria and fungi.

A mixture of anti-fungal and antibacterial agents was used in an aqueous pre-treatment solution to treat the various mature explants of *Taxus* spp. The various anti-

fungal agents were not added to the media because of their well-known phytotoxicity and also because of their growth-regulator like effects *in vitro* (Shields et al., 1984; Watt et al., 1996). **Table 3.10** tabulates the results obtained with pre-treatment solutions of different compositions to minimize the contamination percentage in cultures of *Taxus* spp. In pilot experiments, the pre-treatment period was varied from 4 hours upto 36 hours. Since no statistically significant differences were found in the pre-treatment time duration upto 16 hours and the pre-treatments lasting longer than 16 hours showed a definite phytotoxic effect in the form of browning of explants, overnight pre-treatment of explants (16 hours) with an optimized pre-treatment solution was preferred to minimize the contamination in cultures of *Taxus* spp. (**Table 3.10**).

It is clear from **Table 3.10** that the presence of bavistin (1%) in the pre-treatment solution caused significant reduction in contamination percentage. When it was supplemented with aureofungin or 8-hydroxy-quinoline hemisulphate (8-HQS) and augmentin and streptopenicillinin, further statistically significant reduction in the contamination percentage was achieved. Since a mixture of anti-microbial agents is being used, the possibility of development of resistant strains to a particular anti-microbial agent is minimized, if not eliminated. Bavistin and 8-HQS together were found to be better as compared to Bavistin and Aureofungin. Since pre-treatment no. 3 with 0.1% 8-HQS gave statistically comparable results with pre-treatment no. 3a with 0.25% 8-HQS, the pre-treatment no. 3a with lower percentage of 8-HQS was chosen in the attempts to further reduce the contamination with 500 $mg.l^{-1}$ each of augmentin and streptopenicillin. In this composition, the contamination percentage was further reduced to 14.33%, which is significantly lower than all the other treatments.

The efficient control of contamination may be due to presence and action of following anti-microbial agents such as:

- Bavistin (50% Carbendazim) is a systemic fungicide belonging to the benzimidazole group of fungicides. It is the most wide spectrum systemic fungicide deve loped so far with the maximum activity against Ascomycota, Phycomycota and Fungi Imperfecti. It contains methyl-3-benzimidazole carbamate (MBC) with fungicidal activity (Singh, 1990). It interferes with DNA production and post-DNA synthesis aspects of fungal cell replication. It protects both the medium and the plant tissues (Thurston et al., 1979) from fungal contamination.

- 8-HQS has antimycotic and bactericidal properties and is very useful in fruit tree micropropagation and is also used in humans (Laimer da Camara Machado et al., 1991 and references therein). The compound has a capacity to chelate metal ions present in essential biological systems of the microbial cell and is effective against both gram positive bacteria as well as fungi (Grayson, 1982). It has proved very beneficial in *Taxus* throughout the collection period without adversely affecting the *in vitro* response.
- Aureofungin, an heptaene antibiotic, controls Pythiaceous fungi while Augmentin, another heptaene antifungal agent, contains potassium clavulanate and amoxycillin.
- Streptopenicillin is a mixture of Streptomycin, an aminoglycoside and Penicillin, a β lactam type of antibiotic. It is antibacterial against both gram positive and negative genera as well as mycobacteria and antifungal against Phycomycetes (Singh, 1990).

Table 3.10 Effect of Different Pre-treatments on % Contamination (Fungal and Bacterial) of Mature Explants of *Taxus* spp.

Pre-treatment No.	Composition of Pre-treatment Solution	% Contamination (Mean ± S.D.)
Control	Without pre-treatment, with **"Routine Surface Sterilization Protocol"** .	100 ± 0^{a}
1	1% bavistin	81.21 ± 7.51^{b}
2	1% bavistin + 0.5% aureofungin	55.33 ± 13.64^{c}
3	1% bavistin + 0.1% 8-HQS	32.92 ± 6.48^{cd}
3a	1% bavistin + 0.25% 8-HQS	40.08 ± 2.17^{d}
4	1% bavistin + 0.1% 8-HQS + 500 $mg.l^{-1}$ each of augmentin, streptopenicillin.	14.33 ± 5.06^{e}

a, b, c, d, e Treatment means with different letters are significantly different from each other at 95% confidence level by t-test.

Use of the Quinolone group of antibiotics in the pre-treatment solution for *Taxus* spp. Along with Antimicrobial Agents

Preliminary experiments showed that when the effective antibiotics, ofloxacin, pefloxacin and ciprofloxacin, which were very specific for various identified bacteria (**Section 3.3.2.2, Table 3.9**), were used in the pre-treatment solution without the anti-fungal agents, all the cultures of *Taxus* spp. were overcome by the faster growing fungi and the relatively slower growing bacteria did not appear. All the cultures were lost due to fungal contamination (**Table 3.11**). Hence it was decided to use the identified effective antibiotics at their bactericidal concentrations in the presence of antifungal agents. Since the antibiotics to be used in the pre-treatment solution were for a shorter duration (as compared to incorporation in the medium and continuous exposure of the explants to the antibiotics), the antibiotics at ten times the effective bactericidal concentration determined by liquid bioassays (**Table 3.9**) were used. To the optimized composition of pre-treatment solution containing various antimicrobial agents (See **Table 3.10, Treatment No. 4**), antibiotics such as 25 $mg.l^{-1}$ each of ofloxacin, pefloxacin and ciprofloxacin were added, based on the earlier results of optimization of MBC with solid and liquid Antibiogramme assays (See **Sections 3.3.2.1** and **3.3.2.2** of the present chapter for the details) to eliminate bacterial contaminants of *Taxus* spp. cultures *in vitro*. Ofloxacin, Pefloxacin and Ciprofloxacin are Quinolone antibiotics and inhibit the action of DNA gyrases (C. f. **Table 3.2**).

The best pre-treatment solution (**No. 6 in Table 3.11**) comprising 1% bavistin, 0.1% 8-HQS, 500 $mg.l^{1}$ each of augmentin and streptopenicillin and 25 $mg.l^{-1}$ each of ofloxacin, pefloxacin and ciprofloxacin has proved to be very beneficial to control both bacterial and fungal contamination of *Taxus* explants cultured throughout the collection period of shoots (May-October) without adversely affecting the *in vitro* response. The contamination percentage with this pre-treatment solution, applied for a period of 16 hours followed by surface sterilization of mature explants of *Taxus* spp., could be successfully reduced to 10%. This is significantly noteworthy compared to contamination percentage obtained with and without pre-treatment employing various mixtures of anti-fungal and anti-bacterial agents (**Tables 3.10 and 3.11**).

Table 3.11 Effect of Different Pre-treatments on % Contamination (Fungal and Bacterial) of Mature Explants of *Taxus* spp.

Pre-treatment No.	Composition of Pre-treatment Solution	% Contamination (Mean ± S.D.)
Control	Without pre-treatment, with **"Routine Surface Sterilization Protocol"**.	100 ± 0^{a}
4	1% bavistin + 0.1% 8-HQS + 500 $mg.l^{-1}$ each of augmentin, streptopenicillin.	14.33 ± 5.06^{b}
5	25 mg.l-1 each of ofloxacin, pefloxacin and ciprofloxacin.	-*
6	**1% bavistin + 0.1% 8-HQS + 500 $mg.l^{-1}$ each of augmentin, streptopenicillin + 25 $mg.l^{-1}$ each of ofloxacin, pefloxacin and ciprofloxacin.**	**$10.00 + 0.25^{c}$**

* In the absence of antifungal agents in the pre-treatment solution, all the explants were overcome by fungal contamination within two days and the effect of antibiotics could not be scored.

a, b, c Treatment means with different letters are significantly different from each other at 95% confidence level by t-test.

3.3.4.2 Control of Contamination in *Withania somnifera* (L.) Dun.

Endophytic fungal contaminants were not observed in cultures of *Withania* spp., probably because they were derived from juvenile seedling tissues. Therefore antifungal agents were not used in the pre-treatment solution. Instead, the pre-treatment solution consisted only of 25 $mg.l^{-1}$ each of ofloxacin, pefloxacin and ciprofloxacin, which were specific to the bacterial genera identified (Section 3.3.2.2, Table 3.9). When the internodal explants were pre-treated overnight in this solution, no bacterial contamination was observed and the internodes could produce callus and regenerate *de novo* shoots as described in **Chapter 6.**

3.3.5 Control of phenolic browning/oxidation – the strategy

The effect of different chemical treatments on the percentage of explants of *Taxus* spp. browning due to phenolic oxidation is presented in **Table 3.12**. All explants of *Taxus* spp. died due to phenolic browning in the Control experiments when the explants were not pre-treated with anti-oxidizing agents (Treatment No. 1 in **Table 3.12**). When the explants were pre-treated overnight with various anti-oxidizing agents such as ascorbic and citric acid, soluble PVP, each at 2%, the percentage incidence of phenolic oxidation decreased significantly to 52% (Treatment No. 2 in **Table 3.12)**. However the percentage of explants browning due to phenolic oxidation increased significantly to 63.75% when full strength liquid WPM was used for their culture, despite the use of pre-treatment (Treatment No. 3 in **Table 3.12)**. On the contrary, the percentage of explant browning decreased significantly to 40% (Treatment No. 4 in **Table 3.12**) when WPM was used at half strength of major salts (McCown and Sellmer, 1987). When AC at 0.5% was incorporated in the nutrient medium (solid or liquid), the explants browning due to phenolic oxidation was not observed (Treatment No. 5 in **Table 3.12**). AC probably nullified the effect of phenolic oxidation on explants by adsorbing the polyphenols released into the medium and accounted for healthier and faster growth of the shoots of *Taxus* spp. (3 cm in 15 days). These results are in tune with the earlier observations reported for Conifers (Thorpe et al., 1991). Thus the problem of phenolic oxidation in *Taxus* cultures has been overcome completely by a treatment of explants with anti-oxidizing agents for 16 hours at 26 $\pm$ 2°C with constant shaking at 200 rpm in dark. This strategy of pre-treatment of explants with anti-oxidizing agents has been further combined to include the anti-bacterial and anti-fungal agents to control both the bacterial and fungal contamination as well as phenolic browning observed with mature explants of *Taxus* spp. in culture. For further details see **Section 3.3.6** .

Table 3.12 Effect of Different Chemical Treatments on % Explants Browning due to Phenolic Oxidation in *Taxus* spp.

Treatment No.	Chemical Treatments	% Explants Showing Phenolic Oxidation Mean ± S. E.
1.	Control, No pre-treatment of explants.	100 ± 0^{a}
2.	Use of anti-oxidants like soluble PVP, ascorbic acid and citric acid, each at 2%, in a pre-treatment of explants for 16 hours.	52.66 ± 3.3^{b}
3.	Same as treatment No. 2 but explants cultured on Liquid medium at full strength.	63.75 ± 5.54^{c}
4.	Same as treatment No. 2 but explants cultured on Solid/Liquid medium at half strength.	40.25 ± 1.84^{d}
5.	Same as treatment No. 2 but explants cultured on nutrient medium with activated charcoal (AC) at 0.5% (w/v).	0 ± 0^{e}

[a, b, c, d, e] Treatment means with different letters are significantly different from each other at 95% confidence level by t-test.

3.3.6 Strategy to control bacterial and fungal contamination as well as phenolic browning/oxidation

Based on the results presented in **Tables 3.10, 3.11 and 3.12**, a pre-treatment solution of the following composition has been evolved:

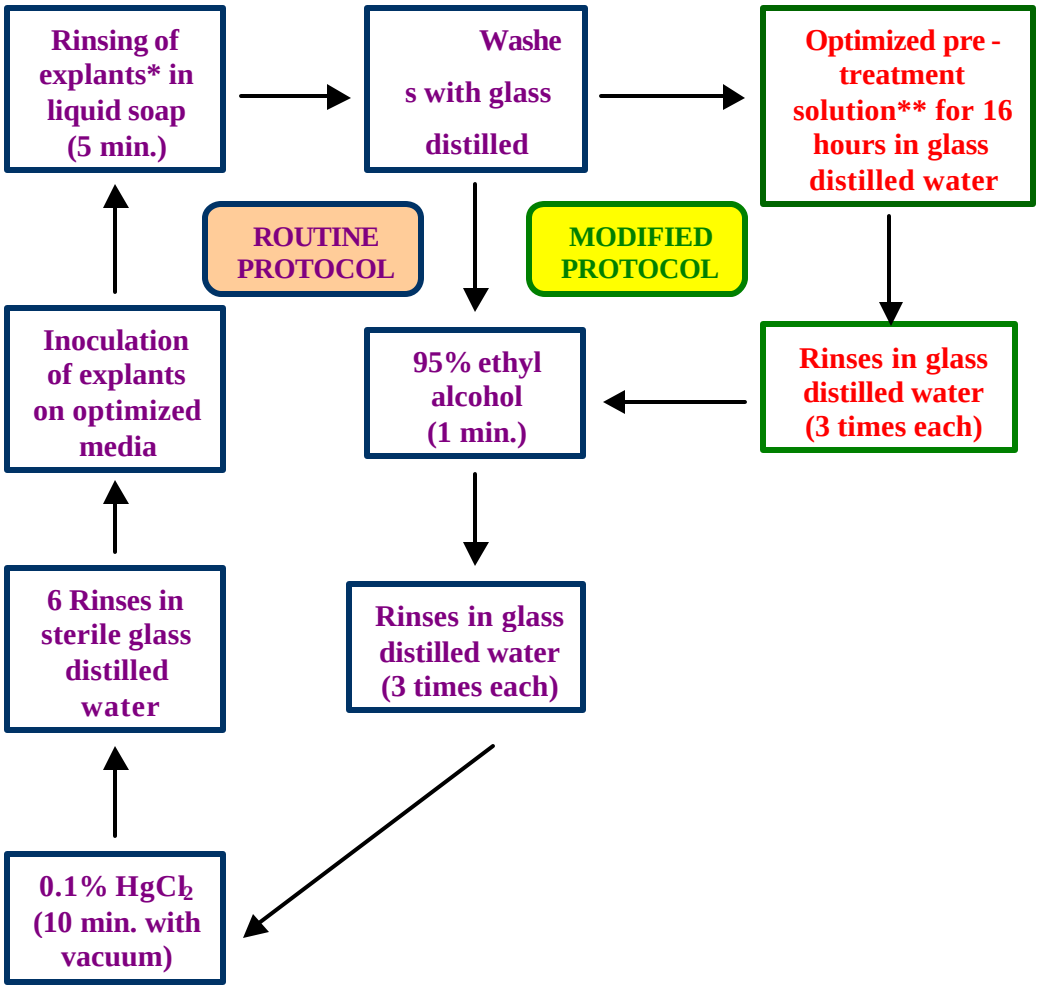
1% bavistin + 0.1% 8-HQS + 500 mg.l^{-1} each of augmentin, streptopenicillin + 25 mg.l^{-1} each of ofloxacin, pefloxacin and ciprofloxacin + 2% each of soluble PVP, citric acid and ascorbic acid in glass distilled water.

The treatment of explants of *Taxus* spp. and *Withania* spp. with the above-mentioned optimized pre-treatment solution for 16 hours at 26 ± 2ºC on a rotary shaker at 200 rpm before **"Routine Surface Sterilization Protocol"** is a part and parcel of the overall strategy adopted not only to control fungal, bacterial contamination but also

phenolic oxidation of explants and is referred hereinafter as the **"Modified Surface Sterilization Protocol"**.

A comparison of **"Routine Surface Sterilization Protocol"** and **"Modified Surface Sterilization Protocol"** is presented here in **Flow Chart 3.3**.

Flow Chart 3.3 Routine and Modified Surface Sterilization Protocols for Stem and Needle Explants of *Taxus* spp. and Internodal Explants of *Withania* spp.



* Explants: Stem bits and needles of *Taxus* spp. and internodal explants of *Withania* spp.

** Pre-treatment solution for *Taxus* explants: 1% Bavistin + 0.25% 8-HQS + 500 mg.l^{-1} each of streptopenicillin and augmentin + 25 mg.l^{-1} each of ofloxacin, pefloxacin and ciprofloxacin + 2% each of soluble PVP, citric acid and ascorbic acid for 16 hours at 26 ± 2ºC on a rotary shaker at 200 rpm.

Pre-treatment solution for *Withania* explants: 25 mg.l^{-1} each of ofloxacin, pefloxacin and ciproflo xacin for 16 hours at 26 ± 2ºC on a rotary shaker at 200 rpm.

3.3.7 "Acid test" for the Effectiveness of the "Modified Surface Sterilization Protocol" (Depicted in Flow Chart 3.3) on Establishment of Sterile Cultures of *Taxus* spp. and *Withania* spp.

To check whether the **"Modified Surface Sterilization Protocol"** employing a number of diverse chemicals in the pre-treatment solution is harmful to the explants and whether the *in vitro* response of *Taxus* spp. and *Withania* spp. are adversely affected, the explants of *Taxus* spp. and *Withania* spp. were surface sterilized using the **"Modified Surface Sterilization Protocol"** and the explants were cultured on media and incubated as described earlier in **Section 3.2.3**. **Table 3.13** presents the data on the *in vitro* responses of *Taxus* spp. and *Withania* spp.

Table 3.13 Effect of "Modified Surface Sterilization Protocol" on % Contamination, Phenolic Browning and *in vitro* Response.

Plant Species	Percentage of Contamination	Percentage of Phenolic Browning	Response of Sterile Cultures
Taxus baccata ssp. *wallichiana* Zucc. Pilg. with Routine Surface Sterilization Protocol.	100%	100%	-
Taxus baccata ssp. *wallichiana* Zucc. Pilg. with Modified Surface Sterilization Protocol.	10-11%	0%	100% of sterile stem and needle explants formed callus on optimized media. Also presence of **Taxanes** was detected in cultures.
	10-11%	0%	60-100% of sterile stem explants showed enhanced primary and secondary sprouting on optimized media.
	<10%	0%	90% of sterile embryos germinated precociously and callus cultures derived from endosperm showed presence of **Taxanes**.

Contd…..

Table 3.13 Contd.

Plant Species	Percentage of Contamination	Percentage of Phenolic Browning	Response of Sterile Cultures
Withania somnifera (L.) Dun. with Routine Surface Sterilization Protocol.	30%	0%	70% of sterile internodes regenerated.
Withania somnifera (L.) Dun. with Modified Surface Sterilization Protocol.	<10%	0%	All the sterile internodes (>90%) gave rise to *de novo* shoots which could be elongated, rooted and acclimatized with 100% success and exhibited normal morphology.

It is clear from **Table 3.13** that while incidence of phenolic browning has been reduced to 0%, the incidence of contamination has also been significantly reduced to 10-11% for stem explants and less than 10% for embryo and endosperm explants of *Taxus* spp. The incidence of bacterial contamination has been reduced to less than 10% in internodal explants of *Withania* spp while phenolic browning has not been observed. The explants subjected to the **"Modified Surface Sterilization Protocol"** were sterile and the response of sterile cultures was very good (**Table 3.13**). Thus the **"Modified Surface Sterilization Protocol"** which has been standardized in the present work was found to be non-phytotoxic to explants and was used routinely.

3.4 Conclusions

1) **The prevalent bacterial contaminants of *Taxus* spp. and *Withania* spp. belong to the genera such as *Pseudomonas*, *Enterobacter-Erwinia* complex and *Serratia* spp.**
2) **The fungal contaminants of *Taxus* spp. and *Withania* spp. belong to the class Ascomycota.**
3) **The bacterial and fungal contamination (endophytic as well as occasional/opportunistic) associated with mature explants of *Taxus* spp. and juvenile explants of *Withania* spp. can be effectively controlled with the help of specific anti-microbial agents in a pre-treatment solution such that no phytotoxicity problems could be encountered.**
4) **To control the bacterial contamination observed with internodal explants of *Withania* spp., a 16 hour long pre-treatment of internodes with 25 mg.l^{-1} each of ofloxacin, pefloxacin and ciprofloxacin is adequate without adversely affecting the regeneration capacity of the internodes. Phytotoxic nature of the optimized pre-treatment on the explants was not observed.**
5) **It is necessary to employ a 16 hour long pre-treatment in an aqueous solution containing 1% bavistin + 0.1% 8-HQS + 500 mg.l^{-1} each of augmentin, streptopenicillin + 25 mg.l^{-1} each of ofloxacin, pefloxacin and ciprofloxacin to overcome the fungal and the bacterial contamination associated with mature explants of *Taxus* spp., to obtain 90% sterile cultures.**
6) **The phenolic browning of stem and needle explants of *Taxus* spp. derived from mature trees can be controlled by incorporation of anti-oxidants in the pre-treatment solution along with the antimicrobial agents, use of half strength nutrient medium and use of activated charcoal at 0.5% in the solid medium.**
7) **A pre-treatment solution containing 1% bavistin + 0.1% 8-HQS + 500 mg.l^{-1} each of augmentin and streptopenicillin + 25 mg.l^{-1} each of ofloxacin, pefloxacin and ciprofloxacin + 2% each of soluble PVP, Citric acid and Ascorbic acid is used for 16 hours to completely overcome**

phenolic browning of mature explants of *Taxus* spp. and to obtain 90% sterile cultures free from bacterial and fungal contaminants.

8) **All the chemicals used for overcoming the problems of contamination and phenolic browning were not phytotoxic as all the treated explants had high success rate of *in vitro* response and the regenerants were normal in morphology.**

CHAPTER 4

Studies on Callus Induction from Various Explants of *Taxus* spp. and Analysis of Taxane Content of *In Vitro* and *In Vivo* Tissues.

4.1 Introduction

Research interest in *Taxus* spp. has become more prominent and pertinent in recent years because it has been realized that plants belonging to *Taxus* spp. (Commonly referred to as the Yews) contain a diverse group of phytochemicals showing a range of biological activities (Parmar et al., 1999). Prominent among these are **Taxane** class of diterpenoid compounds especially **Taxol, 10-DAB** and **Baccatin-III** with antitumor and anticancer activity *in vivo* as well as *in vitro*. This interest is amply evident by the fact that many multinationals are joining the rat race, spending billions of dollars to develop the product (**Taxol**) either by extraction from bark of trees of *Taxus* spp. or by the method of semi-synthesis from naturally occurring precursors such as **10-DAB** and **Baccatin-III**.

The phylogenetic position of the yew family, **Taxaceae** (**Order: Coniferales, Class: Gymnosperms**) has been very controversial (Sahni, 1920; Chaw et al., 1993). The family includes five genera and twenty species characterized by presence of a single ovule, not organized in seed cones, as opposed to other conifers. The name *Taxus* is derived from its Greek name *toxus*, which is made from two words: *toxon* meaning bow and *toxicon* meaning poison. Most authors consider *Taxus* to contain eight species: *Taxus baccata* L., *Taxus brevifolia* Nutt., *Taxus canadensis* Marshall, *Taxus chinensis* (Pilger) Rehder, *Taxus cuspidata* Sieb. et Zucc., *Taxus floridana* Chapm., *Taxus globosa* Schltdl., *Taxus wallichiana* Zucc. Furthermore, there are two hybrids: *Taxus x media* Rehder is a cross between *T. baccata* and *T. cuspidata* while *Taxus x hunnewelliana* Rehder is a cross between *T. cuspidata* and *T. canadensis* (Van Rozendaal et al., 1999).

Today there is an increased tendency to consider various *Taxus* species as intraspecific and geographic variants of a single species *T. baccata*, descending from *Paleotaxus rediviva*, a fossil tree abundant in the Triassic, 200 million years ago; since all the species have identical chromosome numbers of 2n=24 and they readily undergo hybridization with each other (Hartzell, 1991).

4.1.1 Distribution Range and Botany of the Himalayan Yew (Saxena and Sarbhai, 1971-72; Dubey, 1997; Chatterjee and Dey, 1997; http://willow.ncfes.umn.edu/silvics_manual/Volume_1/)

The Himalayan region in India extends for about 2500 km and occupies 18% of the total area of this country. It is divided into different provinces physiographically and biogeographically. The forest cover in the entire area has dwindled to about 21% (Bisht et al., 1998 and references therein). This is viewed with concern because the Himalayas are one of the chief centers of biodiversity and contain an immense wealth of flora and fauna, with many endemic genera, which has not been properly documented. Tissue culture can serve as an important tool in saving many of these endangered plant genera.

The Himalayan Yew, *Taxus baccata* ssp. *wallichiana* Zucc. Pilg., also referred to as *Taxus wallichiana* Zucc. as well as *Taxus baccata* L. in some publications, is one such endangered plant in the Himalayas, occurring at elevations of 1,800 m-3,000 m in the evergreen and coniferous forests. Even now, there is no unanimity amongst the botanists about these names. **In the present thesis, the name *Taxus baccata* ssp. *wallichiana* Zucc. Pilg. (Dr. S. Bhattacharya, personal communication) has been used throughout. Similarly the name Taxol is used instead of Paclitaxel, due to its better familiarity.**

In the earlier days, the wood of these plants was used for making bows, bedsteads and other wooden articles of daily use. The bark was used for procuring a red dye, important in religious ceremonies and for making tea. In addition, the trees were also used for medicinal purposes (Hartzell, 1991).

The plants grow in well-drained soils and can tolerate deep shade and moist habitats. Usually they form the understory component in a broad-leaved (*Quercus* spp., *Rhododendron* spp., *Acer* spp.) or Conifer (*Abies* spp., *Cupressus* spp.) forests. But the trees rarely grow in clusters or as pure strands (Dubey, 1997; Chatterjee and Dey, 1997). The trees have a wide range of adaptability and can grow even under exposed conditions after overstory removal, by undergoing a number of morphological and anatomical changes like increase in the quantity of photosynthetic tissue, establishment of multi-layered branch arrangement etc. Also the strobilus production is shown to be positively

associated with overstory openness with respect to light and microenvironment rather than with branch mass or branch bifurcations (DiFazio et al., 1997).

The Himalayan Yew is an evergreen, slow-growing and long-living tree or shrub. Whether the differences in size and form are genetic traits or the results of environment and stand history is not known. The root system is deep and wide spreading. The roots display a diarch structure and the tracheids display spiral thickenings.

It has wide spreading horizontal branches (for catching maximum, available light) forming a canopy, unlike other conifers. The branches have only unlimited growth. There are no short shoots as in *Pinus* spp. The stems show a number of anatomical peculiarities like absence of resin canals, small cortex and pith as compared to the vascular cylinder, xylem tracheids with opposite bordered-pits and spiral thickenings, absence of xylem parenchyma in the wood, uniseriate medullary rays, conjoint, collateral, endarch and open vascular bundles and abundant secondary growth.

Unlike *Pinus*, the needles are not dimorphic. They are 2.5-3.8 cm long, linear, flattened, distichous, alternate, acute, narrowed in a short petiole decurrent along the twig, dark green and shining above and pale yellowish-brown or rusty-red below. The needles are shed in May-June. Anatomically the needles show a dorsiventral structure where the mesophyll is well differentiated into an upper pallisade and a lower spongy layer. There is a single vascular bundle signifying the mid-rib. A number of xerophytic structures like sunken stomata restricted to the lower epidermis, a thick cuticle above the upper epidermis and a continuous upper epidermis and a discontinuous lower epidermis are present.

The Himalayan Yew is dioecious where male and female trees are identical but distinct. Male strobili appear from March to May in the axils of leaves of the previous year, on the lower side of the branches, in subglobose and solitary catkins. They are stalked, pale yellow with a dozen, spirally arranged, basal bract scales. 6-12 filamentous stamens/microsporophylls are present at the top, which bear 5-9 anthers/microsporangia. The sperms in *Taxus* spp. have no cell membrane or cell wall and hence are not cells. Instead the sperms are nuclei with associated cytoplasm and organelles from the spermatogenous cell (Anderson and Owens, 1999).

There is no female cone in *Taxus* spp. It is very much reduced and is represented by a single erect ovule surrounded by four imbricate bracts. These solitary structures arise in the axils of the leaves of the previous year on the underside of the branches. Absence of seed cones, presence of a rudimentary pollen chamber, absence of a ventral canal cell in the archegonia, a short free nuclear stage (16-32 free nuclei) and a short period of gametophyte and embryo development can be taken as the advanced evolutionary features of *Taxus* spp. (Brukhin and Bozhkov, 1996). Wind pollination and fertilization occur in the same year and fertilization occurs two months after pollination. The fruits ripen from August/September to November of the same year. The fruit comprises of a single, olive-green seed, 8 mm long, almost completely enclosed by a juicy red aril developing from the enlarged disc-like base of the ovule. The embryo has two cotyledons. The seeds are shed in the same year, approximately 12 months after ovule initiation. The seeds have a dormancy requirement of two years before natural germination can occur.

4.1.2 Vegetative Propagation of *Taxus* spp.

Normally, vegetative propagation is achieved through cuttings. The percentages of rooting varied between cultivars and species depending on temperature, misting, use of IBA. 63-100% rooting was observed with many species and cultivars of *Taxus* spp. (Eccher, 1988). Chee (1995a) reported that stem cuttings of *T. cuspidata* treated with an aqueous solution of IBA (0.2%) + NAA (0.1%) + Thiamine (0.08%) produced roots at a frequency of 73.5%. However the success rate was not very high for the Himalayan Yew (20-30%) and the cuttings took at least three months to root. Success of grafting and layering was also not very encouraging (Chatterjee and Dey, 1997).

4.1.3 Development of Taxol and Related Class of Taxane Molecules from *Taxus* spp.

Plants belonging to *Taxus* spp. have been investigated since 1856, mostly for the poisonous alkaloids present in various plant parts. But the intensive study of the plants of this genus started in the early 1960s, when the National Cancer Institute (NCI) at USA began an intensive screening program for plant compounds with anti-cancer activities. **Taxol** was found to be one of the most promising anticancer agents, among the 120,000 compounds screened. After 1969 (elucidation of structure of **Taxol**), these studies diversified into biochemical, biotechnological and clinical arenas. Important milestones in this long and illustrious journey are tabulated in **Table 4.1.**

Table 4.1. Major Milestones in the Discovery and the Development of Taxol.

(Biochemical, Biotechnological, Chemical and Clinical).

Year	Devebpment
1856	**Taxine**, an ill-defined alkaloidal substance isolated from *Taxus baccata* by Lucas H.
1921, 1923	Determination of structure of **Winterstein's Acid**, a major constituent of **Taxine**.
1925	Isolation of neutral, nitrogen-free **Taxinine**.
1953	**Gametophyte Cultures** of *Taxus* spp. initiated by LaRue C. D.
1957, 1958	**Taxine** separated into pure **Taxine -A** and **Taxine -B**.
1959	**Pollen Cultures** of *Taxus* spp. initiated by Tuleke W.
1963	Isolation of **Baccatin-I**.
1964	**9 KB cytotoxic activity** detected **in bark extract of *Taxus brevifolia***
1966	**Baccatin-III** isolated by Chan et al. but structure not determined.
1969-1975	Isolation of **Taxusin** by various workers.
1970-1975	Structures of **Baccatins -I, III, IV, V, VI and VII** confirmed by X-ray crystallography by Della Casa De Marcano and Halsall.
1971	**Isolation and structure** of **Taxol** (the cytotoxically active principle, occurring in miniscule quantities of 0.004% of dry plant matter) from *T. brevifolia* elucidated and published by Wani et al.
1970-1973	Series of papers by Le Page -Degivry M. T. describing **Embryo Germination** in *Taxus baccata* by leaching out ABA from seeds.
1974	First **Callus Culture** initiated from *Taxus baccata* by David and Plastira.
1975	**Taxol activity against B16 melanoma assay** confirmed at NCI, USA.
1977	**Pre-clinical development** of **Taxol** initiated by NCI.
1979	Schiff et al. showed that **Taxol binds to microtubules** and stabilizes them against depolymerization.
1983	**Phase I clinical trials** showed **effectivity of Taxol against** a number of **refractory tumors**.
1985-till date	**Phase II clinical trials** showed **effectivity of Taxol against refractory breast, ovarian and lung cancers**.
1989	First abstract by Christen et al. demonstrating **Taxol Production *in vitro* by Callus and Cell Suspension Cultures**.
	Development of a **New Taxol analogue** : **Taxotere** by semi-synthesis from 10 DAB, by Mangatal et al. It is water-soluble and shows better activity than **Taxol** in some assays.
	First report of **clinical activity of Taxol,** in patients **with relapsed ovarian cancer** by McGuire et al.

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Table 4.1 Contd.

Year	Development
1990-1994	Various groups developed **Semi-synthesis of Taxol from 10-deacetyl-baccatin-III (10-DAB).**
1991	First US patent awarded to USDA (Christen et al.) for demonstrating **Taxol Production *in vitro* by Callus and Cell Suspension Cultures**.
	Activity of Taxol demonstrated **against breast cancer** by Holmes et al.
1992	**Activity of Taxol** demonstrated **against non-small cell lung cancer** by Murphy et al.
	Taxol approved for **Treatment of Advanced Ovarian Cancer** by FDA, USA.
	Tishler et al. showed **radiosensitizing properties** of **Taxol**.
1993	**Taxol** and **Taxane production** demonstrated **in *Taxomyces andreanae*, an endophytic fungus isolated from inner bark of *Taxus brevifolia*** by Stierle et al.
	Taxol showed **good activity against advanced head and neck cancers.**
1994	**Total Synthesis of Taxol** by Nicolau et al. and Holton et al.
	Taxol produced by semi-synthesis from 10-DAB, available for clinical use.
	Taxol approved for **Treatment of Breast Cancer** by FDA, USA.
1995	Hall et al. Showed that **Taxol inhibited osteoclastic bone resorption**.
	The **semisynthetic Taxol approved for marketing by FDA**.
1996	Pengsuparp et al. showed **Cytotoxic activity of Baccatin-III against a number of cancer lines.**
1997	**Anti-proliferative and Apoptotic effects of Taxol in Human Prostate Tumors** demonstrated.
	Semisynthetic Taxol approved for second-line treatment of AIDS related Kaposi's Sarcoma by FDA.
1997-2000	**Much of the new information is in the form of Patents and not published.**

4.1.4 Taxane Class of Molecules

The chemical constituents of different *Taxus* spp. belong to alkaloids, diterpenoids with **Taxane** skeleton, lignans, biflavonoids, steroids, sugar derivatives, and diterpenoids with tropane skeletons. These various compounds exhibit diverse functions such as anticancer, antitumor and antileukemic **Taxanes**, moulting hormones, toxic alkaloids, antineoplastic and antifungl diterpenes and so on (Young et al., 1992; Parmar et al., 1999 and references therein). Because of this, plants of this genus have been intensively studied in the recent years.

Upto 1999, over 350 taxoids have been isolated and characterized. The basic structure for most of the taxoids consists of a pentamethyl tricyclopentadecane skeleton, which is often called as the **Normal Taxane Skeleton (Fig. 4.1)**. In addition, a number of **Taxanes** with rearrangements in this basic skeleton are also known (See the review by Baloglu and Kingston, 1999 for details). Out of all these **Taxanes**, clinically and/or chemically the most important **Taxanes** are: **Taxol/Paclitaxel**, **10-deacetylTaxol**, **Cephalomannine**, **Baccatin-III**, **10-deacetylbaccatin-III** (**10-DAB**) and **Taxol C** (C. f. **Table 4.1**). There are a number of **Taxol**-like derivatives derived from **Baccatin-III**, esterified at C-13 with phenylisoserines having different N-acyl and N-alkyl groups, which are collectively known as **'Taxols'**. Five major structural types of **Taxols**: **Taxols A-D** and **Taxuspinanane** are known till date. Overall 28 natural **Taxols** have been reported from different *Taxus* spp. (Das et al., 1999 and references therein). These compounds possess an unusual oxetane ring at C-5, C-20 and a phenylisoserine side chain at C-13, both of which are necessary for the anticancer and other activities of **Taxol**. **Fig. 4.2** depicts the structures of some of the important taxoids.

The contents of **Taxanes** in plants of *Taxus* spp. are known to vary based on geographical locations, environmental and agricultural influences, harvesting and storage conditions (Vidensek et al., 1990; Kelsey and Vance, 1992; Wheeler et al., 1992; ElSohly et al., 1994). The average yield of **Taxol** (the most important and efficient anti-cancer agent identified till date) in bark of *Taxus brevifolia* ranges between 0.014-0.017% (Vidensek et al., 1990; Patel, 1998) and 16,000 lb. bark is needed to produce 1 kg purified **Taxol** (Patel, 1998 and references therein). Other species of *Taxus* are reported to contain **Taxol** in the range of 0.001-0.0832 $mg.g^{-1}$ DW (Arteca and Wickremesinhe, 1993).

Fig. 4.1 Basic Taxane skeleton

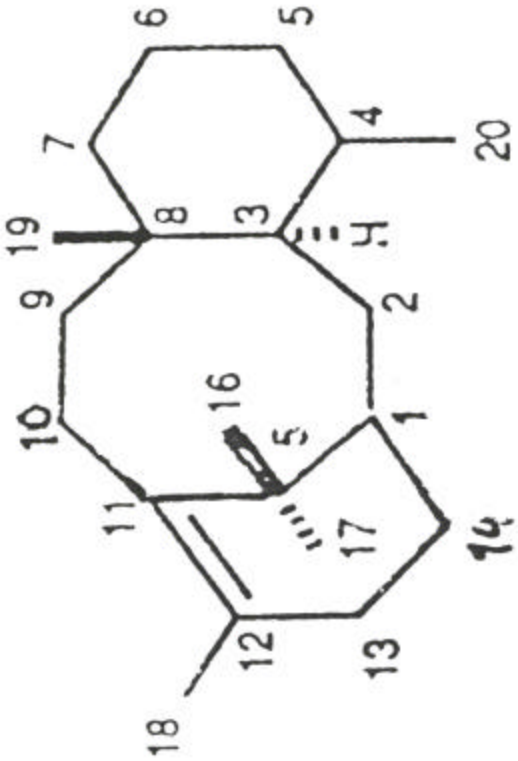

Table 4.2 represents some of the physico-chemical properties of **Taxol**.

Table 4.2 Physicochemical Properties of Taxol (Sohn and Okos, 1998).

IUPAC Systematic Name	[2aR-(2a,4,4a,6,9,11,12,12a,12b)]-Benzoylamino-hydroxy benzenepropanoic acid 6,12b-bis(acetyloxy)-12-(benzoyloxy)-2a,3,4,4a,5,6,9,10,11,12,12a,12b-dodecahydro-4,11-dihydroxy-4a,8,13,13-tetramethyl-5-oxo-7,11-methano-1H-cyclodeca[3,4]benz[1] ester
Molecular Formula	$C_{47}H_{51}NO_{14}$
Molecular Weight	853.92 g/mole
% Composition	C 66.51%, H 6.02%, N 1.64% O 26.33%
Solubility in Water	4 mg/L at room temperature
Melting Point	213~216°C
Specific Rotation	-49° in methanol
TLC	R_f = 0.125 (Silica 50% ethyl acetate in hexane)
UV Max.	227, 273 nm in methanol
IR (Thin film)	V_{max}: 3472, 2937, 1720, 1652, 1520, 1241/cm
Mass Spec	*m/e* = 854.3360 M+H calculated for $C_{47}H_{51}NO_{14}$ = 854.3360

4.1.5 Development of Taxol as an Anti-tumor and Anti-cancer Agent

After the isolation of **Taxol** in pure form by Wall's group, its activity was tested against a number of cell lines *in vivo*. It showed antitumor activity against KB cells (carcinoma of nasopharynx), P-1534 leukemia, P-388 leukemia, L-1210 leukemia, Walker 256 carcinoma in the primary screening and against B-16 melanoma in the secondary screening (Patel, 1998 and references therein). In spite of these results, development of **Taxol** was very slow due to very low contents in the natural bark of *Taxus brevifolia* and the concomitant difficulties in the extraction procedure, water insolubility and resulting difficulty in formulation and patient administration (Ibid.).

Results of Phase I - Phase III clinical trials of **Taxol** showed its efficacy against a number of cancer types (C. f. **Table 4.1**): refractory ovarian cancer (McGuire et al., 1989), primary metastatic breast cancer (Holmes et al., 1991), non-small cell lung cancer

(Murphy et al., 1992) etc. **Taxol** is also combined with a number of other drugs such as Doxorubicin (O'Shaughnessy et al., 1994) and Navelbine/Vinorelbine (Chang and Garrow, 1995) to increase its efficacy against various types of cancers.

Taxol is shown to inhibit growth of parasites such as *Plasmodium falciparum*, *Trypanosoma* and *Physarum* (Schrével et al., 1994 and references therein). **Taxol** inhibits osteoclastic bone resorption (Hall et al., 1995) and acts as a radiation sensitizer (Tishler et al., 1992).

4.1.6 Taxol-Tubulin Interactions and Mode of Action of Taxol

Taxol binds to and stabilizes both the 2-dimensional zinc sheets of tubulin as well as the 3-dimensional microtubule against low temperature and alkalinity-induced depolymerization and polymerization can proceed even in the absence of guanidine triphosphate (GTP) and microtubule associated proteins (MAPs). **Taxol** promotes both the nucleation and elongation phases of polymerization and can reduce the critical concentration of tubulin to almost zero (Patel, 1998 and reference therein). **Taxol** binds into a pocket in the second globular domain of β tubulin, facing the central hole in the microtubule. Here it makes close contact with the shorter S9-S10 loop, the core helix and the M loop between S7 and H9 (**Fig. 4.3,** adapted from Amos and Löwe, 1999). Mutations in this loop reduce the toxicity of **Taxol** to cancer cells.

4.1.7 Mode of Action of Taxol

Taxol stabilizes the microtubules against depolymerization and hence cells are arrested at the G2-M phase of cell division and cell division cannot proceed.

Because of the high efficacy of **Taxol**, it is believed that mitotic inhibition can not be the sole process responsible for its clinical activity and tubulin can not be its only target. Following alternatives have been suggested:

1. **Taxol** also binds to a protein: Bcl-2, which normally blocks the process of cell death or apoptosis. When **Taxol** binds to Bcl-2, apoptosis is allowed to proceed (Fang et al., 1998).
2. Another mechanism suggested for **Taxol**-induced apoptosis is a phosphorylation pathway possibly involving the $p34^{cdc2}$ kinase (Donaldson et al., 1994).

3. **Taxol** is believed to induce cytokines via signal transduction pathways and has been shown to transcriptionally activate expression of a number of genes. The C-1 to C-4 positions on the **Taxol** molecule appear to be important (Watson et al., 1998 and references therein).
4. **Taxol** is also shown to activate other regulatory activities such as induction of MAP kinases, induction of protein-tyrosine phosphorylation, tumor necrosis factor (TNF) and interleukin (IL-1) etc. (Watson et al., 1998 and references therein).
5. **Taxol** is a potent platelet antiaggregant (Canizares et al., 1997).

4.1.8 Rationale for Use of Plant Tissue and Cell Cultures of *Taxus* spp. for Production of Taxane Class of Molecules

It has become imperative to find alternative sources for **Taxol** production due to the dwindling natural resources of *Taxus* spp. because of their overexploitation for **Taxane** extraction from bark, stem and needles. Naturally, the plants belonging to *Taxus* spp. never form the major component of any forest (Dr. O. Singh, Personal communication) and due to overexploitation; these plants have attained an endangered status.

1) Normally, synthetic chemistry proves to be an important tool in such endeavors as the molecules can be synthesized totally in the laboratory. But in case of **Taxol**, it has proved to be an uphill task for synthetic chemists. The two total syntheses reported so far (Nicolau et al., 1994; Holton et al., 1994) employ more than 35 steps and hence cannot be used for economical production of **Taxol**.
2) Some methods of semi-synthesis of **Taxol** from **10 DAB-III** or **Baccatin-III** (**Taxol** precursors found in large quantities in *T. baccata* and *T. wallichiana*) by attachment of side chain also have been proposed (Denis et al., 1988; Lavelle et al., 1993). These methods may prove to be viable alternatives for **Taxol** production.
3) Stierle et al. (1993) reported the production of **Taxol** (25-50 ng/L) by an endophytic fungus *Taxomyces andreanae* isolated from an identified tree of *T. brevifolia*. It appears unlikely that strain improvement by mutagenesis or genetic engineering will result in spectacular yield enhancement necessary for bioreactor/fermentor scale-up (Patel, 1998).
4) Since vegetative propagation of *Taxus* spp. by use of cuttings has not yielded significant results (**Section 4.1.2**), especially for the Himalayan Yews, use of plant

tissue culture methods seems to be the best alternative amongst various approaches as outlined below.

Plant tissue culture methods have proved to be an important alternative wherein a number of *Taxus* spp. have shown production of **Taxane** class of compounds *in vitro* (C. f. **Table 4.3**). Such cultures can be scaled up for large-scale production of **Taxanes**. Cultures producing **Taxol** can be used for direct scale-up. Even cultures producing **Taxol** precursors like **10 DAB-III** and **Baccatin III** can be scaled up for their subsequent use in semi-synthetic production of **Taxol**. Also these **Taxol** precursors by themselves have antitumor/cytotoxic properties like **Taxol** and hence can be used independently (Pengsuparp et al., 1996). Natural, field-grown populations of *Taxus* spp. plants show considerable seasonal, environmental and epigenetic variation in **Taxane** contents (Kelsey and Vance, 1992; Wheeler et al., 1992). Using defined conditions in a cell culture system, **Taxane** production can be closely controlled to supply consistent product quality. Cell cultures could eliminate the need to use the limited natural resources of *Taxus* spp., thus preserving the largely unexplored genetic diversity within the genera for selecting high yielding variants for cell culture process (Gibson et al., 1995). The cell cultures can also be used for selection of elite variants and for elicitation, immobilization and permeabilization studies as well as for genetic manipulations. They can also be used for elucidating the biosynthetic pathway of **Taxol** so that very specific manipulations for improving **Taxol** yields could be carried out. In addition, the rate limiting steps can be identified and can be modified by genetic manipulations (Wildung and Croteau, 1996; Hefner et al., 1996; Pennington et al., 1998). **Table 4.3** presents various studies carried out so far in the establishment of undifferentiated callus and cell cultures of *Taxus* spp. and production of **Taxol** therefrom. The table also lists genetic transformation studies with *Agrobacterium tumefaciens* and *A. rhizogenes.*

Table 4.3 Establishment of Undifferentiated Callus and Cell Cultures from Various *Taxus* spp. and Taxane Production Therefrom.

No.	*Taxus* spp.	Explants	Medium	Plant Growth Regulators & Additives	Amount of Taxol Produced	Reference
1.	*T. baccata*	Pollen grains	-	-	-	Tuleke (1959)
2.	*T. baccata*	Gametophyte	B & N	No hormones	-	Rohr (1973)
3.	*T. baccata*	Old stem	Heller	2,4-D/KIN + Thiamine + Tobacco **Nurse Culture**	-	David & Plastira (1974, 1976)
4.	*T. cuspidata*	Young needles	B5	2,4-D + PVP	200 µg/g dry weight callus, 55 days old	Fett-Netto et al. (1992) (1994a, b)
5.			B5 with 3 times the organic stock	2,4-D + KIN +Sol. PVP	4-14 µg/g dry weight cells in suspension & 0.15 µg/ml medium after 38 days	
					120 µg/g dry weight immobilized cells	
6.	*T. cuspidata*	Young needles	B5	2,4-D + PVP + **Phenylalanine** as **Metabolic Precursor**	400 µg/g dry weight callus, 55 days old	Fett-Netto et al. (1993a, b)
7.	*T.*x *media, T. cuspidata, T. brevifolia, T. baccata*	Stems, Needles	B5	2,4-D/NAA/IBA + KIN	1.7-14.2 µg/g dry weight callus, more than 18 months old	Wickreme-sinhe & Arteca (1993)
8.	*T.*x *media*	Stem	B5	No hormones + CH	0.1-13.1 µg/g dry weight callus, 4-6 years old	Wickreme-sinhe & Arteca (1993)

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Table 4.3 Contd.

No.	*Taxus* spp.	Explants	Medium	Plant Growth Regulators & Additives	Amount of Taxol Produced	Reference
9.	*T. brevifolia*	Bark, Stem, Needles	B5	2,4-D + CA (Casamino acids)	Not detectable by HPLC	Gibson et al. (1993)
10.	*T.*x *media*	Stem	B5	No hormones (**Habituated Callus**) + CH (Casein Hydrolysate)	1-283 µg/g dry weight cells in suspension, 1 year old from 4-6 years old callus	Wickreme-sinhe & Arteca (1994)
11.	*T. cuspidata*	Young Stems, Needles	MS	NAA + KIN/BAP, 2,4-D + KIN	4.5-9.6 µg/g dry weight callus, 6 months old	Tachibana et al. (1994)
12.	*T. baccata*	Stem	WPM	NAA + BAP	8 µg/g dry weight callus, 4 months old	Han et al. (1994)
13.	*T. baccata*	Stem	WPM	No hormones	**0.8-4 mg/g dry weight transformed callus, 9 months old**	Han et al. (1994)
14.	*T. brevifolia*	Young stem	SH	NAA + BAP	1.43 µg/ml cell suspension	Kim et al. (1995)
15.	*T. brevifolia*	Cambium	B5	Picloram (P) + KIN + ABA + GA3+ **Aspartic acid** + **Arginine** + **Glycine** + **Proline** as **Metabolic Precursors**	-	Ketchum et al. (1995)
16.	*T. baccata*	Twigs, Needles	B5	NAA, P	-	Furmanowa et al. (1995)
17.	*T. cuspidata*	Needles, Young stems	B5	2,4-D + KIN, NAA + KIN	**3.2 mg/g dry weight cells & 0.38 mg/ml in medium in continuous culture**	Seki et al. (1995)

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Table 4.3 Contd.

No.	*Taxus* spp.	Explants	Medium	Plant Growth Regulators & Additives	Amount of Taxol Produced	Reference
18.	*T. cuspidata*	Young stem	B5	NAA + CH + PVP	0.0297-0.0362 µg/ml cell suspension, **Immobilized**	Park & Chung (1995)
19.	*Taxus* spp.	-	1/4 strength B5	2,4-D + Zeatin + **Elicitors** after 10 days	4-6 µg/ml cell suspension	Ciddi et al. (1995)
20.	*T. baccata*	-	Modified B5	NAA + BAP + Glutamine + Ascorbic Acid	**1.5 mg/ml cell suspension in pneumatically mixed Bioreactors**	Srinivasan et al. (1995)
21	*T. baccata*	-	Modified B5	NAA + BAP + Glutamine + Ascorbic Acid	0-15 µg/ml cell suspension after 26 days	Hirasuna et al. (1996)
22.	*T. canadensis*	Embryo	B5	NAA + BA/TDZ, 2,4-D + BA/TDZ	5.9-21 µg/ml cell suspension	Ketchum & Gibson (1996)
23.	*T. cuspidata*	Embryo	B5	IAA + BAP	1.6-69 µg/ml cell suspension	Ketchum & Gibson (1996)
24.	*T. cuspidata*	-	B5	NAA + BAP + Glutamine + Ascorbic Acid	**22 mg/ml cell suspension in shake flasks & Wilson type Bioreactor in 20 days**	Pestchanker et al. (1996)
25.	*T. brevifolia*	Young stems, Needles	B5	2,4 -D + NAA	170 ng/ml cell suspension	Heinstein et al. (1996)
26.	*T. baccata*	Stem	Modified SH	2,4-D/NAA/+ BAP/KIN + Glutamine + Asparagine + PVP + Ascorbic acid	3-10 µg/g dry weight callus, 6 months old	Brukhin et al. (1996)

Contd…..

Table 4.3 Contd.

No.	*Taxus* spp.	Explants	Medium	Plant Growth Regulators & Additives	Amount of Taxol Produced	Reference
27.	*T. media*	Embryos, Stems	WPM	NAA	**33 mg/ml cell suspension = 2.75 mg/g dry weight cells**	Yukimune et al. (1996)
				NAA + Methyl **Jasmonate**/ ± **Jasmonic acid** as **Elicitors**	**104-110 mg/ml cell suspension = 8.66 -9.16 mg/g dry weight cells**	
28.	*T. baccata*			NAA	29 µg/ml cell suspension	
				NAA + **Methyl Jasmonate**/ ± **Jasmonic acid** as **Elicitors**	**3.45 mg/ml cell suspension**	
29.	*T. brevifolia*			NAA + Methyl **Jasmonate**/ ± **Jasmonic acid** as **Elicitors**	14 µg/ml cell suspension 36 µg/ml cell suspension	
30.	*T. cuspidata*	Young needles from shoot cultures	Modified WPM	NAA + 2-iP + Riboflavin + Biotin + Folic Acid + D-Pantothennic acid + Choline chloride (**Anti-metabolite**)	**12 mg/g dry weight of nodule**	Ellis et al. (1996)
31.	*T. cuspidata*	Bark, stem, needles	B5	2,4-D + CA + **Ethylene** + **Methyl jasmonate** (**Elicitor**)	3.4 µg/ml cell suspension	Mirjalili & Linden (1996)
32.	*T. cuspidata* Sieb et Zucc.	Needles	B5 (Modified)	2,4-D/NAA + KIN	3.2 mg/g DW of cells	Seki and Furusaki (1996)

Contd…..

Table 4.3 Contd.

No.	*Taxus* spp.	Explants	Medium	Plant Growth Regulators & Additives	Amount of Taxol Produced	Reference
33.	*T. cuspidata* Sieb et Zucc.	Needles	B5 (Modified)	CA + NAA	0.2 µg-excreted/ml cell suspension	Seki et al. (1997)
				CA + NAA + KIN	**0.3 mg/g dry weight cells per day.**	
34.	*T. chinensis*	-	MS	2,4-D + NAA + BAP + Ascorbic acid	13-32.7 µg/g dry weight cells in suspension & 0.045-0.091 µg/ml extracellular medium	Wang et al. (1997)
35.	*T. cuspidata*	Cell culture from habituated callus line	Modified SH	Plant growth regulators absent, No adsorbent	0.15 mg/L in medium	Kwon et al. (1998)
				Plant growth regulators absent, **with Adsorbent**	0.23 mg/L in medium + adsorbent	
36.	*T. baccata*	-	B5	NAA	40 µg/g dry weight cells in suspension	Laskaris et al. (1999)
37.	*T. canadensis*	Embryos	B5	NAA + BAP/TDZ, 2,4-D + BAP/TDZ	0.29-1.4 µg/ml cell suspension	Ketchum et al. (1999)
				NAA + BAP/TDZ, 2,4-D + BAP/TDZ + **Methyl Jasmonate (Elicitor)**	2.89-16.4 µg/ml cell suspension	
38.	*T. cuspidata*	Young stems	Ericksson's E6	2,4-D + KIN + BAP	51 µg/g DW in cells and 8 µg/L of medium in a 6.2 L **Bioreactor**	Vanek et al. (1999)

Contd…..

Table 4.3 Contd.

No.	*Taxus* spp.	Explants	Medium	Plant Growth Regulators & Additives	Amount of Taxol Produced	Reference
39.	*T. baccata*	Young stems	Modified B5	2,4-D + KIN + GA3	5.2 µg/g DW in cells	Cusidó et al. (1999)
				2,4-D + KIN + GA_3 + VSO_4 (**Elicitor**)	13.1 µg/g DW in cells and excretion in medium increased	
40.	*T. chinensis*	-	MS	2,4-D + NAA + BAP	14 mg/g DW of cells with < 30 $g.l^{-1}$ sucrose	Wang et al. (2000)
					274.4 mg/L with sucrose feeding in fed-batch cultures	

In view of the above literature survey, it is quite evident that alternative approaches should be explored for production of **Taxol** and its related **Taxanes** through callus and cell suspension culture technology so that the dependence on extraction of **Taxanes** from bark, stems and needles of *Taxus* spp. could be minimized, if not totally eliminated. The development of such methods has become increasingly more relevant and important in view of the dwindling natural resources of *Taxus* spp. in their natural habitats and because of increase in demands of **Taxol** and related **Taxanes** in the anti-cancer therapy. The present chapter is therefore devoted to the work on callus induction, growth and **Taxane** production *in vitro* from tissues of the Himalayan Yew.

The specific objectives were as follows:

1. When this work was started, very few reports were available on tissue cultures of *Taxus* spp. and production of **Taxanes** therefrom. Therefore, the work was initiated right from optimization of the basal nutrient media so as to achieve better callus growth and high **Taxol** and/or **10-DAB** production in one and the same medium. The results obtained by use of the standardized methodology could prove useful in their scale-up for production of desired **Taxanes**.
2. Identification of hyper-producer cell lines of **Taxanes**.
3. Analysis of growth and **Taxane** production from both diploid and haploid callus lines of the Himalayan Yew.
4. Extrapolation of the optimized protocol to develop callus cultures from different collection locations in the Himalayas.
5. Preliminary work on establishment of cell suspension cultures from callus cultures of *Taxus* spp. and to study for the possible increased production of **Taxanes** under such conditions of liquid culture.

4.2 Material and Methods

4.2.1 Collection of plant material

Taxus baccata ssp. *wallichiana* Zucc. Pilg. is an endangered Himalayan Gymnosperm. The collections of plant material were done at different locations above 2000 m in the Himalayas like Darjeeling (West. Bengal, W. B.), Narkanda (Near Shimla), Khajjiyar (Himachal Pradesh, H. P.) and Almora (Uttar Pradesh, U. P.) (**Fig. 4.4**) during the months of March to October. (It was not possible to collect the material during remaining part of the year because of very heavy snow-fall in this area and the resultant inaccessibility to these areas by road). The plants were at least 80-130 years old, fully mature (all the female plants were producing seeds) and open to the vagaries of the nature (**Fig. 4.5 A**). The branches were cut from the lower regions of the plant and their cut ends were sealed with 'Parafilm-M' ("American National Can", USA). These were then packed in cool packs and transported to the laboratory. In the laboratory, the branches along with needles and seeds (when present), (**Fig. 4.5 B**) were stored in dark in a walk-in cold room at 4-8^{o}C until processed, within a fortnight.

4.2.2 Surface sterilization of explants

Stem bit and needle explants of *Taxus* spp. were surface sterilized according to **"Modified Surface Sterilization Protocol"** described in **Chapter 3, Flow Chart 3.3**. Endosperm explants were taken out by cutting open the seeds that were surface sterilized according to **"Modified Surface Sterilization Protocol"** described in **Chapter 3, Flow Chart 3.3** (For details refer to **Chapter 5, Section 5.5.2**).

4.2.3 Inoculation of explants

4.2.3.1 Initiation of callus cultures

Stem bit explants (5-7 mm long), needle explants (3-6 mm long) and endosperm halves taken out from seeds were cultured in a laminar airflow cabinet. Explants were cut to desired size (as above) on sterile filter papers using autoclaved forceps and surgical blade holders and these were transferred to 55 mm diameter plastic dishes containing various nutrient media and plant growth regulator combinations for initiation of callus

Fig. 4.4 Collection locations for plant material of *Taxus baccata* ssp. *wallichiana* Zucc. Pilg. in Northern India and location of our lab at Pune, India..

Fig. 4.5

A. A full-grown mature tree of *Taxus baccata* ssp. *wallichiana* Zucc. Pilg. (~100 years old). Indicated with an arrow.
B. A branch from a tree of *Taxus* spp. with vegetative buds (arrows). The scale is in cm.

cultures. The forceps and blade holders fitted with sterile surgical blades (No. 11 and 23, "Surgeon", India) were dipped in ethyl alcohol and flamed after each transfer.

4.2.3.2 Initiation of suspension cultures

For initiating cell suspension cultures in *Taxus* spp., 200 mg fresh weight (FW) of callus was placed in 20 ml of optimized liquid medium in each of the 100 ml capacity Erlenmeyer flasks on a magnetic shaker ("Infors AG", Germany) at 100 rpm for varying periods of time before **Taxane** analysis.

4.2.4 Media and culture conditions

A number of basal media were used for callus induction experiments such as Schenk and Hidebrandt's SH medium (1972) with major salts reduced to 1/2 strength (1/2 SH), Gamborg's B5 medium (1968) at full strength, Lloyd and McCown's Woody Plant Medium: WPM (1981) at full strength and Gupta and Durzan's DCR medium (1985) at full strength. Their compositions are described in **Chapter 2, Table 2.1** . The media were modified by addition of different concentrations of various plant growth regulators such as Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), α-naphthalene-acetic acid (NAA), Picloram (P), 2,4-dichlorophenoxyacetic acid (2,4-D), N^6 benzylaminopurine (BAP), Kinetin (KIN) and N^6-(2-isopentyl)-adenine (2-iP) individually or in permutations and combinations. All these details regarding concentrations and combinations of plant growth regulators used are listed in **Tables 4.4** and **4.5**.

All the media contained 2% sucrose and 0.3% phytagel. All the plant growth regulators were added before autoclaving of the media at 121°C and at a pressure of 1.1 $kg.cm^{-2}$. pH of the media was adjusted to 5.8 before autoclaving as described in detail in **Chapter 2, Section 2.4**.

For initiating suspension cultures from **Taxane** hyper-producing calli, only an optimized medium B5 supplemented individually with P at 2, 4, 6, 8 or 10 $mg.l^{-1}$ was used. The media were prepared as described above and contained 2% sucrose without any gelling agent.

All callus and suspension cultures were incubated in the dark at a temperature of 25 ± 2°C.

4.2.5 Observations and subculture schedules

Callus cultures were observed routinely and subcultures were carried out at intervals of 21-35 days depending upon the growth rate, phenolic browning (if any) and nature of the developing callus tissues. At the time of subcultures, fresh weight determinations of promising cell lines were done only under sterile conditions. For this, the callus derived from a single needle explant was dried between sheets of sterile filter paper till no moisture was released from the callus. The callus mass was then weighed on a pre-weighed sterile piece of aluminium foil kept on a micro-balance ("Anamed", India), placed inside the laminar airflow cabinet. Dry weights were determined only at the time of extraction of callus tissues for **Taxane** content determination by TLC and HPLC. Here the callus derived from a single needle explant was dried at 60°C in an oven till constant weight was obtained. Usually this took 16-24 hours. The spent solid medium from various nutrient media and plant growth regulator combinations was saved after subculture of the calli to fresh media. These spent media were analyzed independently for presence of **Taxanes** by HPLC.

Suspension cultures for preliminary studies were raised from randomly selected callus lines (Also see **Table 4.11**) in three replicate flasks at day 0. These were subcultured in fresh media of identical compositions after every 4 days. Routine growth determinations in suspension cell cultures were carried out by centrifuging 8 ml of culture broth in a graduated, conical centrifuge tube at 5000 rpm for 5 min. to determine Packed Cell Volumes (PCV).

Out of the three replicate flasks of suspension cultures, at day 9, one flask was harvested for each of the media and cell line combinations (See **Table 4.11**) and cells and media were extracted independently and analyzed by HPLC for **Taxane** contents (**Sections 4.2.7.4, 4.2.7.5 and 4.2.8.2**). At the same time, fresh and dry weight measurements of cells in suspension cultures were also carried out. For this, the cells were separated from the liquid media by centrifuging them at 5000 rpm for 5 min. The liquid medium was pipetted out and the cell pellet was dried between sheets of filter paper till no moisture was released from the cell pellet. The fresh weight of pellet was then determined on the analytical balance. Dry weight determinations were carried out after drying the cells at 60°C in an oven till constant weight was obtained. Usually this

took 16-24 hours. The liquid medium separated from the cells was used for direct extraction of **Taxanes**. Then at day 18, the contents of one flask were harvested for each of the media and cell line combinations. The fresh and dry weights of cells were determined as described above. The dried cell mass and media were extracted independently and analyzed by HPLC for **Taxane** contents (**Sections 4.2.7.4, 4.2.7.5 and 4.2.8.2**). The above procedure was repeated with the other remaining two replicate flasks and analysis was done by HPLC (**Section 4.2.8.2**) on separate days to check for the reproducibility of the experiments.

4.2.6 Extraction of *in vitro* grown callus tissues for analysis of Taxanes by TLC

Various callus lines were extracted and screened by TLC as a primary screen to identify **Taxane** producing, fast growing callus lines. Promising cell lines of various ages were then screened by HPLC. Only 50 mg (DW) of callus tissue was used for extraction as TLC was used only as a qualitative screen.

4.2.6.1 Extraction of callus tissues

Extraction of callus tissues was carried out by the method of Vidensek et al. (1990) modified during the course of the present work. All the solvents used were of HPLC grade and the steps involved in the extraction procedure were as follows:

1. Calli of different age groups growing on various basal media and plant growth regulator combinations were selected for extraction (C. f. **Table 4.5**).
2. 50 mg dry weight (DW) of each type of calli was taken for analysis.
3. The dried tissues were ground in a pre-cooled mortar and pestle with liquid nitrogen and extracted overnight in methanol (10 times w/v) on a rotary shaker at 25°C and 100 rpm.
4. The procedure was repeated three times and the methanolic extracts were pooled together.
5. The extracts were filtered through Whatman No.1 filter paper and dried in a Centrivap Concentrator ("Labconco", USA).
6. The residue was redissolved in chloroform : water (1:1 v/v) and then centrifuged at 7000-8000 rpm, at 16°C to separate the layers of chloroform and water.

7. The lower chloroform layer was collected and the upper layer of water was discarded. The layer at the interphase was re-extracted with chloroform and again the chloroform layer was collected.
8. The chloroform fractions from both the separations were combined and dried in the Centrivap Concentrator as described earlier.
9. The residue was redissolved in 1 ml methanol and used for spotting on TLC plates.

4.2.7 Extraction of *in vivo* as well as *in vitro* grown tissues and cells and spent medium for analysis of Taxanes by HPLC

4.2.7.1 Extraction of *in vivo* tissues (Bark and Needles)

For extraction of *in vivo* tissues, bark shavings and needles from all the identified trees of the Shimla region were pooled together and the extraction for determination of the content of **Taxanes** was carried out by the method of Vidensek et al. (1990) modified during the course of the present work. All the solvents used were of HPLC grade and the steps involved in the extraction procedure were as follows:

1. Bark shavings and needles were dried in an oven at 60°C, till constant weight was obtained.
2. The dried tissues were ground in a mortar and pestle and the powder was sieved through a metal sieve of pore size 0.8 mm.
3. 10 g of bark and needle powders were extracted with 10 volumes of methanol (100 ml) on a rotary shaker at 100 rpm and 25°C for 16 hours.
4. The extraction procedure using methanol was repeated thrice and the methanolic extracts were pooled together for bark and needle tissues respectively.
5. The methanolic extracts were filtered through Whatman No.1 filter paper and dried on a Büchi Rotavapor-R ("Laboratoriums-Technik AG", Switzerland) at 40°C, at a vacuum equivalent to 25" Hg.
6. The residue was redissolved in chloroform : water (1:1 v/v) and then centrifuged at 7000-8000 rpm at 16°C in a centrifuge ("Remi instruments", India) to separate the layers of chloroform and water.
7. The lower chloroform layer was collected and upper layer of water was discarded. The layer at the interphase was re-extracted with chloroform and again the chloroform fraction was collected.

8. The chloroform fractions from both the separations were combined and dried on a Büchi Rotavapor-R at 35°C, at a vacuum equivalent to 25" Hg.
9. The residue was redissolved in 2 ml of methanol and was filtered through 0.2 or 0.4 µM nylon filters before analysis by HPLC.
10. 20 µL of extract suspended in methanol was analyzed by HPLC.

4.2.7.2 Extraction of callus tissues

Extraction of callus tissues for determination of the content of **Taxanes** was carried out by the method of Videnšek et al. (1990) modified during the course of the present work. All the solvents used were of HPLC grade and the steps involved in the extraction procedure were as follows:

1. Calli of different age groups growing on various basal media and plant growth regulator combinations were selected for extraction.
2. 200-400 mg dry weight of each type of calli was taken for analysis. (The tissues were dried in an oven at 60°C, till constant weight was obtained).
3. The dried tissues were ground in a pre-cooled mortar and pestle with liquid nitrogen and extracted overnight in methanol (10 times w/v) on a rotary shaker at 25°C and 100 rpm.
4. The procedure was repeated three times and the methanolic extracts were pooled together.
5. The extracts were filtered through Whatman No.1 filter paper and dried in the Centrivap Concentrator as described earlier.
6. The residue was redissolved in chloroform : water (1:1 v/v) and then centrifuged at 7000-8000 rpm, at 16°C to separate the layers of chloroform and water.
7. The lower chloroform layer was collected and the upper layer of water was discarded. The layer at the interphase was re-extracted with chloroform and again the chloroform layer was collected.
8. The chloroform fractions from both the separations were combined and dried in the Centrivap Concentrator at conditions as described earlier.
9. The residue was redissolved in 1 ml of methanol and was filtered through 0.2-0.4 µM nylon filters before analysis by HPLC.

10. 20 µL of extract suspended in methanol, from each callus line, was analyzed by HPLC.

4.2.7.3 Extraction of spent solid medium (with phytagel) from callus cultures

All the solvents used during extraction were of HPLC grade and the extraction procedure involved the following steps modified from Fett-neto et al. (1992).

1. Solid nutrient media of identical compositions and combinations, used for growing callus cultures derived from the same parent tree, were pooled together and their fresh weights were determined as described earlier.
2. These media were then extracted with 10 volumes of methanol (10 times w/v) on a rotary shaker at 100 rpm and 25°C for 16 hours.
3. The procedure was repeated three times and the methanolic extracts were pooled together.
4. The extracts were filtered through Whatman No.1 filter paper and dried on a Büchi Rotavapor-R at 40°C and a vacuum equivalent to 25" Hg.
5. The residue was directly dissolved in 2 ml methanol and filtered through 0.2-0.4 µM nylon filters before analysis by HPLC.
6. 20 µL of extract suspended in methanol, from each type of medium was analyzed by HPLC.

4.2.7.4 Extraction of cells grown in suspension cultures

Extraction of cells grown in suspension cultures for determination of the content of **Taxanes** was carried out by the method of Vidensek et al. (1990) modified during the course of the present work. All the solvents used were of HPLC grade and the steps involved in the extraction procedure were as follows:

1. Cells growing in suspension cultures were separated from the liquid nutrient medium on day 9 and day 18, from separate but identical replicate flasks, by centrifuging them at 7000-8000 rpm, at 16°C.
2. The cells were blotted dry on filter papers and dried in oven at 60°C, till constant weight was obtained.
3. All the other steps followed were identical to the ones described above in **Section**

4.2.7.2 for extraction of callus tissues.

4.2.7.5 Extraction of spent medium (without cells) from suspension cultures

All the solvents used during extraction were of HPLC grade and the extraction procedure involved the following steps modified from Fett-neto et al. (1992).

1. Cells growing in suspension cultures were separated from the liquid nutrient medium on day 9 and day 18, from separate but identical replicate flasks, by centrifuging them at 7000-8000 rpm, at 16°C.
2. The spent medium was extracted overnight with equal volumes (v/v) of chloroform on a rotary shaker at 100 rpm and 25°C.
3. These extractions were repeated for three days and all the chloroform extracts were combined.
4. Since the medium contained a lot of water, it formed two layers with chloroform automatically. The two layers were separated by centrifuging them at 7000-8000 rpm, at 16°C.
5. The chloroform fraction was dried in the Centrivap Concentrator as described earlier
6. The residue was resuspended in 1 ml of methanol, filtered through 0.2-0.4 μM nylon filters before analysis by HPLC.
7. 20 μL of extract from each type of medium was analyzed by HPLC.

4.2.8 Analytical techniques

4.2.8.1 Thin Layer Chromatography (TLC)

TLC was carried out by using handmade glass plates of size 10 cm x 15 cm x 0.2 mm coated with either Silica Gel G or Silica Gel F254 (“Merck”, India). In addition, pre-coated plates of size 10 cm x 15 cm x 0.2 mm, with aluminum backing (“Merck”, India) and coated with either Silica Gel G or Silica Gel F254 were used. Routine procedures for analytical TLC were followed as given in Fried and Sherma (1994). The actual procedure followed is described here in brief. Starting line was marked at a distance of 1.5 cm from the bottom of the plate and the finishing line was marked at a distance of 2 cm from the top of the plate. Spots of standard and sample solutions in methanol were applied with the help of microcapillaries. Each spot contained 50 μL of the standard solution of a strength

0.5 mg/ml (2.5 µg of standard chemical applied effectively) and 50 µL of the sample solution from callus extracts. Chromatogram development was done in vertical glass chambers saturated with chloroform : methanol (7:1 v/v) using the method of Strobel et al. (1992), upto the finishing line marked on the TLC plate. At this time, the plate was removed from the development chamber and the mobile phase was allowed to dry. The developed plates with the normal Silica Gel G were sprayed with H_2SO_4 and kept in an oven at 100°C for 5 min for visualization of spots of both standard and sample while the Silica Gel F254 plates were observed under a short wavelength UV torch of 312 nm ("Vilber Lonrmat", Cedex, France) for detection of UV quenching **Taxanes** in the sample and their comparison with the standards. R_f was calculated as per the formula (Fried and Sherma, 1994):

$$R_f = \frac{\text{Distance travelled by the applied solute mixture front (cm)}}{\text{Distance travelled by the solvent (mobile phase) front (cm)}}$$

4.2.8.2 High Performance Liquid Chromatography (HPLC)

All routine procedures for analytical HPLC were followed (Melander and Horvath, 1980; Scott, 1996). The actual procedure followed for HPLC analysis was modified from the protocol given by ElSohly et al. (1994).

HPLC was carried out on a Merck-Hitachi instrument with a series D-7000 interface with HSM Manager, L-7100 pump, L-7420 diode array detector, a rheodyne injector with a 20 µL injection loop. RP-18 column ("Merck", India) of dimensions 250 x 4.6 mm with particle size of 5µM was used. The column was operated in the **reverse-phase** mode. The data were recorded at a wavelength of 225 nm on a computer and the chromatograms and data reports were printed on HP Deskjet 200 printer ("Hewlett Packard", India). Photographs of selected runs were recorded on color films. External standard method of quantitation was used.

All the solvents used were of HPLC (Lichrosolv) grade ("Merck", India). The in-house purified Milli-Q water was used ("Millipore Corporation", USA). All the solvents and samples were filtered through 0.22 or 0.45 µm pore size filters ("Millipore Corporation", USA). Degassing of solvents was done under vacuum (equivalent to 20-

25" Hg). The mobile phase consisted of a mixture of Methanol (65%) and Water (35%) under isocratic conditions for analysis of **Taxanes**. The flow rate was 1.0 ml/min. and the run was continued for 30 minutes for complete resolution and detection of all the **Taxanes**. All the samples (extracts of callus tissues, cells from suspension cultures and solid as well as liquid spent media) were prepared and filtered as described in the preceding section **4.2.7** and 20 µL was used for injection. The standard solutions of **Taxol** and **10-DAB** were prepared in HPLC grade methanol at a strength of 0.5 mg/ml. These were also filtered before HPLC analysis and 20 µL was used for injection. Injection volume of 20 µL was kept constant during the experimentation. Spiking of the samples was accomplished by addition of authentic standard such that 2.5 µg of the standard was present in the spiked injection. Thus 10 µL from standard solution of a strength of 0.5 mg/ml was added during each injection.

Since there was no change in back-pressure after many injections, it was not necessary to develop any column regeneration procedures as has been described earlier by many authors (Kopycki et al., 1994 and references therein). The column was flushed after every injection by mobile phase for 10 min. to re-establish the initial conditions.

Plotting of graphs and calculations of amounts of **Taxanes** in the tissues by the external standard method of quantification were done using routine protocols as described in the "Instruction Manual for HSM Manager" as well as using the formula given by Scott (1996):

$$C_{p(s)} = \frac{a_{p(s)}}{a_{p(st)}} \times C_{p(st)}$$

where, $C_{p(s)}$ is the concentration of solute in the mixture.

$a_{p(s)}$ is the area of the peak for the solute (p) in the sample chromatogram.

$a_{p(st)}$ is the area of peak for solute (p) in the reference chromatogram.

$C_{p(st)}$ is the concentration of the standard in the reference solution.

The linearity of detector response for standards of **Taxol** and **10-DAB** was observed by injecting 20 µL from solutions of standards ranging in concentration from 0.625 µg-10 µg/ml (ElSohly et al., 1995) and plotting the standard curve (**Fig. 4.11 B, C**). Identification of **Taxol** and **10-DAB** in callus and cell samples was confirmed by

retention time, co-chromatography with the standard and peak purity by wavelength (Fett-neto et al., 1993a, b).

4.2.9 Statistical analysis

In experiments to optimize nutrient media for callus growth, each medium and plant growth regulator combination (experimental unit) consisted of 10 plastic dishes (replicate), each dish containing 15 needle pieces derived from a single parent tree. The experiments were repeated over four successive years for each of the trees. Fresh and dry weight determinations were performed with five callus pieces from a single plastic dish and the means with standard errors of means were taken for comparisons (Wardlaw, 1985).

Cell suspension culture experiments were carried out in optimized media and only three replicates were used for each experiment and each experiment was repeated thrice. The rate of cell suspensions growth was monitored by PCV at each subculture, for all the three replicate flasks and the mean PCV was used to determine the growth of cell suspensions. Fresh and dry weight determinations were performed with cell pellets derived on centrifugation of cells from three replicate flasks and the means were taken for comparisons.

The bark and needle tissues of the parental trees and the cultures derived from them were used for *in vivo* and *in vitro* tissue extractions for **Taxane** contents in duplicate and the whole procedure was repeated at least thrice.

TLC spottings and HPLC injections for each of the callus and cell lines and standards were done at least in duplicate and the injections were repeated on different days to check for the repeatability of the experimental procedure. Day to day variations, if any, were also checked with the standard solutions. The variations in peak areas from the respective experiments were tabulated and the mean and standard errors of means were calculated (Wardlaw, 1985).

Wherever the standard errors of means are less than 2% of the average mean values, they are not mentioned individually in the tables and only the mean values are represented (Ray and Jha, 1999).

4.3 Results and Discussion

4.3.1 Callus initiation and growth studies in stem and needle explants of *Taxus* spp.

During the initial experiments, all the cultures were lost due to heavy bacterial and fungal contamination and phenolic browning as the explants were derived from mature trees. In order to get sterile cultures, the explants (stem bits and needles as well as entire seeds with endosperm) were surface sterilized by the **"Modified Surface Sterilization Protocol"** presented in **Chapter 3, Flow Chart 3.3**. By use of this protocol, more than 90% sterile cultures were consistently obtained (**Table 3.13**).

In the present study, callus cultures were initiated on a large number of combinations of nutrient media and plant growth regulator combinations, from stem bit and needle explants of *Taxus* spp. These are referred to as **'Diploid Callus Cultures'** hereinafter. In addition, endosperm halves derived from seeds were also used for callus initiation, the details of which are described in **Chapter 5, Section 5.6.3**. These are referred to as **'Haploid Callus Cultures'** hereinafter. All **'Diploid Callus Cultures'** derived from different parent trees were used in all the experiments to optimize nutrient media which support better callus growth and all the cultures were maintained independently. For details of deriving callus cultures from endosperm refer to **Chapter 5, Part B.**

The main criterion for optimization of nutrient media was to select those media with low ionic strength so as to reduce further phenolic browning and to obtain good callus growth. On this basis, SH medium was used at 1/2 strength of major salts (1/2 SH) since the ionic strength of SH medium is quite high at 69.9 mM (C. f. **Chapter 2, Table 2.2**). In addition, low salt media such as B5, WPM and DCR at full strengths of major salts were also used (C. f. **Chapter 2, Table 2.2**). In the pilot experiments, these media were initially appended with 5 concentrations of 2,4-D (2, 4, 6, 8, 10 mg.l^{-1}) since it is the most commonly used auxin for callus induction. **Table 4.4** presents the results obtained in this experiment.

Table 4.4 Response of Stem and Needle Explants* for Callus Formation on Various Basal Media Combined with Different Concentrations of 2,4-D, after Two Months of Callus Growth.**

No.	Basal Medium	Plant Growth Regulator	Concentration ($mg.l^{-1}$)	Amount of Callus***	Nature of Callus
1.	1/2 SH	Absent (Control)	-	-	Only swelling of explants.
2.		2,4-D	2	++	Light yellowish green callus.
3.			4	++	
4.			6	+++	
5.			8	+++	
6.			10	+++	
7.	B5	Absent (Control)	-	-	Only swelling of explants.
8.		2,4-D	2	++	Callus showing different areas of white, yellow and brown colored cells.
9.			4	++	
10.			6	+++	
11.			8	+++	
12.			10	+++	
13	WPM	Absent (Control)	-	-	Only swelling of explants.
14.		2,4-D	2	++	Whitish, light brown callus.
15.			4	+++	Greenish, light brown callus.
16.			6	+++	
17.			8	+++	Light brown callus having white islands of new growth.
18.			10	+++	

Contd…..

Table 4.4 Contd.

No.	Basal Medium	Plant Growth Regulator	Concentration ($mg.l^{-1}$)	Amount of Callus*	Nature of Callus
19.	DCR	Absent (Control)	-	-	Only swelling of explants.
20.		2,4-D	2	++	Fast growing, light yellow to light brown colored callus.
21.			4	++	
22.			6	+++	
23.			8	+++	
24.			10	+++	

***:** The general trend of callus formation on different nutrient media with 2,4-D was the same for stem bit and needle explants. The frequency of callus formation was 20% in stem explants while in needle explants, the frequency of callus formation was 100%.

****:** Callus formation and growth in terms of callus fresh weight was scored at every monthly subculture. The standard errors of means for the fresh weight values were always less than 5 for all the experiments.

*****:** Amount of Callus = Callus fresh weight (FW) is symbolized as follows:

+ : Callus FW ~ 70-120 mg.
++ : Callus FW ~ 120-170 mg.
+++ : Callus FW ~ 170- 220 mg.
- : Absence of callus formation.

Callus induction occurred in 20-40 days on all the above-mentioned nutrient media with 2,4-D from both stem bit and needle explants. The stem bit explants were extremely poor in terms of callus induction in all the media combinations. Hardly 20% of stem bit explants produced calli within 20-40 days and out of these only 10% survived in subsequent subcultures. On the other hand, needle explants formed callus with 100% frequency within 20-40 days and most of these cultures (>95%) could be subcultured routinely.

It is clear from **Table 4.4** that all basal media with 6, 8 and 10 $mg.l^{-1}$ 2,4-D, gave good callus growth (FW in the range of 175-225 mg) irrespective of the source of explant (stem bits or needles). The calli were subcultured on fresh, identical media at monthly intervals after callus initiation. The differences in calli obtained on various basal media and 2,4-D combinations became apparent at this stage. Calli obtained on 1/2 SH, WPM and DCR media supplemented with 2,4-D could not be maintained beyond their fourth monthly subculture (140-180 days after callus initiation) and the callus survival deteriorated gradually from one passage to another. This was more pronounced in callus cultures obtained from stem bit explants. No new growth of cells was apparent on these calli and subsequently these calli became brown due to phenolics, became necrotic and died. The reason for non-survival of callus obtained from both the stem bit and needle explants beyond four subcultures on these media is unclear. Hence these media were not tried in further experiments with different plant growth regulators. On the contrary, B5 medium was found to be the best in terms of callus growth as well as callus survival over the monthly subcultures. Calli growing in B5 medium with 2,4-D and obtained from needle explants alone are still being maintained in our laboratory over the last 4 years. B5 medium was therefore chosen for further optimization of plant growth regulators. Preece (1995) observed that when the nutrient salts are optimized for *in vitro* tissues of a plant, the nutrient level and their balance in the medium makes tissues to be under less stress and *in vitro* performance gets dramatically improved. This is evident in our observation that callus cultures of *Taxus* spp. in B5 medium do not exude any phenolics.

Large scale studies on callus induction and growth, with the ultimate objective of their use in **Taxane** production, were therefore carried out with needle bit explants and all the subsequent data refers to needle derived calli.

B5 medium was further modified with individual additions of auxins like 2,4-D, IAA, IBA, NAA and P at various concentrations and combinations of IAA + IBA, IBA + 2-iP, 2,4-D + KIN and NAA + KIN (See **Table 4.5**) for callus induction from needle explants. Good callus growth and callus fresh weight (FW) in the range of 120-225 mg was obtained on B5 medium supplemented individually with 2,4-D or NAA, depending on its concentration. Callus growth and fresh weight of callus were at their best in B5 medium supplemented individually with 2, 4, 6, 8, 10 $mg.l^{-1}$ P. At the end of six months, the calli consistently weighed more than 250 mg (FW) and were very fast growing. B5 medium supplemented individually with IAA or IBA gave poor callus response. Hence initially IBA was combined with 2-iP to see if a combination of an auxin and a cytokinin could lead to good callus initiation and growth. But the results were not encouraging (**Table 4.5**). Hence IBA and 2-iP were not used in further experiments, in combination with other auxins/cytokinins. Instead, IAA and IBA were used together in concentrations ranging 0.5-10 $mg.l^{-1}$. These auxins in many combinations supported good callus growth and the fresh weights of calli ranged between 175-225 mg (**Table 4.5**). Growth stimulation at relatively high exogenous auxin concentrations has been reported earlier for other Gymnosperms like *Cephalotaxus harringtonia* (Delfel and Rothfus, 1977). KIN at low concentration of 0.2 $mg.l^{-1}$ alone was tried with 2,4-D and NAA independently as KIN is known to exert an inhibitory effect on callus growth of *Taxus* spp. at higher concentrations (Fett-Neto et al., 1993a). However no significant improvement in growth of calli was obtained in these combinations (**Table 4.5**). **Figures 4.6-4.9** represent the results obtained on callus induction and growth from needle explants on B5 medium and different plant growth regulator combinations.

In none of the cultures, presence of distinctly organized structures such as roots or shoots has been observed. However, 5% of callus cultures developed from needle explants on B5 medium supplemented with 6 $mg.l^{-1}$ 2,4-D and 0.2 $mg.l^{-1}$ KIN showed presence of root-like outgrowths (**Fig. 4.7 C**). But these did not develop further.

The calli were friable, white to light yellow to light yellow-green to light brown in color on all the growth media and plant growth regulator combinations tried (**Table 4.5**). None of the calli were hard, nodular or green in color.

Portions of calli showing active growth and friability were separated from the rest of the explant and used for subculturing of calli in their respective media combinations.

After transfer of such callus pieces to fresh media, the explants were reinoculated in their respective fresh growth media to produce another batch of callus formation while at the same time the spent media were pooledand used further for extraction of **Taxanes**.

Table 4.5 Response of Needle Explants for Callus Formation* on B5 Basal Medium Supplemented with Various Plant Growth Regulators Individually or in Combinations, after Six Months of Callus Growth.

No.	Plant Growth Regulator(S)	Concentration ($mg.l^{-1}$)	Amount of Callus**	Nature of Callus
1.	-	-	-	Only swollen explants.
2.	2,4-D	2	++	Callus showing different areas of white, yellow and brown colored cells.
3.		4	++	
4.		6	+++	
5.		8	+++	
6.		10	+++	
7.	2,4-D + KIN	2.0 + 0.2	++	Slow growing, light yellow callus.
8.		4.0 + 0.2	++	Light yellow to medium brown callus.
9.		6.0 + 0.2	+++	Light yellowish-brown callus.
10.		8.0 + 0.2	+++	Fast growing, light yellowish-greenish-brown callus.
11.		10.0 + 0.2	+++	Fast growing, light yellowish-greenish-brown callus.

Contd…..

Table 4.5 Contd.

No.	Plant Growth Regulator(S)	Concentration ($mg.l^{-1}$)	Amount of Callus*	Nature of Callus
12.	NAA	2.0	++	Callus showing different areas of white and brown colored cells.
13.		4.0	++	
14.		6.0	++	
15.		8.0	+++	
16.		10.0	+++	
17.	NAA + KIN	2.0 + 0.2	++	Yellowish-white friable callus with elongated cells.
18.		4.0 + 0.2	++	Yellowish-white friable callus with elongated cells.
19.		6.0 + 0.2	+++	Yellow moist callus.
20.		8.0 + 0.2	+++	Yellow moist callus.
21.		10.0 + 0.2	+++	Light brown, friable callus.
22.	IAA	2	-	Absence of callus formation.
23.		4	Less than 20 mg FW.	Very little callus just from the cut end of needles.
24.		6		
25.		8		
26.		10		
27.	IBA	2	-	Absence of callus formation.
28.		4	Less than 20 mg FW.	Very little callus just from the cut end of needles.
29.		6		
30.		8		
31.		10		

Contd…..

Table 4.5 Contd.

No.	Plant Growth Regulator(S)	Concentration ($mg.l^{-1}$)	Amount of Callus*	Nature of Callus
32.	2-iP + IBA	0.5 + 0.5	-	-
33.		0.5 + 1.0	+	Very slow growing light yellow callus.
34.		0.5 +3.0	+	Very slow growing light yellow callus.
35.		0.5 + 5.0	++	Light yellow callus.
36.		1.0 + 0.5	-	-
37.		1.0 + 1.0	+	Very slow growing yellow callus.
38.		1.0 + 3.0	+	Slow growing yellow callus.
39.		1.0 + 5.0	++	Yellow callus.
40.		3.0 + 0.5	++	Yellowish-brown callus.
41.		3.0 + 1.0	+	Light yellow, slow growing callus.
42.		3.0 + 3.0	+	White to light yellow callus.
43.		3.0 + 5.0	++	Light yellow callus
44.		5.0 + 0.5	+	Whitish yellow callus.
45.		5.0 + 1.0	+	Yellow-brown callus.
46.		5.0 + 3.0	++	Yellow-brown callus.
47.		5.0 + 5.0	++	Brown callus.

Contd…..

Table 4.5 Contd.

No.	Plant Growth Regulator(S)	Concentration ($mg.l^{-1}$)	Amount of Callus*	Nature of Callus
48.	IAA + IBA	0.5 + 10.0	++	White to light yellow to light brown, fast growing callus.
49.		1.0 + 10.0	++	White to light brown, fast growing callus.
50.		5.0 + 10.0	++	Light brown, fast growing callus.
51.		10.0 + 10.0	+++	Profuse callus showing white, yellow and brown colored cells.
52.		0.5 + 5.0	++	Fast growing callus showing white, yellow and brown colored cells.
53.		1.0 + 5.0	+++	Profuse callus showing white, yellow and brown colored cells.
54.		5.0 + 5.0	+++	Light yellow to dark brown, fast growing callus.
55.		10.0 + 5.0	+++	Light yellow to dark brown, fast growing callus.
56.		0.5 + 1.0	++	Light to dark brown, slow growing callus.
57.		1.0 + 1.0	++	Light to dark brown, slow growing callus.
58.		5.0 + 1.0	+++	Light to dark brown, faster growing callus.
59.		10.0 + 1.0	+++	Light to dark brown, faster growing callus.

Contd…..

Table 4.5 Contd.

No.	Plant Growth Regulator(S)	Concentration ($mg.l^{-1}$)	Amount of Callus*	Nature of Callus
60.	IAA + IBA	0.5 + 0.5	+	Brown callus.
61.		1.0 + 0.5	+	Brown callus.
62.		5.0 + 0.5	++	Whitish yellow callus.
63.		10.0 + 0.5	++	Light yellow to light brown callus.
64.	P	2.0	+++	Fast growing, light yellow to light brown colored callus.
65.		4.0	+++	
66.		6.0	+++	
67.		8.0	++++	
68.		10.0	++++	

*** :** Callus formation and growth in terms of callus fresh weight was scored at every monthly subculture. The standard errors of means for fresh weights were always less than 5 for all the experiments.

****: Amount of Callus = Callus mean fresh weight (FW) is symbolized as follows** :

+ : Callus FW ~ 70-120 mg.
++ : Callus FW ~ 120-175 mg.
+++ : Callus FW ~ 175- 225 mg.
++++ : Callus FW ~ 225-275 mg and more.
- : Absence of callus formation.

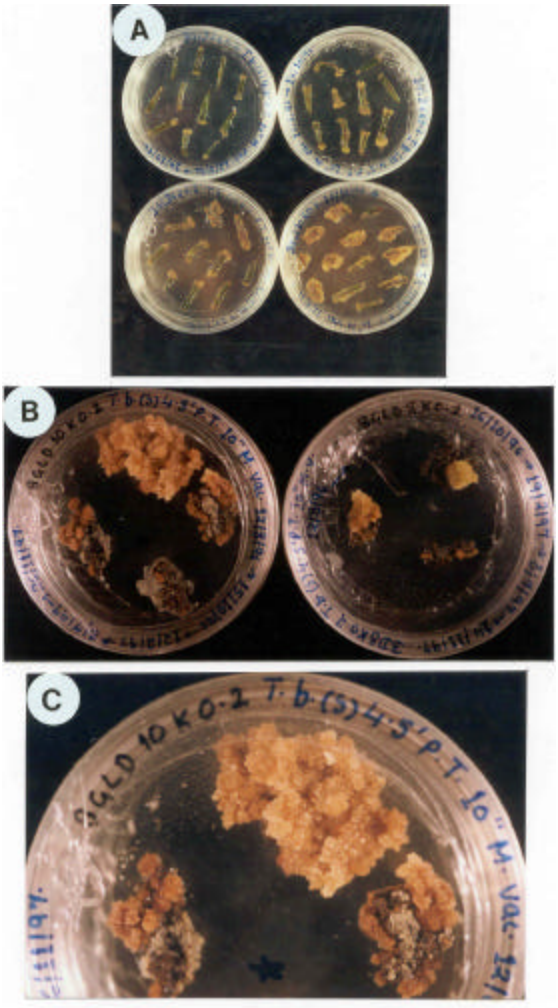


Fig. 4.6 Callus induction from needle explants of *Taxus baccata* ssp. *wallichiana* Zucc. Pilg. on B5 basal nutrient medium supplemented with various concentrations and combinations of KIN (0.2 mg.l^{-1}, constant) and 2,4-D (2, 4, 6, 8, 10 mg.l^{-1}).

A. All the needle explants showing light yellow colored callus induction in 30 days after inoculation. Note the differences in amount of callus based on growth media composition. Least amount of callus is present on 2 mg.l^{-1} 2,4-D and 0.2 mg.l^{-1} KIN and highest amount of callus is present on 10 mg.l^{-1} 2,4-D and 0.2 mg.l^{-1} KIN.

B. One year old, profuse needle derived calli growing on B5 medium supplemented with 8 mg.l^{-1} 2,4-D and 0.2 mg.l^{-1} KIN and 10 mg.l^{-1} 2,4-D and 0.2 mg.l^{-1} KIN.

C. A closer view of calli developed on B5 medium supplemented with 10 mg.l^{-1} 2,4-D and 0.2 mg.l^{-1} KIN. The new growth on callus is friable and light in color than older areas of calli.

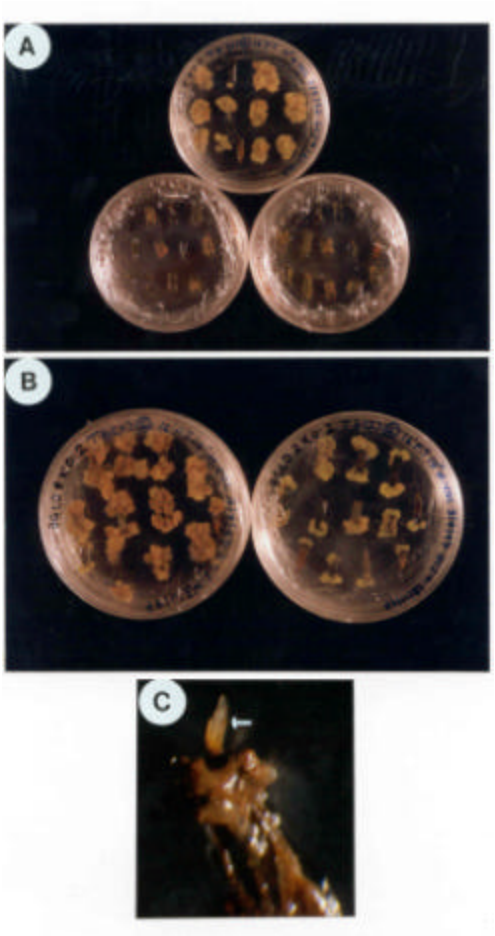


Fig. 4.7 Callus proliferation from needle explants of *Taxus baccata* ssp. *wallichiana* Zucc. Pilg. on B5 basal nutrient medium supplemented with various concentrations and combinations of KIN (0.2 mg.l^{-1}, constant) and 2,4-D (2, 4, 6, 8, 10 mg.l^{-1}).

A. & B. Profuse callus formation and proliferation in 60 days on B5 basal nutrient medium supplemented with various concentrations and combinations of KIN (0.2 mg.l^{-1}, constant) and 2,4-D (2, 4, 6, 8, 10 mg.l^{-1}). Note callus induction from cut edges of needle explants and eventual proliferation all over the needle explant. The callus is light yellow, friable and relatively fast growing.

C. A root like outgrowth from callus grown on B5 basal nutrient medium supplemented with KIN at 0.2 mg.l^{-1} and 2,4-D at 6 mg.l^{-1}.

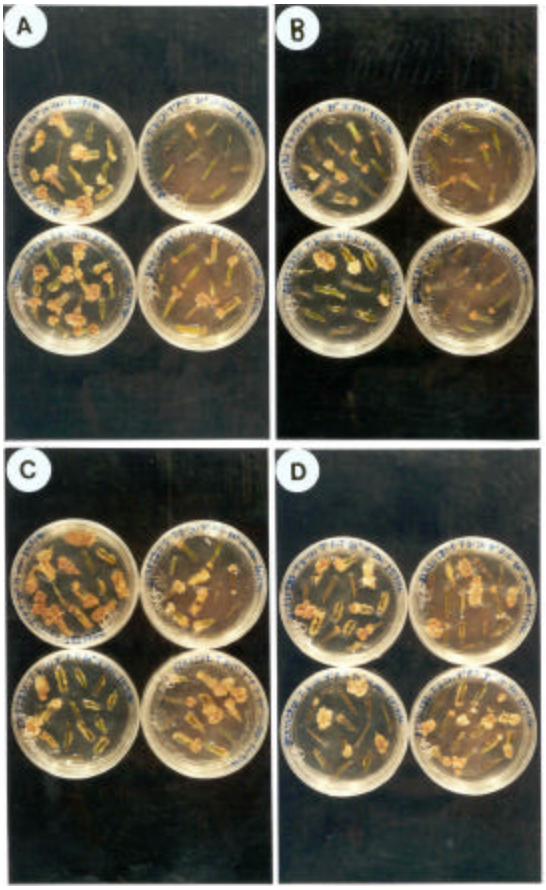
Fig. 4.8 Callus proliferation from needle explants of *Taxus baccata* ssp. *wallichiana* Zucc. Pilg. on B5 basal nutrient medium supplemented with various concentrations and combinations of IAA and IBA (30 days old cultures).

A. Induction of light yellow colored callus on B5 medium supplemented with 0.5 $mg.l^{-1}$ IAA (Constant) supplemented with 0.5, 1.0, 5.0 and 10.0 $mg.l^{1}$ IBA.

B. Induction of light yellow colored callus on B5 medium supplemented with 1.0 $mg.l^{-1}$ IAA (Constant) supplemented with 0.5, 1.0, 5.0 and 10.0 $mg.l^{1}$ IBA.

C. Induction of light yellow to light brown colored callus on B5 medium supplemented with 5.0 $mg.l^{-1}$ IAA (Constant) supplemented with 0.5, 1.0, 5.0 and 10.0 $mg.l^{1}$ IBA.

D. Induction of light yellow colored callus on B5 medium supplemented with 10.0 $mg.l^{-1}$ IAA (Constant) supplemented with 0.5, 1.0, 5.0 and 10.0 $mg.l^{1}$ IBA.



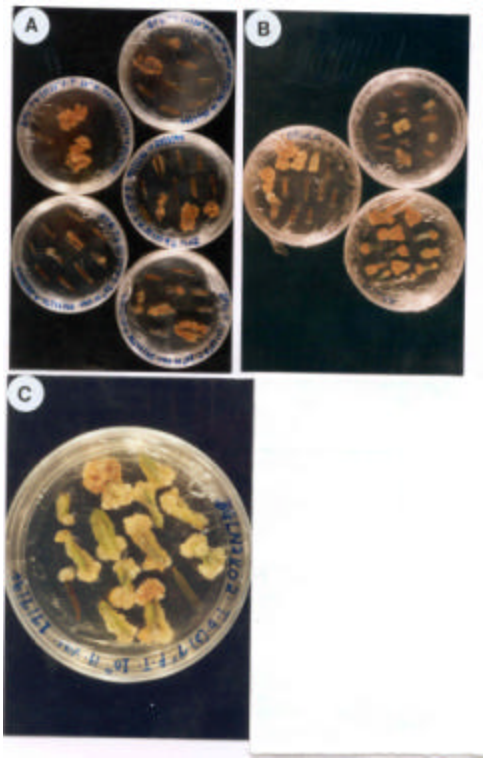


Fig. 4.9 Callus induction and proliferation from needle explants of *Taxus baccata* ssp. *wallichiana* Zucc. Pilg. on B5 basal nutrient medium supplemented with various concentrations and combinations of a number of plant growth regulators (30-60 days old cultures).

A. Callus induction on B5 medium supplemented with 2, 4, 6, 8 and 10 mg.l^{-1} Picloram (P). The 30 day old callus is light yellowish brown in color.

B. Further growth of callus on B5 medium supplemented with 2 and 6 mg.l^{-1} P. The light yellow callus is 60 days old.

C. Profuse greenish yellow callus developed on B5 medium supplemented with 2 mg.l^{1} NAA and 0.2 mg.l^{1} KIN. The callus is 55 days old.

A graphical representation of growth of 15 callus lines, starting from initiation of callus on optimized media (B5 medium supplemented with 2, 4, 6, 8 and 10 mg.l^{-1} P, referred to in the figure as BP2, BP4, BP6, BP8 and BP10) over a period of three months, in three monthly transfers is presented in **Fig. 4.10**. Callus initiated in B5 medium supplemented with 2 mg.l^{-1} P had a high initial FW of 67 mg but it did not increase significantly during the next two transfers of one month duration each. Calli initiated on B5 medium supplemented with 4, 6 and 8 mg.l^{-1} P did not have initial high FW and the growth rate was not very fast in the next two monthly transfers except for calli growing on B5 medium supplemented with 10 mg.l^{-1} P. The callus lines on B5 medium supplemented with 10 mg.l^{-1} P (Line Nos. 11, 12, 13, 14 and 15) had an initial FW ranging from 11-35 mg that increased to 21-49 mg in the third month of culture. Such a comparatively faster growth rate of callus in the first three months with high concentrations of P is similar to observations reported earlier (Gibson et al., 1995 and references therein). The rate of callus growth increased further during the next three months of culture and the final weights obtained at the end of six months are mentioned in **Table 4.5**.

4.3.1.1 Repeatability and Applicability of Optimized Callus Induction Medium for Callus Induction from Plant Material Collected from Khajjiyar and Almora.

The optimized medium B5 supplemented with various concentrations of P was also used for callus induction from needle explants of plant material collected from Khajjiyar and Almora. Here also 100% needle explants formed calli but with lower fresh weights than calli derived from needles of plant material collected from Shimla (**Table 4.5**). However the calli grew well and could be maintained by regular monthly subcultures. The results are presented in **Table 4.6**.

Table 4.6 Response of Needle Explants for Callus Formation* on B5 Basal Medium Supplemented with Various Concentrations of Picloram (P), after Six Months of Callus Growth.

No.	Location	Plant Growth Regulator(S)	Concentration ($mg.l^{-1}$)	Amount of Callus*	Nature of Callus
1.	Khajjiyar	Picloram (P)	2	++	Light yellow colored callus.
2.			4	++	
3.			6	++	
4.			8	++	
5.			10	++	
6.	Almora	Picloram (P)	2	+++	Light yellow colored callus.
7.			4	+++	
8.			6	+++	
9.			8	+++	
10.			10	+++	

*** :** Callus formation and growth in terms of callus fresh weight was scored at every monthly subculture. The standard errors of means were always less than 5 and hence only the mean fresh weights are mentioned everywhere.

****: Amount of Callus = Callus mean fresh weight (FW) is symbolized as follows** :
++ : Callus FW ~ 120-175 mg.
+++ : Callus FW ~ 175- 225 mg.

It is clear from Table 4.6 that needles collected from Almora showed better callus formation in all the concentrations of P than needles collected from Khajjiyar during six months of callus growth. In addition, the fresh weights of calli remained more or less constant in the range of 175-225 mg. Unfortunately, due to paucity of time, Taxane analysis of these cultures could not be carried out but they are being maintained in the laboratory.

4.3.2 Extraction and Analysis of Taxanes in *in vivo* and *in vitro* tissues of *Taxus* spp

Methanol was chosen for primary extraction of **Taxanes** because it has been shown to give high yields of polar compounds like **10-DAB** and related structures as compared to extractions with solvents like chloroform (Theodoridis et al., 1998). After primary extraction, liquid-liquid extraction was performed using chloroform and water which has been shown to recover 95-100% of **Taxol** and **Baccatin-III** and 70-90% of **10-DAB-III** (Cass et al., 1997).

Since the **Taxanes** display a wide range of polarity and molecular size, their separation upto baseline in HPLC needs to be properly adjusted. A number of reports have appeared in literature using a variety of columns and solvent systems for HPLC analysis of **Taxanes** and it is not possible and pertinent to review all of those with respect to present work. The present system of reverse phase HPLC analysis using methanol and water was chosen because both the **Taxanes** of interest namely **Taxol** and **10-DAB** were baseline separated and the run time required was only 30 min. Methanol has a high solvent strength parameter $\varepsilon^{o} = 0.95$ and hence increased the solubility of hydrophobic **Taxanes** (Kopycki et al., 1994).

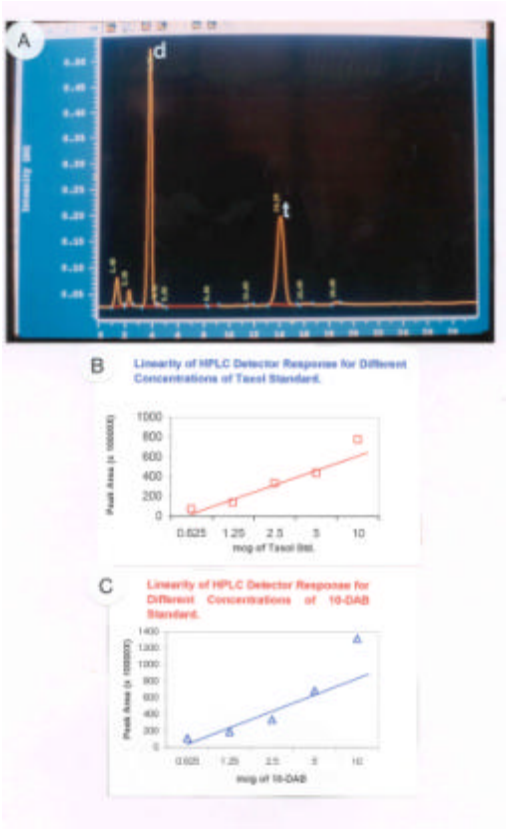
The HPLC run of standard solutions of **Taxol** and **10-DAB** was performed under identical conditions to those employed for analysis of samples. **Fig. 4.11 A** shows the standard chromatogram for **10-DAB** (d) and **Taxol** (t) while **Fig. 4.11 B** and **C** show the linearity of UV-VIS detector response for concentrations of standards ranging from 0.625 5.0 µg.

Fig. 4.11

A. HPLC chromatogram of standards of 10-DAB (d) and Taxol (t). It shows two distinct, baseline-resolved peaks at retention times of 3.86 min. and 14.10 min. respectively corresponding to 10-DAB and Taxol.

B. A graph showing linearity of HPLC UV detector response for various concentrations of Taxol standard. The graph is plotted as peak area versus mcg of Taxol standard. Note that the detector response is linear for all the concentrations of Taxol standard tried.

C. A graph showing linearity of HPLC UV detector response for various concentrations of 10-DAB standard. The graph is plotted as peak area versus mcg of 10-DAB standard. Note that the detector response is linear for all the concentrations of 10-DAB standard tried.



4.3.2.1 Extraction and Analysis of Taxanes in *in vivo* tissues of *Taxus* spp.

The **Taxane** content of pooled bark and needle samples from all the six chosen trees from Shimla, are as presented in **Table 4.6**. As reported by earlier authors (Denis et al., 1988), the bark contained higher amounts of **10-DAB** than **Taxol** and **Taxol** content was significantly higher than that of needles. Surprisingly the needles did not contain any **10-DAB** and had very small amount of **Taxol**. Chattopadhyay et al. (1994) reported that out of *Taxus* spp. plant material collected from Assam, Arunachal Pradesh, Uttar Pradesh and Himachal Pradesh, only the needles of the material collected from Himachal Pradesh had **Taxol** and **10-DAB** while plant material from other locations contained only **10-DAB**. However, the authors have not reported quantitative amounts of the **Taxanes** in their publication and thus limits comparison of their data with the quantitative data obtained here.

Chattopadhyay et al. (1997) also reported that the heartwood of the Himalayan Yew, *T. wallichiana* showed a number of unique **Taxanes** besides **Taxol** and **10-DAB,** and is similar to the results obtained in the present study. This can be seen from the chromatogram in **Fig. 4.12 A** wherein a number of peaks besides **Taxol** and **10-DAB** could be seen (green curve in the chromatogram) but were not identified as standards were not available.

Table 4.6 Amounts of Taxanes in *in vivo* tissues of *Taxus* spp.

Tissues	Amount of Taxanes (mg.g^{-1} DW) Mean ± S. E.	
	Taxol	**10-DAB**
Needles	000.0941 + 0.08	-
Bark	134.0600 ± 3.20	1495.0 ± 24.7

-: Absence of **10-DAB**.

4.3.2.2 Extraction and analysis of Taxanes in selected callus lines of *Taxus* spp.

For extractions, methanol was the preferred solvent due to reasons outlined above and additional extractions with hexane or other apolar solvents was not necessary to remove lipophilic compounds (Theodoridis et al., 1998). This was proven during the

HPLC analysis because there was no significant increase in back-pressure of the column and the chromatograms sometimes showed only an initial broad peak of polar impurities which did not interfere with the **Taxane** analysis. For any successful analysis of plant secondary metabolism, it is necessary to have a separation technique providing efficient separation of metabolites as well as good selectivity and sensitivity of detection and capacity to provide important structural information on-line. TLC and HPLC are the most commonly used analytical techniques. These techniques were used for callus cultures raised on defined media to identify the **Taxane** hyper-producer lines which could be further used for preliminary studies in raising cell suspension cultures.

4.3.2.2.1 Use of TLC in Taxane analysis

In the present work, TLC was used as a preliminary screen to check for the presence of **Taxol** in callus lines raised on B5 media supplemented with various plant growth regulators. In all, 58 diploid callus lines and 10 haploid callus lines were screened by TLC. Out of these, 20 diploid callus cultures and 5 haploid callus cultures showed spots having identical R_f value of 0.67 to the standard of **Taxol**. Only these promising callus lines derived from needles (diploid) and endosperm (haploid) were extracted on a larger scale (C. f. **Section 4.2.7.1**) and used for HPLC analysis (C. f. **Table 4.8**). In addition, one **Taxane** non-producer callus (based on negative TLC results) growing on B5 medium supplemented with 10 $mg.l^{-1}$ P (Callus Line No. 5 in **Table 4.8**) and chosen at random was also analyzed by HPLC. The spots of **Taxol** were visualized by spraying the TLC plate with H_2SO_4 and then heating it. This is a general method followed for visualization of secondary metabolites. Visualization under UV light also showed the fluorescing spots with R_f of 0.67. But some additional spots in the vicinity were also visible on the plates, which might represent other **Taxanes** with similar structure to **Taxol**.

4.3.2.2.2 Use of HPLC in Taxane analysis

HPLC is the most appropriate technique for an efficient separation of crude plant extracts and can be coupled with different spectroscopic detection methods (Hostettmann and Wolfender, 1997). In the present endeavor, HPLC coupled with UV photodiode array detection was used for the UV spectra of natural compounds providing useful information about the type of constituents and their structures (Hostettmann and Wolfender, 1997).

The TLC positive needle-derived diploid calli (Callus Line Nos. 1-13 of different ages), a TLC negative diploid Callus Line No. 5 and the endosperm-derived haploid Callus Line Nos. 1-5 (C. f. **Tables 4.8 and 4.9**), growing on B5 or DCR media respectively, supplemented with various plant growth regulators were used further for quantitative **Taxane** analysis by HPLC. The quantitative amounts of **Taxanes** in all these different callus lines analyzed by HPLC are presented in **Tables 4.8 and 4.9**. **Figs. 4.12-4.14** represent the chromatograms of various callus lines.

While carrying out the HPLC analysis of cell and callus cultures of *Taxus* spp., everyday at least two independent injections of the samples were performed and an average of the peak areas was calculated. The standard error of the mean (S. E.) obtained was always less than 6.0. The standard and sample injections were repeated on successive days and again the S. E. for the peak areas of **10-DAB** and **Taxol** were respectively 6.86 and 3.89. S. E. for peak areas of samples were between 3 and 10.

HPLC analysis of TLC positive Needle-derived calli for Taxane production

The TLC positive, needle-derived callus cultures (except Callus Line No. 5 which is TLC negative) growing on B5 medium supplemented with P had relatively faster growth (Callus Line Nos. 1-4 of different ages, **Table 4.8**) compared to callus cultures growing on B5 medium supplemented with 2,4-D + KIN (Callus Line Nos. 10-13, **Table 4.8**) and NAA + KIN (Callus Line Nos. 6-9, **Table 4.8**). The TLC positive, endosperm-derived callus cultures (**Table 4.9**) on DCR medium supplemented with P grew faster as compared to those on B5 medium supplemented with P (**Table 5.14**). The details pertaining to needle and endosperm derived callus culture initiation, growth and nature of callus are described in **Tables 4.5 and 5.14**. By comparison of **Tables 4.5, 5.14** and **4.8** it is clear that higher **Taxane** contents were detected in relatively faster growing callus cultures. This will become evident in the following discussion.

It is clear from **Table 4.8** that B5 medium supplemented with 0.2 $mg.l^{-1}$ KIN along with 2,4-D concentrations of 2, 4, 6 and 8 (Referred to as BD2K0.2-BD8K0.2, Callus Line Nos. 10-13) and B5 medium supplemented with 0.2 $mg.l^{-1}$ KIN along with NAA concentrations of 2, 4, 6 and 8 (Referred to as BN2K0.2-BN8K0.2, Callus Line Nos. 6-9) did not produce detectable levels of **Taxanes** in needle-derived calli. This was true for three year old calli of Callus Line Nos. 6-9 and 10-13.

On the other hand, B5 medium supplemented with various concentrations of P (Referred to as BP, Callus Line Nos. 1-4 of different ages) showed appreciable amounts of **Taxanes**. In addition, there is a general trend in increase of **Taxol** and **10-DAB** in all callus lines maintained at different concentrations of P with the age of the callus line from 3 months to 2 years with minor deviations in the trend. For example, the amount of **10-DAB** in 3 month old callus growing on medium BP8 was 138.10 µg/g DW of callus. This amount increased to 2585 µg **10-DAB**/g DW of callus in 1 year old callus and then decreased to 2282 µg **10-DAB**/g DW of callus in the second year (**Table 4.8**). In contrast, the amount of **Taxol** was 76.0 µg/g DW of callus in 3 month old callus growing on medium BP6. This amount decreased to 60.4 µg **Taxol**/g DW of callus in 1 year old callus and then increased to 540.0 µg **Taxol**/g DW of callus in the second year (**Table 4.8**). This pattern of **Taxane** content observed with our cultures is in contrast to earlier reports where P induced good callus growth but inhibited **Taxane** production (Shuler, 1994 and references therein; Gibson et al., 1995 and references therein). Also in contrast to earlier reports (Shuler, 1994 and references therein), both faster growth of callus and high **Taxane** production was achieved here in the same medium.

2 year old callus growing on BP6 medium (B5 medium with 6 $mg.l^{-1}$ P) produced the maximum level of **Taxol** (540 µg/g DW of callus) which is ~ 6000 times more than in the *in vivo* needles. Concomitantly it had intermediate contents of **10-DAB** at 1836 µg/g DW of callus. Also another callus of the same age growing on BP4 medium (B5 medium with 4 $mg.l^{-1}$ P) had 517 µg **Taxol**/g DW of callus. Yet another callus of the same age growing on BP8 medium (B5 medium with 8 $mg.l^{-1}$ P) had 344 µg **Taxol**/g DW of callus and **10-DAB** content at 2282 µg/g DW of callus. Surprisingly, callus of the same age growing on BP10 medium (B5 medium with 10 $mg.l^{-1}$ P) had no detectable levels of **Taxol** and **10-DAB**. A relatively fast rate of growth of this callus compared to other callus lines might be the reason for absence of **Taxanes** in the callus (Also see **Fig. 4.10**).

1 year old callus growing on BP8 medium produced a maximum level of **10-DAB** (2585 µg/g DW of callus). This is surprising in the light of the fact that **10-DAB** is absent in *in vivo* needles. However **Taxol** was absent in this callus line although **Taxol** was detected in needles *in vivo*.

The pattern of **10-DAB** and **Taxol** accumulation reported here in callus cultures *in vitro* is the actual picture present in the parent tree(s) or is a manifestation of the culture conditions needs further study and elucidation.

This is the first report of production of Taxol and 10-DAB in *in vitro* cultures of the Himalayan Yew. An earlier report by Banerjee et al. (1996) reported presence of only minor taxoids in the *in vitro* cultures of the Himalayan Yew.

Table 4.8 Amount of Taxanes in Needle -derived Callus Cultures of Selected Lines of *Taxus* spp. with Age of Callus Lines on Different Basal Media and Plant Growth Regulator Combinations.

Serial No.	Callus Line No.	Medium	Age of Callus Line	Amount of Taxol (µg/g DW of callus)	Amount of 10-DAB (µg/g DW of callus)
1.	1.	BP2	2 years	200.00	60.70
2.	2.	BP4		517.00	95.70
3.	3.	BP6		540.00	1836.00
4.	4.	BP8		344.00	2282.00
5.	5.	BP10		-	-
6.	1.	BP2	1 year	-	98.39
7.	2.	BP4		-	-
8.	3.	BP6		60.4	453.00
9.	4.	BP8		-	2585.00
10.	5.	BP10		-	-
11.	1.	BP2	3 months	ND	ND
12.	2.	BP4		ND	ND
13.	3.	BP6		76.00	-
14.	4.	BP8		245.02	138.10
15.	5.	BP10		-	-
16.	6.	BN2K0.2	3 years	Traces, < 2	Traces, < 2
17.	7.	BN4K0.2			
18.	8.	BN6K0.2			
19.	9.	BN8K0.2			
20.	10.	BD2K0.2	3 years	Traces, < 2	Traces, < 2
21.	11.	BD4K0.2			
22.	12.	BD6K0.2			
23.	13.	BD8K0.2			

ND: Not done.
- : Absence of **Taxanes**.

Fig. 4.12

A. HPLC chromatogram showing presence of 10-DAB (d) and Taxol (T) in pooled bark sample from trees of Shimla of *Taxus* spp. The blue colored graph shows the chromatogram for the standards of 10-DAB (d) and Taxol (t) while the green colored graph represents the spiked extract of bark sample showing presence of both the d and t peaks.

B. HPLC chromatogram showing presence of 10- DAB (d) and Taxol (t) in callus cultures of *Taxus* spp. growing for 2 years in B5 medium supplemented with 2 mg.l^{-1} P (BP2). The blue colored graph shows the chromatogram for the standards of 10-DAB (d) and Taxol (t) while the green colored graph represents the spiked extract of BP2 callus cultures showing presence of both the d and t peaks.

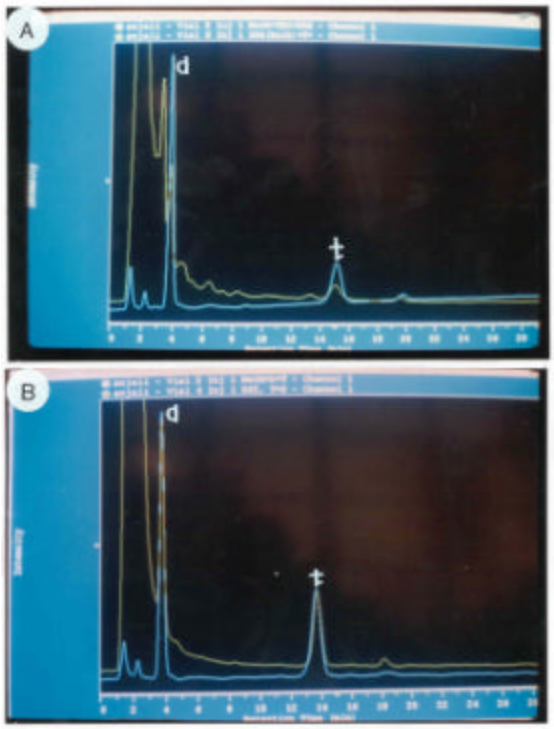
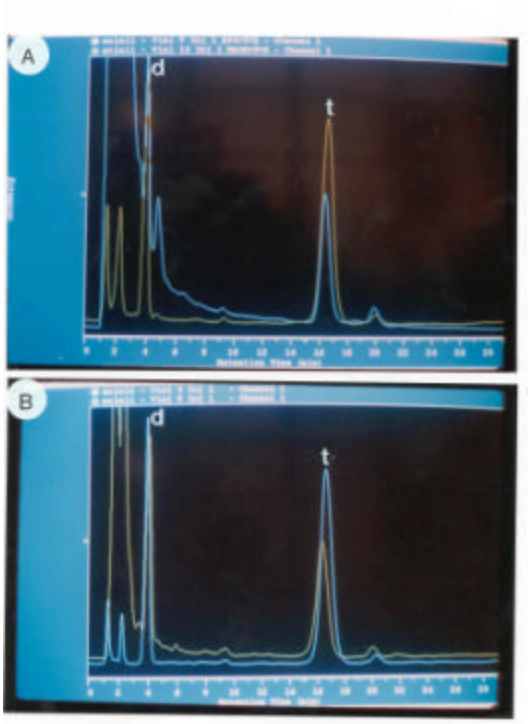


Fig. 4.13

A. HPLC chromatogram showing presence of 10-DAB (d) and Taxol (T) in callus cultures of *Taxus* spp. growing for 2 years in B5 medium supplemented with 4 mg.l^{-1} P (BP4). The green colored graph shows the chromatogram for the standards of 10-DAB (d) and Taxol (t) while the blue colored graph represents the spiked extract of BP4 callus cultures showing presence of both the d and t peaks.

B. HPLC chromatogram showing presence of 10- DAB (d) and Taxol (t) in callus cultures of *Taxus* spp. growing for 2 years in B5 medium supplemented with 8 mg.l^{-1} P (BP8). The green colored graph shows the chromatogram for the standards of 10-DAB (d) and Taxol (t) while the blue colored graph represents the spiked extract of BP8 callus cultures showing presence of both the d and t peaks.



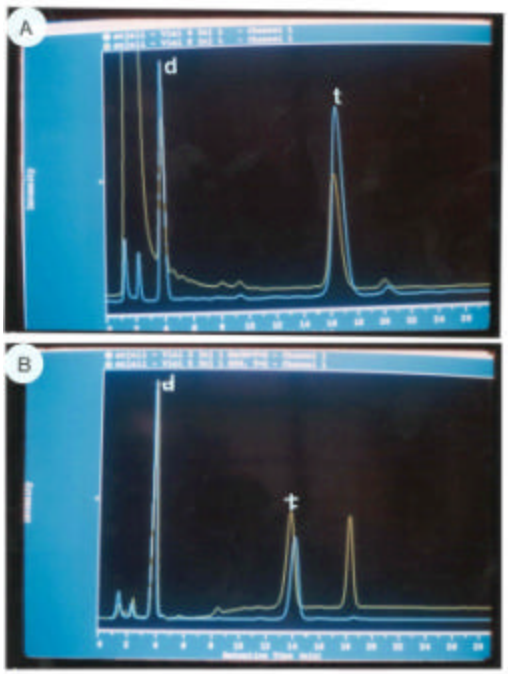


Fig. 4.14

A. HPLC chromatogram showing presence of 10-DAB (d) and Taxol (T) in callus cultures of *Taxus* spp. growing for 2 years in B5 medium supplemented with 6 $mg.l^{-1}$ P (BP6). The blue colored graph shows the chromatogram for the standards of 10-DAB (d) and Taxol (t) while the green colored graph represents the spiked extract of BP6 callus cultures (Serial No. 3) showing presence of both the d and t peaks.

B. HPLC chromatogram showing presence of 10- DAB (d) and Taxol (t) in callus cultures of *Taxus* spp. growing for 1 year in B5 medium supplemented with 6 $mg.l^{-1}$ P (BP6). The blue colored graph shows the chromatogram for the standards of 10-DAB (d) and Taxol (t) while the green colored graph represents the spiked extract of BP6 callus cultures (Serial No. 8) showing presence of both the d and t peaks. Also not an extra peak eluting after Taxol. Its identity is not known.

HPLC analysis of TLC positive Endosperm-derived callus cultures and culture medium for Taxane production.

External standard method of quantification gave following values of **Taxane** contents for selected endosperm-derived callus lines (found to be positive for **Taxanes** in TLC) as shown in **Table 4.9**. Even though the growth of endosperm-derived callus lines was equally fast in both B5 and DCR media supplemented with various concentrations of P, calli growing on B5 medium supplemented with various concentrations of P did not show presence of **Taxanes** in TLC. On the other hand, calli growing on DCR medium supplemented with various concentrations of P showed presence of **Taxanes** in TLC. Hence only the latter calli were quantitatively analyzed by HPLC. These calli were either white or yellow in color, moist and fast-growing with high FW values. For details on the characterization of these lines please refer to **Chapter 5, Section 5.7.3 and Table 5.14)**. **Taxol** content was very low in all of these callus lines. **10-DAB** content was in traces in callus lines grown on DP2, DP4 and DP 10 media (DCR medium supplemented with 2, 4 and 10 $mg.l^{-1}$ P respectively). But callus grown on DP6 medium (DCR medium supplemented with 6 $mg.l^{-1}$ P) showed **10-DAB** content of 203 µg/g DW of callus while callus grown on DP8 medium (DCR medium supplemented with 8 $mg.l^{-1}$ P) had **10-DAB** content of 89.7 µg/g DW of callus.

This is the first report of detection of Taxanes (10-DAB in particular) in appreciable quantities in haploid endosperm-derived callus cultures of *Taxus* spp.

Table 4.9 Amounts of Taxanes in Selected Callus Lines (Age: 6-12 months) of Endosperm Calli from Almora.

Callus Line No.	Callus Line	Taxol Content (mg/g DW)	10-DAB Content (mg/g DW)
1.	DP2	Trace (<2)	Trace (<2)
2.	DP4	Trace (<2)	Trace (<2)
3.	DP6	-	203
4.	DP8	2.27	89.7
5.	DP10	Trace (<2)	Trace (<2)

-: Absence of **Taxol**.

The culture media of these Callus Line Nos. 1-5 of **Table 4.9** showed presence of some exudates and hence were also extracted to see if the **Taxanes** were exuded from the callus into the medium. These results are presented in **Table 4.10**.

Table 4.10 Amounts of Taxanes in Medium Extracts from Selected Endosperm Callus Lines (Age: 6-12 months) from Almora (C. f. Table 4.9).

Serial No.	Callus Medium	Taxol Content (mg/g FW)	10-DAB Content (mg/g FW)
1.	DP2	Trace (<2)	Trace (<2)
2.	DP4	-	13.96
3.	DP6	-	1.11
4.	DP8	Trace (<2)	Trace (<2)
5.	DP10	Trace (<2)	Trace (<2)

-: Absence of **Taxanes**.

Analysis of the spent solid media for the presence of **Taxanes** showed traces of **Taxol** and **10-DAB** in DP2, DP8 and DP10 media. DP6 medium showed 1.11 µg **10-DAB**/g FW of extracted medium while DP4 medium showed 13.96 µg **10-DAB**/g FW of extracted medium Thus these **Taxanes** were not released into the medium in significant amounts as per observations during the course of the present work, even though earlier reports have described release of **Taxanes** in the growth media (Jaziri et al., 1996 and references therein; **Table 4.3** of the present thesis). On the other hand, Wickremesinhe and Arteca (1994) had reported that less than 10% of total **Taxanes** were detected in the culture medium and 90% remain sequestered within the cells. Our cultures also show a similar behavior in the sense that majority of the **Taxanes** remained sequestered inside the cells while very low amounts were into the nutrient medium.

4.3.3 Initiation and growth determinations in suspension cell cultures

In the course of the present work, only preliminary studies with cell suspension cultures were carried out to see if some of the callus lines obtained during the course of this work are amenable for liquid culture and to see if the **Taxane** contents could be increased by cell suspension culture.

Cell suspension cultures were initiated as described earlier in **Section 4.2.3.2** only on B5 medium supplemented with 2-10 mg.l^{-1} P (Optimized medium for best growth and **Taxane** content) by random selection of calli (Serial Nos. 8, 9, 14 and a non-producer callus with Serial No. 5 of **Table 4.8**). **Fig. 4.15** shows microscopic view of cell suspension grown in BP6 and BP8 media (Cell Suspension Line Nos. 1 and 2).

Dry weight (DW) analyses of cell cultures were done at the time of extraction for HPLC analysis. **Fig. 4.16** is a representative graph showing changes in DW with time in selected cell suspension cultures. It is clear from the graph that except for the cell suspension grown in BP10 medium, all the other three cell suspensions showed the typical growth curve of a liquid cell suspension culture. The lag phase of growth was over by day 9 when the cells entered the log phase of growth and even at day 18 this growth was not declining towards stationary phase. On the contrary, cell suspension grown in BP10 medium exhibited a relatively flat pattern of growth.

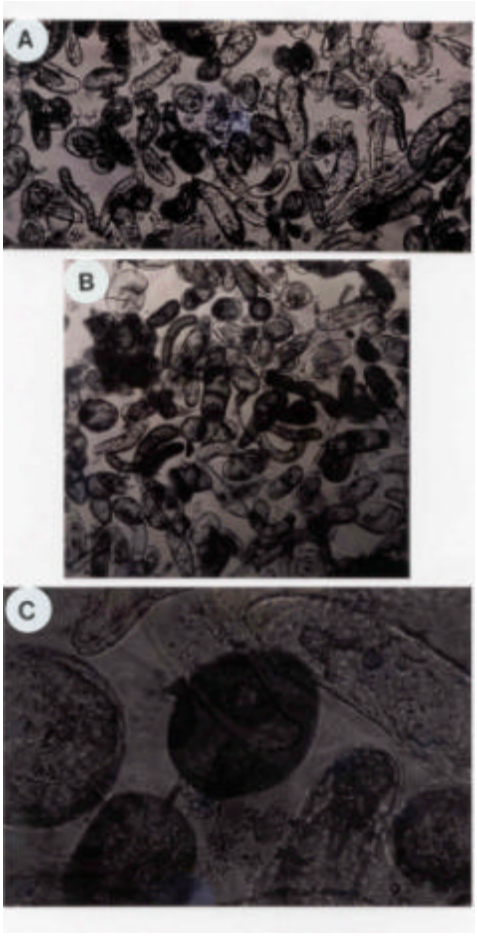


Fig. 4.15 Microscopic observation of cells from suspension cultures of callus lines of *Taxus* spp. Magnification is described in the bracket.

A. Suspension culture derived from callus cultures growing in B5 medium supplemented with 6 $mg.l^{-1}$ P (Suspension Line No. 1) showing globular and elongated cells. Both the cell types are rich in cytoplasm (40x).

B. Suspension culture derived from callus cultures growing in B5 medium supplemented with 8 $mg.l^{-1}$ P (Suspension Line No. 2) showing globular and elongated cells. Both the cell types are rich in cytoplasm. Here there are relatively more number of elongated cells (40x).

C. A magnified view of suspension cultured cells. Some fluorescent/translucent inclusions are seen in the cytoplasm of both the types of cells (100x).

4.3.4 Analysis of Taxanes in selected cell suspension cultures of *Taxus* spp.

Table 4.11 depicts the results of HPLC obtained with cell suspension cultures derived from some of the randomly selected callus lines. The suspension cultures initiated from these callus lines are numbered as in **Table 4.11**. A dramatic increase (almost 10 times) in **Taxol** content was evident in Cell Suspension Line No. 1 from day 0 to day 9 and then its total disappearance at day 18. **10-DAB** content decreased steadily from day 0 to day 18 in this line. **Fig. 4.17 B** represents the chromatogram for BP6 cell suspension culture (Cell Suspension Line No. 1) derived from Callus Line with Serial No. 8 in **Table 4.8** while **Fig. 4.17 A** represents the chromatogram of the standard solutions of **10-DAB** and **Taxol**. Cell Suspension Line No. 2 showed an abrupt decrease in **Taxol** and **10-DAB** contents from day 0 to day 9 itself in liquid medium. Cell Suspension Line No. 3 initially had undetectable levels of **Taxol** but contained very high levels of **10-DAB**. Over the culture period of 18 days, **10-DAB** content decreased rapidly and steadily while **Taxol** contents increased concomitantly. In contrast, the most surprising observation was that **the Taxane non-producer Cell Suspension Line No. 4 (TLC and HPLC results were negative for parent callus culture), exhibited presence of both Taxol and 10-DAB in appreciable quantities in suspension culture and they were still on the upward spiral even at day 18.** More attention is required in continuing these cell suspension lines in culture beyond 18 days to elucidate the pattern of **Taxane** production versus the age of cell suspension culture.

The spent liquid media analyzed by HPLC showed extremely low amounts of **Taxanes** (< 2 µg/ml liquid medium) (**Table 4.11**). This proves that all the **Taxanes** are sequestered within the cells. One possible reason is that since **Taxol** is a lipophilic compound, it is not getting excreted in the aqueous extracellular medium. Russin et al. (1995) have shown by immunohistochemistry that **Taxol** is localized in cell walls of xylem, phloem and vascular cambium and hence cannot be easily released into the medium.

Table 4.11 Content of Taxanes in Cell Suspension Cultures at 0, 9 and 18 Days of Their Growth in Liquid Medium.

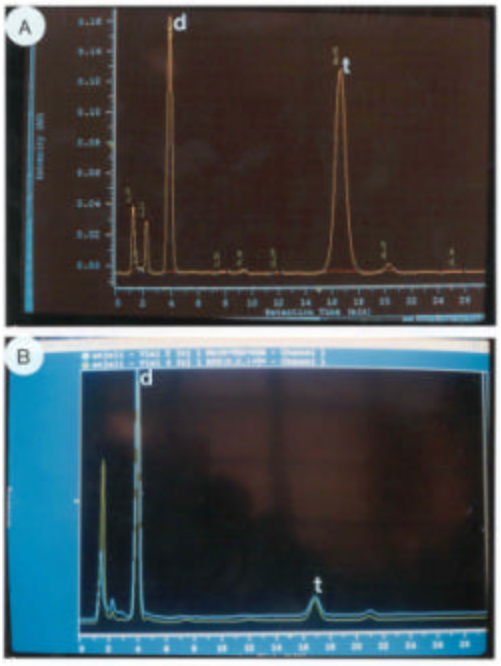
Cell Suspension Lines Raised from	Cell Suspension Line No.	Days of Growth	Amount of Taxol (µg/g DW of cells)	Amount of 10-DAB (µg/g DW of cells)
BP6 (Callus Line with Serial No. 8 in **Table 4.8**)	1.	0**	60.40	453.00
		9	594.90	255.10
		18	-	86.60
BP 8 (Callus Line with Serial No. 14 in **Table 4.8**)	2.	0**	245.02	138.10
		9	50.00	-
		18	ND	ND
BP8 (Callus Line with Serial No. 9 in **Table 4.8**)	3.	0**	-	2585
		9	ND	ND
		18	1040	-
BP10 (Callus Line with Serial No. 5 in **Table 4.8**)	4.	0**	-	-
		9	< 2	240.00
		18	147.52	311.36
*	**Spent media analyzed from all of these cell suspensions, over the 18 day growth period showed <2 µg Taxanes/ml liquid medium.**			

- : Absence of compound.
ND : Not Done.
** : The amount of **Taxol/10-DAB** at day 0 represents the respective amounts in the callus line from which cell suspensions have been raised.

Fig. 4.17

A. HPLC chromatogram of standards of 10-DAB (d) and Taxol (t). It shows two distinct, baseline-resolved peaks at retention times of 3.93 min. and 16.73 min.

B. HPLC chromatogram showing presence of 10- DAB (d) and Taxol (t) in suspension cultures of *Taxus* spp derived from callus cultures growing for 2 years in B5 medium supplemented with 6 mg.l^{-1} P (BP6) (Suspension Line No. 1). The blue colored graph shows the chromatogram for the standards of 10-DAB (d) and Taxol (t) while the green colored graph represents the spiked extract of Suspension Line No. 1 showing presence of both the d and t peaks after 9 days of growth in liquid medium.



4.4 Conclusions

1) **Callus cultures were raised from stem bits and needle (diploid calli) explants of mature trees of *Taxus baccata* ssp. *wallichiana* Zucc. Pilg. and from endosperm in seeds (haploid calli)**
2) **Callus initiation was achieved on 1/2 SH, B5, DCR and WPM media each supplemented individually with 2, 4, 6, 8 and 10 mg.l^{-1} 2,4-D from both stem bits and needle explants.**
3) **The stem bit explants formed callus with 20% efficiency as opposed to 100% for needle explants.**
4) **B5 medium with a number of growth regulators was the best medium for survival and good growth of needle-derived calli over monthly subcultures for more than 4 years.**
5) **Needle explants gave rise to callus with fast growth rate and high FW on B5 medium supplemented individually with high concentrations (6, 8, 10 mg.l^{-1}) of auxins like 2,4-D, NAA and P. The best callus growth rate and high FW was obtained in calli grown on P containing media.**
6) **B5 medium supplemented individually with 2, 4, 6, 8 and 10 mg.l^{-1} of auxins like IAA and IBA individually, produced insignificant amounts of callus from needles.**
7) **Callus induction from needle explants and the subsequent callus growth were very good on B5 medium supplemented with auxins like IAA and IBA together in various concentrations.**
8) **Callus growth of needle-derived calli did not improve on B5 medium with 2-iP combined with IBA and KIN combined with 2,4-D and NAA.**
9) **Taxane production was not detected in needle-derived calli cultured on B5 medium supplemented with various combinations of 2,4-D + KIN and NAA + KIN. Taxane production did not improve with age of such calli.**
10) **B5 medium supplemented with Picloram was found to be the best medium for good growth of needle-derived callus cultures as well as Taxane production therefrom.**
11) **Taxane contents increased with increasing age of callus tissues in culture on the optimized B5 medium with P. The highest Taxol content**

was 540 µg/g DW of callus in 2 year old needle-derived diploid callus line with Serial No. 3 growing on B5 medium supplemented with 6 mg.l^{-1} P.

12) Needle-derived Callus Line with Serial No. 9 on B5 medium supplemented with 8 mg.l^{-1} P had the highest 10-DAB content of 2585 µg/g DW of callus.

13) Higher P concentrations above 4 mg.l^{-1} are conducive for both Taxol and 10-DAB production.

14) Endosperm-derived callus growing on DCR medium supplemented with 8 mg.l^{-1} P produced 203 µg 10-DAB/g DW of callus.

15) Taxanes were not detected in the spent solid or liquid nutrient media used for growing either haploid or diploid callus cultures.

16) Taxane contents increased many-fold in three out of the four cell suspension lines analyzed.

17) Taxol and 10-DAB were detected in Taxane non-producer Callus Line with Serial No. 5 on raising a cell suspension from such a callus. The contents of both these compounds increased with the increase in cell suspension culture period from 0 days to 18 days.

CHAPTER 5

In vitro Studies on Micropropagation from Mature and Juvenile Explants of *Taxus* spp.

PART A: MICROPROPAGATION STUDIES USING MATURE EXPLANTS OF *TAXUS* SPP.

5.1 Introduction

The goal of **'Clonal Propagation'** or **'Micropropagation'** is to obtain a large number of genetically identical, physiologically uniform and developmentally normal plantlets, preferably with a high photosynthetic or photoautotrophic potential to survive the harsh *ex-vitro* conditions, in a reduced time period and at a lowered cost (Jeong et al., 1995). Although true-to-type plantlets are the desired goal of clonal propagation, due to involvement of tissue culture component, many times, somaclonal variants are obtained. These can be used for selection of new useful variants, which can then be propagated on a large scale by micropropagation. E.g. stress and disease tolerant variants.

There are many advantages of clonal propagation (Vasil and Vasil, 1980) like:

a. Uniform genetic background.
b. Production of uniformly superior seeds.
c. Improved progeny evaluation of breeding experiments.
d. Improved vigor and quality.
e. Elimination of pathogens.
f. An improved aesthetic value due to richness and fullness.
g. Facilitation of international transport and exchange of germplasm without quarantine.
h. Fast release of newly developed cultivar.

There are three methods of 'Clonal Propagation or Micropropagation' (Vasil and Vasil, 1980):

(1) Enhanced Axillary Bud Breaking – It is the slowest method as compared to the other two methods but is widely used in commercial set-ups due to assured genetic uniformity of newly derived plants.

(2) Production of Adventitious Buds/Organogenesis – This involves *de novo* formation of meristematic loci on the explant, leading to development of root or shoot meristems, depending upon the balance of plant growth regulators in the growth medium and a number of other physical and physiological factors. Although a large number of plants can be regenerated by this procedure, it does not have a wider applicability

since many of the economically important plant species do not undergo organogenesis *in vitro*.

(3) Somatic Embryogenesis – These are the asexual, bipolar embryos produced from somatic cells of the explant due to hormonal stimuli. This method of propagation provides the fastest route for production of a large number of clonal plants since the somatic embryos contain both the shoot and the root poles. But till date very few species have demonstrated their potential for direct somatic embryogenesis. Especially in Gymnosperms, few successes have been achieved only from zygotic embryos.

The method of enhanced axillary bud breaking produces the least number of plantlets as the number of shoots produced is controlled by the number of axillary buds cultured. But this is the most widely used method of micropropagation because:

I. It produces true-to-type plantlets with a uniform genetic background (Brown and Thorpe, 1995).

II. The method is relatively simpler with a reasonably good rate of propagation (Pierik, 1987).

III. The growth of the resulting plants is very good either due to rejuvenation and/or the lack of internal infections (Pierik, 1987).

This method of enhanced axillary bud breaking is widely used for angiospermic hardwoods (Bonga and Von Aderkas, 1992) but rarely used for conifers (Thorpe et al., 1991). Very few successes have been achieved in conifers starting from mature material and even in successful cases, rarely more than three shoots have been obtained from a single explant and rooting has been problematic as has been described in the following examples: 30 year old *Larix* spp. (Bonga and Pond, 1991), 19 year old *Larix decidua* (Diner, 1995) 4-16 year old *Psuedotsuga* spp. (Pullman and Timmis, 1992), 90 year old *Sequoia sempervirens* (Bon et al., 1994), 30 year old *Juniperus brevifolia* (Moreira da Silva and Debergh, 1996) and 20 year old *Pinus* spp. (Nadgauda et al., 1993). However It is necessary to use explants from elite, mature trees in order to obtain the maximum genetic gains in terms of desired characters like wood/pulp making quality, production of high value secondary metabolites, reforestation potential etc.

But such mature explants are rarely responsive in culture due to a number of factors such as (Modified from Pierik, 1987):

(1) **Chronological, physiological and ontogenetical aging of mature explants** (Fortanier and Jonkers, 1976); where a number of morphological and physiological attributes are affected unidirectionally and many times irreversibly. Hence for such explants to respond, a reversion in their maturation-related characters that is **"Rejuvenation"** needs to occur *in vitro* at the meristematic regions. The induction of rejuvenation is extremely difficult in woody spp.
(2) In general, woody plants have relatively weak regenerative capacity and lower multiplication rates.
(3) Because of strong dormancy observed in woody plants, buds do not open and stem elongation fails to take place.
(4) **"Topophysis"** (The phenomenon whereby the position of the explant in the plant affects its *in vitro* growth and response after isolation and culture due to gradients of regeneration) plays a greater role in woody plants.
(5) Sterilization of mature explants, especially those grown under field conditions, is difficult to achieve successfully.
(6) The explants derived from field grown plants exhibit considerable variations *in vitro*, due to the different growth conditions and annual fluctuations in the climate *in vivo*.
(7) The adult trees generally have a great degree of genetic variation as compared to agricultural and horticultural plants and hence the results obtained are highly variable and many times difficult to repeat (Pierik, 1987).

In addition, explants of mature origin have high rates of contamination and phenolic oxidation as described earlier in **Chapter 3** in detail.

The success of micropropagation also depends on following factors *in vitro* such as:

- **Biological Factors** (Pierik, 1987) such as genotype, age, health and physiology of the parent plant, environmental conditions pertaining to parent plants, position, size and method of inoculation of the explant.

- **Physical Factors** (Pierik, 1987; Jeong et al., 1995) such as light including day length, spectral composition and irradiance, temperature, water availability, gaseous components such as oxygen, carbon dioxide, ethylene and other hydrocarbons, electric currents, medium composition and volume.

5.1.1 Vegetative propagation of *Taxus* spp.

Normally, vegetative propagation is achieved through cuttings. In case of *Taxus* spp., the percentage of rooting varied between cultivars and species depending on the conditions of temperature, misting and use of IBA. 63-100% rooting was observed with many species and cultivars (Eccher, 1988). Chee (1995) reported that stem cuttings of *T. cuspidata* treated with an aqueous solution of IBA (0.2%) + NAA (0.1%) + Thiamine (0.08%) produced roots at a frequency of 73.5%.

But the success rate was not very high for the Himalayan Yew (20-30%) and the cuttings took at least three months to root. Success of grafting and layering was also not very encouraging (Chatterjee and Dey, 1997). A variety of reasons have been attributed for this low rate of success in rooting (Mitchell, 1997 and references therein):

- Great age of parent trees (more than 100 years) and the associated low growth vigor.
- Variation in rooting among parent trees from different geographical locations due to variations in a number of ecological and environmental factors.
- Variation in rooting percentage between different species and cultivars as well as between male and female trees.
- The yews tend to grow in stressful environments (too cold, too exposed) which might reduce the shoot vigor, finally adversely affecting rooting success.

The roots of mature trees of Yews are associated with ectomycorrhizal mats (ECM) and vesicular arbuscular mycorrhiza (VAM) in their natural habitats. ECM and VAM might play important roles in rooting and establishment of cuttings derived from mature trees by suppressing root pathogens and increasing the available nutrients (Griffiths et al., 1995).

5.1.2 *In vitro* propagation of *Taxus* spp.

Even though success with vegetative propagation has not been significant in *Taxus* spp., not much work has been done with respect to micropropagation studies *in vitro* in *Taxus* spp. There are no full-length papers on micropropagation from mature explants of *Taxus* spp. in the literature. Only two abstracts report formation of 2-4 shoots in *Taxus* spp. (Amos and McCown, 1981; Barnes, 1983) without much information on the media used and the culture conditions. Also there are no reports on micropropagation in the Himalayan Yew. Precocious germination from seeds of *Taxus* spp., *de novo* adventitious bud development and somatic embryogenesis are some of the studies carried out in *Taxus* spp. so far and are presented in **Table 5.1**. In addition, much of the information has been in the form of patents thus restricting its use and availability in the public domain.

Table 5.1 *In vitro* Culture Methods Applied to Propagation of *Taxus* spp.

Culture Methods	*Taxus* spp.	Explants	Media	Plant Growth Regulators	*Features*	References
Embryo Culture / Germi-nation.	**T. baccata**	Mature Embryos	Heller	-	Leaching of mature embryos. Role of ABA implied in seed dormancy.	LePage-Degivry (1970, 1973a, b, c)
	*T.*x *media*	Immature Embryos	White/ MS	-	70% germination. 30% full seedlings after 2 weeks.	Flores and Sgrignoli (1991).
	T. brevifolia	Embryos	White/ MS/ DCR		60-70% germination 30% full seedlings after 2 weeks.	Flores et al. (1993).
	Taxus spp.	Immature and Mature Embryos	B5	GA3	63% full seedlings after 2 weeks.	Hu et al. (1992).
	T. baccata	Mature Embryos	Modi-fied MS/ Heller	-	Leaching of whole seeds for 1 week. 100% full seedlings after 2 weeks.	Zhiri et al. (1994).
	Taxus spp.	Mature Embryos	B5	-	36% full seedlings after 2 weeks.	Chee (1994).

Contd…..

Table 5.1 Contd.

Culture Methods	*Taxus* spp.	Explants	Media	Plant Growth Regulators	*Features*	References
Embryo Culture / Germi-nation.	*T. mairei, T. baccata, T. canade-nsis, T. media, T. cuspidata*	Mature and Immature embryos	1/2 MS + PVP	GA3	15% germination from immature embryos and 90% germination from mature embryos.	Chang and Yang (1996).
	T. chinensis	Mature embryos	B5, New medium with AC, 20% sugar	-	97% germination	Chien et al. (1998).
Micro-propagation	*Taxus* spp.	Shoot tips	WPM	BAP	2-4 shoots per explant	Amos and McCown (1981).
	T. floridana	Shoot tips	WPM	BAP	2-4 shoots per explant	Barnes (1983).
	T. brevifolia	Embryos	1/2 B5	BAP	Multiple shoots, 58% rooting, plantlets.	Chee (1995b).
	T. mairei	Shoot tips and stem from 6 to 18 months old seedlings	MS	BAP	Both orthotropic and plagiotropic shoots obtained. 60% rooting.	Chang et al. (1998).

Contd…..

Table 5.1 Contd.

Culture Methods	*Taxus* spp.	Explants	Media	Plant Growth Regulators	*Features*	References
Micro-cuttings.	*T.*x *media*	Shoot tips	Hoagland	KIN	-	Cerdeira et al. (1994).
		Cuttings from tips of branches from 3-6 year old plants.	B5	2,4-D	A number of **Taxanes** released in liquid culture medium.	Hoffman et al. (1996)
Somatic Embryo-genesis.	*T. floridana*	Needles	MS	2,4-D	Glutamine induces embryo-genic structures on callus.	Salandy et al. (1993).
	Taxus spp.	Embryos	-	2,4-D/BAP	Two stage culture.	Wann and Goldner (1994).
	T. cuspidata	Immature Embryos	Modified B5, MS, WPM.	NAA, 2,4-D, KIN	Embryo-genesis after transfer to hormone free medium.	Lee and Son (1995).
	Taxus spp.	Immature embryos	DCR	KIN, BAP, 2,4-D for embryo-genic calli. KIN, IBA for globular embryo formation.	Cotyledons formed but no root formation.	Ewald et al. (1995).

Contd…..

Table 5.1 Contd.

Culture Methods	*Taxus* spp.	Explants	Media	Plant Growth Regulators	*Features*	References
Somatic Embryo-genesis.	*T. brevifolia*	Immature Embryos	WPM (MCM)	2,4-D, BAP, NAA for embryo-genic callus. BAP, KIN, NAA for embryoid formation.	5% emblings produced on hormone free 1/2 WPM (MCM).	Chee (1996).
Nodule Culture	*Taxus* spp.	Juvenile needles from *in vitro* shoot cultures.	Modified WPM	NAA, 2-iP	Cohesive cellular units of nodules with differentia-ted internal structures.	Ellis et al. (1996).

From the literature review presented in **Table 5.1**, it is clear that it is extremely important to develop a micropropagation protocol for the Himalayan Yew as it is becoming endangered in its natural habitat due to merciless felling of the trees or unauthorized stripping of the bark for **Taxol** extraction, leading to slow death of the trees. New stocks of plants are required to replenish the natural resources. Even though the juvenile shoots produced *in vitro* are not desirable for planting, they provide an opportunity in the elucidation of production of **Taxane** class of compounds. Micropropagation by use of mature explants is desirable as the advantages far outweigh the disadvantages faced when using mature explants *in vitro*.

In view of the aforesaid, the objectives of the present work were:

1. Optimization of nutrient media, plant growth regulators and culture conditions for achieving enhanced axillary budding from stem explants of mature trees of *Taxus baccata* ssp. *wallichiana* Zucc. Pilg. collected from Shimla.
2. Establishment of primary sprouts and subsequent initiation as well as establishment of secondary sprouts.
3. Extrapolation of the standardized protocol for plant material of *Taxus* spp. collected from other locations in the Himalayas.
4. Preliminary studies on precocious embryo germination, seedling establishment and raising of callus cultures from endosperm halves of seeds of the Himalayan Yew.
5. Analysis of **Taxane** contents in callus cultures derived from endosperm of the Himalayan Yew.

The results achieved on the objectives are presented in this chapter as two parts:

PART A: Deals with the attempts on development of a widely applicable micropropagation protocol for *Taxus* spp. from mature explants, collected from different locations in the Himalayas.

PART B: Describes the results of *in vitro* studies with embryo and endosperm explants of *Taxus* spp.

5.2 Materials and Methods

5.2.1 Collection of plant material

Collection of twigs of *Taxus baccata* ssp *wallichiana* Zucc. Pilg. from various locations in the Himalayas and the preparation of explants for *in vitro* studies were as described in detail in **Chapter 4, Section 4.2.1**.

5.2.2 Pre -treatment and surface sterilization of explants

Stem bit explants were surface sterilized according to the **"Modified Surface Sterilization Protocol"** described in **Chapter 3, Flow Chart. 3.3**.

5.2.3 Media and culture conditions

A number of basal media either at full strength or at half strength (with major salts reduced to half strengths) were used for axillary bud break. In the pilot experiments, a number of basal media at full strength such as: Murashige and Skoog's MS medium (1962) (High salt medium), Gamborg's B5 medium (1968) (Medium salt medium), Lloyd and McCown's Woody Plant Medium, WPM (1981) (Low salt medium) without addition of plant growth regulators were used. The composition details were as described in **Chapter 2, Table 2.1**. WPM was selected for further study based on the sprouting obtained and shoot survival and was supplemented individually with various concentrations of TDZ (0.01, 0.05, 0.1, 0.5, 1.0 $mg.l^{-1}$) and BAP (0.1, 2.0, 5.0 $mg.l^{-1}$).

To further optimize the results obtained, the following media at half strength of major salts were used: WPM (Based on pilot experiment data), Gupta and Durzan's DCR medium (1985), Litvay's Medium, LM (1981) and Modified Litvay's Medium, MLM (Kulkarni, 1997) which was modified to contain 10 mM each of KNO_3, NH_4NO_3 and glutamine. The composition details were as described in **Chapter 2, Table 2.1.** For each experiment, these half strength media without any plant growth regulator served as control treatments (1/2 WPM-H, 1/2 DCR-H, 1/2 LM-H and 1/2 MLM-H). 1/2 WPM and 1/2 DCR media were further modified by individual additions of a number of cytokinins like BAP, KIN, TDZ and 2-iP at concentrations of 0.01, 0.05, 0.1 and 0.5 $mg.l^{-1}$. In one set of experiments, IBA and BAP were used together. All the media contained 2% sucrose and 0.85% agar and 0.5% activated charcoal (AC) in case of solid media. Liquid

media had only 2% sucrose (No agar and AC) and support to the explants was provided with Whatman No. 1 filter paper bridges. Media used for establishment of primary and secondary sprouts were appended with 10% coconut milk (CM) and 1g.l^{-1} of casein hydrolysate (CH). All other details regarding media preparation, inoculation and culture of explants and routine tissue culture practices were as described earlier in **Chapter 2, Sections 2.4, 2.5** and **Chapter 4, Section 4.2.3**.

Stem bit explants with axillary buds and trimmed needles were inoculated independently in each culture tube and incubated either in the dark or at light intensities of 4.6 or 19 $\mu Em^{-2}s^{-1}$ provided by cool white fluorescent tubelights ("Phillips", India) for 16 hours at a temperature of 25 $\pm$ 2^{o}C.

5.2.4 Elongation and establishment of primary sprouts

Initiation of primary sprouts from axillary buds of the stem bit explants was observed in 25-35 days on the optimal media (C. f. **Tables 5.5, 5.5 A**). Elongation of these occurred on the same media to a length of 0.5-1.0 cm during the second monthly transfer. In the third monthly transfer, the elongated sprouts were transferred to the same basal media without plant growth regulators where the sprouts grew into well-developed shoots of a length upto 3 cm. These shoots were then detached from the mother explant and cultured individually either on the same basal medium without plant growth regulators or on the same basal medium supplemented with 10% CM and 1 g.l^{-1} CH for independent establishment of primary shoots (C. f. **Table 5.9**). The cultures were incubated under the conditions described under **Section 5.2.3, Media and culture conditions**.

5.2.5 Secondary sprouting

The elongated primary shoots were cultured either on 1/2 WPM or 1/2 DCR basal media supplemented individually with low concentrations of KIN or BAP (0.01, 0.05 and 0.1 mg.l^{-1}), 10% CM and 1 g.l^{-1} CH, for induction of secondary sprouting.

5.2.6 Statistical analysis

Each culture tube with one shoot explant was considered as one replicate. Each treatment in each set of experiments consisted of 50 such replicates and each experiment was repeated three or more times over successive years. The treatments were analyzed by one-way ANOVA and the individual treatment means were compared by Student's t-test (Wardlaw, 1985).

5.3 Results and Discussion

5.3.1 Primary sprouting from mature shoot explants

5.3.1.1 Plant material of *Taxus* spp. collected from Shimla

Initially stem bit explants were cultured on full strength MS, B5 and WPM basal media without plant growth regulators (compositions described earlier in **Chapter 2, Table 2.1)**. MS medium has a high total ionic strength of salts at 100.48 mM while B5 medium has a total ionic strength of salts at 65.738 mM (C. f. **Chapter 2, Table 2.2**). All the explants (100%) inoculated in these media exuded heavy amount of phenolics due to high ionic strengths of these media and were dead within three days. Hence these media were not used for further studies. Only the explants inoculated in full strength WPM without plant growth regulators, with a total ionic strength of 43.516 mM (C. f. **Chapter 2, Table 2.2**), produced less phenolics and responded to axillary shoot development at a frequency of 42.94% (**Table 5.2**). This medium was therefore chosen for further experiments with cytokinins. Full strength WPM was modified by individual addition of BAP and TDZ at a number of concentrations (**Table 5.2**). BAP and TDZ were chosen for these initial experiments because they had been reported to be useful in Conifer/Woody plants micropropagation (Fiola et al., 1990 and references therein; Thorpe et al., 1991). **Table 5.2** and **Fig. 5.1** describe the results obtained.

Table 5.2 Effect of Different Plant Growth Regulator Concentrations on % Sprouting from Mature Explants Cultured on Full Strength WPM. (Plant material collected from Shimla during 1995).

No.	Plant Growth Regulator	Concentration ($mg.l^{-1}$)	% Sprouting Mean + S. E.
1.	Nil (Control)	-	42.94 ± 1.32^{a}
2.	TDZ	0.01	41.05 ± 1.05^{a}
3.		0.05	25.00 ± 0.00^{a}
4.		0.1	10.55 ± 0.55^{b}
5.		0.5	80.00 ± 20.00^{c}
6.		1.0	8.83 ± 2.17^{d}
7.	BAP	0.1	31.67 ± 1.67^{a}
8.		2.0	37.5 ± 2.5^{a}
9.		5.0	58.3 ± 8.3^{b}

$^{a, b, c, d}$: Values followed by different letters are significantly different from each other at 95% confidence level by t-test.

Although the maximum initiation of primary sprouting was observed with higher concentrations of TDZ (80% at 0.5 $mg.l^{-1}$) and BAP (58.3% at 5.0 $mg.l^{-1}$), further growth and development of primary shoots was hampered and the shoots failed to elongate even upto a length of 1 cm in subsequent two transfers. On the contrary, primary sprouts developed on lower concentrations of BAP (0.1 and 2.0 $mg.l^{-1}$) and TDZ (0.01 and 0.05 $mg.l^{-1}$) could elongate to a length of 2 cm but released phenolic exudates in limited quantities into the nutrient medium. The release of phenolics into the nutrient medium even at low concentrations of BAP (**Fig. 5.1 B**) and on basal medium without plant growth regulators (**Fig. 5.1 C**) is quite evident. This led to browning of shoots and yellowing of leaves and the shoots were not able to survive beyond 2 months. It has been suggested that an improper balance of nutrient salts leads to darkening of media and/or explants (Preece, 1995).

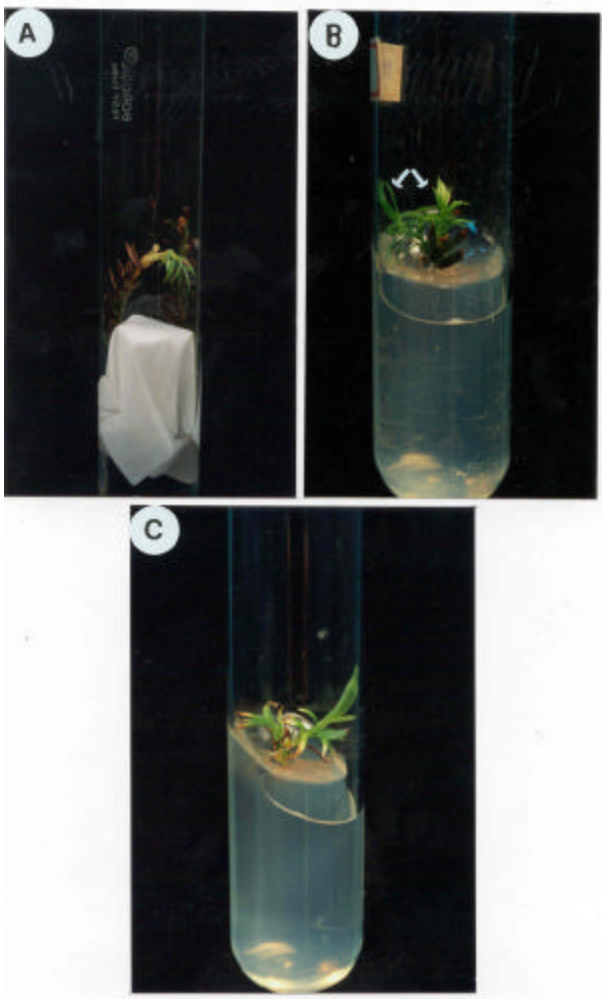


Fig. 5.1 Plant material of Taxus baccata ssp. wallichiana Zucc. Pilg. collected from Shimla, 1995.

Figs. A – C Response of vegetative buds *in vitro.*

A. Elongation of apical bud on full strength WPM without plant growth regulators.
B. Simultaneous sprouting of two axillary buds (arrows) on full strength WPM with 0.1 $mg.l^{-1}$ BAP. Note the browning of the medium due to phenolics.
C. Separation of these two sprouts from the mother plant and their independent growth and establishment on full strength WPM without plant growth regulators. Note the yellowing of new leaves and phenolic exudates in the medium.

Further experiments were therefore carried out with WPM (Total ionic strength of 43.516 mM) and other media such as DCR (Total ionic strength of 36.042 mM), LM (Total ionic strength of 144.185 mM) and MLM (Total ionic strength of 60.736 mM), at half strengths of their major salts, which have been used in Woody plant and/or Conifer micropropagation (Litvay et al., 1981; Gupta and Durzan, 1985). Details of ionic strengths of these media and NH_4^+ to NO_3^- ratios are presented in **Table 5.3**.

Table 5.3 Total Ionic Strengths of Basal Nutrient Media Used in Enhanced Axillary Budding and NH_4^+ to NO_3^- Ratios.

Basal Medium		Total Ionic Strength		NH_4^+ to NO_3^- Ratios
Full Strength	Half Strength	Full Strength	Half Strength	
MS	1/2 MS	100.480	55.937	0.523
B5	1/2 B5	65.738	36.903	0.081
WPM	1/2 WPM	43.516	23.326	0.512
DCR	1/2 DCR	36.042	17.649	0.477
LM	1/2 LM	144.185	84.046	0.523
MLM without Glutamine	1/2 MLM without Glutamine	60.736	31.911	0.500 without glutamine addition.*

*: Contribution of glutamine addition to NH_4^+ to NO_3^- ratios is not indicated due to non-availability of authentic values.

These media (WPM, DCR, LM and MLM) were used at half strength of their major salts, which are known to reduce browning and improve response in mature tissues (McCown and Sellmer, 1987). It has also been shown that suitability of different basal media depends on the genotype, age and type of explant (Franclet, 1991). Preece (1995) suggests that when nutrient level and their balance is correct, explants are under less stress and their *in vitro* performance improves dramatically, which cannot be achieved solely with plant growth regulators.

Initially four media were used at half strength of major salts without plant growth regulators (Designated as "1/2 medium name-H") to determine the best basal medium. As seen from **Table 5.4**, it is clear, that the sprouting percentage in different media varied

significantly. Except 1/2 MLM-H, all the other three media showed high incidence of sprouting. Since only 6.46% sprouting was obtained in 1/2 MLM-H, this medium was not used in further experiments with cytokinins. In MLM, the NH_4^+ to NO_3^- ratio was 0.5 and in addition, organic nitrogen was present in the medium in the form of 10 mM glutamine. Apparently the modification of reducing the major salt concentration to half in MLM is not quite enough for *Taxus* spp. for supporting growth and development of axillary buds. It also appears that organic nitrogen is not required for growth of *Taxus* spp., as sprouting percentage in 1/2 MLM-H is much lower than that observed for 1/2 LM-H. Even though sprouting was the highest (78.95%) on 1/2 LM-H medium, the newly induced shoots failed to elongate further and their apices became necrotic within 15 days. This medium was therefore not used for further experiments with cytokinins. The total ionic strength of LM is 144.185 mM and for 1/2 LM the total ionic strength is approximately 84.046. This further confirmed the observations of pilot experiments of the non-suitability of high ionic strength media for culture of *Taxus* spp. The shoot necrosis of primary shoots, observed in 1/2 LM might therefore be a manifestation of this effect. In addition, LM has a NH_4^+ to NO_3^- ratio of 0.523 that is identical with MS medium, a high salt medium. The other two media namely 1/2 WPM-H and 1/2 DCR-H showed 52.94% and 50% sprouting (statistically similar) and in these media, the primary shoots neither became necrotic nor showed phenolic exudations. This may be due to very low total ionic strengths of approximately 23.326 mM and 17.649 mM respectively of these media. These values are less than half of 1/2 MS (55.937 mM), 1/2 LM (84.046 mM) and 1/2 MLM (31.911 mM). Also the NH_4^+ to NO_3^- ratios for WPM and DCR are 0.512 and 0.477 respectively, lesser than 1/2 LM (0.522), 1/2 MS (0.522) and 1/2 MLM (0.50). The amount of chlorides is also very less in WPM and DCR, thus eliminating the chances of chloride poisoning of the plants. Hence 1/2 WPM and 1/2 DCR media were used in all the subsequent experiments.

Table 5.4 Effect of Different Basal Media on % Sprouting from Mature Explants (Plant material collected from Shimla during 1995-1996).

Medium	% Sprouting Mean ± S.E.
1/2 WPM - H	52.94 ± 5.32[a]
1/2 DCR - H	50.00 ± 0.00[a]
1/2 LM - H	78.95 ± 5.27[b]
1/2 MLM - H	6.46 ± 0.21[c]

[a, b, c] : Treatment means with different letters are significantly different from each other at 95% confidence level by t-test.

1/2 WPM and 1/2 DCR media were further used to test the effects of different cytokinins individually as the conifers are known to induce sprouting of buds in response to exogenously applied cytokinins (Thorpe et al., 1991).

1/2 WPM was chosen for the initial experiments. As a means to avoid any possibility of leaching of phenolics further, it was decided to use this medium in liquid form. 1/2 WPM without plant growth regulators gave 52.94% sprouting. It was then combined with TDZ as TDZ was known to induce sprouting of buds or development of *de novo* shoot buds in woody tissues (Fiola et al., 1990 and references therein).

1/2 WPM with 0.1 mg.l^{-1} TDZ gave low percentage of sprouting (13.75%, **Table 5.5**) while no sprouting was observed in 1/2 WPM with 0.5 mg.l^{-1} TDZ, as has been the case in many other conifers (Rong, 1989; Morte et al., 1992). Although the initiation of sprouting was high at 27.2% and 42.86% with 0.01 and 0.05 mg.l^{-1} TDZ respectively, the newly sprouted buds were highly swollen and failed to elongate beyond 0.5 cm (**Fig. 5.2**). Since the quality of newly initiated primary shoots was not very good in liquid medium with TDZ, liquid medium was not used subsequently. 1/2 WPM solidified with agar and supplemented with AC was therefore used in further experiments with BAP and KIN.

Higher percentage of sprout induction and better shoot quality (ability of shoots to elongate, orthotropism) were observed in 1/2 WPM with BAP and KIN (**Table 5.5 and Figs. 5.3 and 5.4**). 1/2 WPM supplemented with 0.05 mg.l^{-1} BAP gave 83.33% sprouting

that was significantly higher than 40% sprouting obtained with 0.01 $mg.l^{-1}$ BAP and 66.66% sprouting obtained with 0.1 $mg.l^{-1}$ BAP. No sprouting was obtained with 0.5 $mg.l^{-1}$ BAP. Thus higher cytokinin concentrations are not conducive for sprouting. 1/2 WPM supplemented with 0.05 $mg.l^{-1}$ KIN supported significantly higher 75.77% sprouting while 1/2 WPM supplemented with 0.01 and 0.1 $mg.l^{-1}$ KIN gave similar sprouting at 73.13% and 63.33% respectively.

No sprouting was observed with any of the 2-iP concentrations tried.

Table 5.5 Effect of Different Plant Growth Regulator Concentrations on % Sprouting from Mature Explants Cultured on 1/2 WPM (Plant material collected from Shimla during 1996-1998).

No.	Basal Medium	Plant Growth Regulator	Concentration ($mg.l^{-1}$)	% Sprouting Mean ± S.E.	Shoot Quality/ Elongation
1.	1/2 WPM	Absent	-	52.94 ± 5.32^{a}	Good
2.		TDZ	0.01	27.20 ± 2.21^{b}	Shoots did not elongate.
3.			0.05	42.86 ± 7.15^{ac}	
4.			0.1	13.75 ± 1.25^{d}	
5.			0.5	0 ± 0^{e}	-
6.		BAP	0.01	40.00 ± 0^{a}	Good, Shoots elongated well.
7.			0.05	$83.33 + 0^{b}$	
8.			0.1	66.66 ± 0^{a}	
9.			0.5	0 ± 0^{e}	-
10.		KIN	0.01	73.13 ± 8.13^{a}	Very good, Shoots elongated very well.
11.			0.05	$75.77 + 14.23^{c}$	
12.			0.1	63.33 ± 3.33^{a}	
13.			0.5	0 ± 0^{e}	-

$^{a, b, c, d, e}$: Values followed by different letters are significantly different from each other at 95% confidence level by t-test.

Fig. 5.2 Plant material collected from Shimla, 1996.

Figs. A – D Response of vegetative buds *in vitro* on 1/2 strength WPM supplemented with various concentrations of TDZ.

A. Response of primary sprouting and shoot bud induction on 1/2 strength WPM supplemented with various concentrations of TDZ (From left to right: Control (without plant growth regulator), 0.01 $mg.l^{-1}$ TDZ, 0.05 $mg.l^{-1}$ TDZ, 0.1 $mg.l^{-1}$ TDZ and 0.5 $mg.l^{-1}$ TDZ). Note the heavy callus and stunting effect of TDZ on shoot bud elongation.

B. Simultaneous development of an apical bud (a) and three axillary buds (arrows) on 1/2 WPM with 0.05 $mg.l^{-1}$ TDZ.

C. Simultaneous development of an apical bud and three axillary buds on 1/2 WPM with 0.05 $mg.l^{-1}$ TDZ.

D. Two swollen apical buds on 1/2 WPM with 0.1 $mg.l^{-1}$ TDZ.

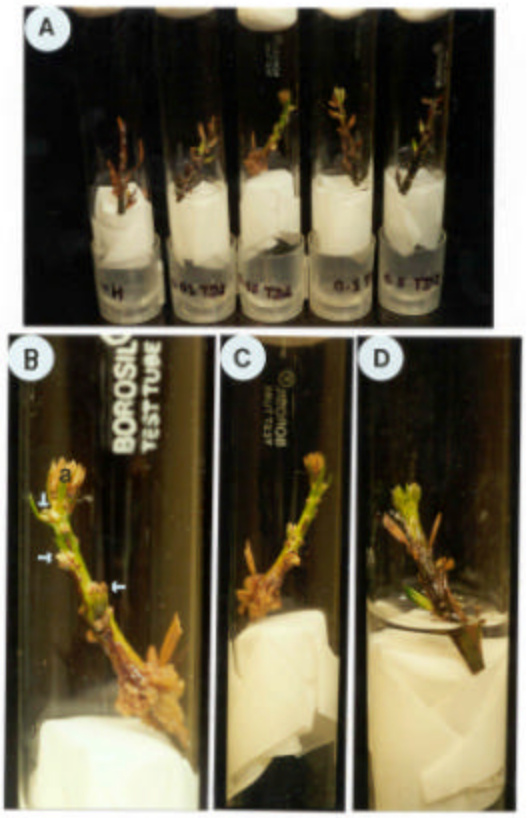


Fig. 5.3 Plant material collected during 1998-1999.

Figs. A - E Initiation of primary sprouting on 1/2 strength WPM basal medium without plant growth regulators (1/2 WPM – H) (Control treatment) and supplemented with various concentrations of KIN.

A. Sprouting of an apical bud on 1/2 WPM – H.
B. Sprouting of an axillary bud on 1/2 WPM – H.
C. Sprouting of four axillary buds (arrows) simultaneously on 1/2 WPM – H.
D. Sprouting of an apical bud on 1/2 WPM + 0.01 $mg.l^{-1}$ KIN.
E. Initiation of sprouting of two axillary buds simultaneously on 1/2 WPM + 0.01 $mg.l^{-1}$ KIN.

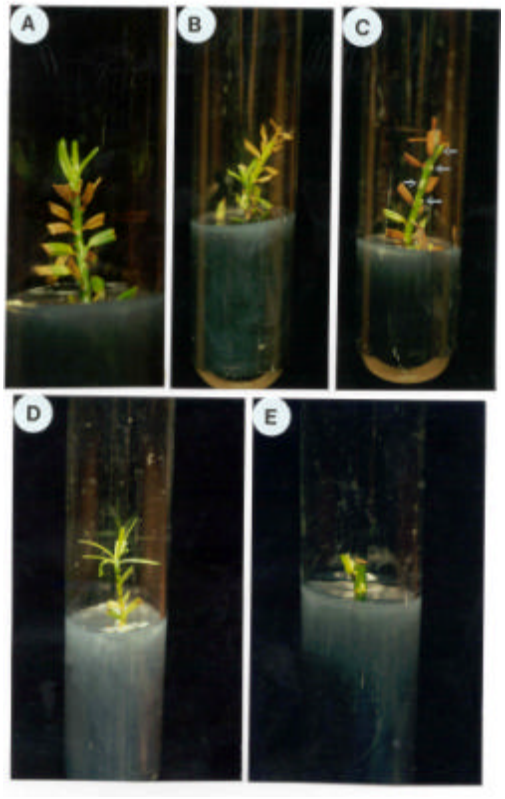
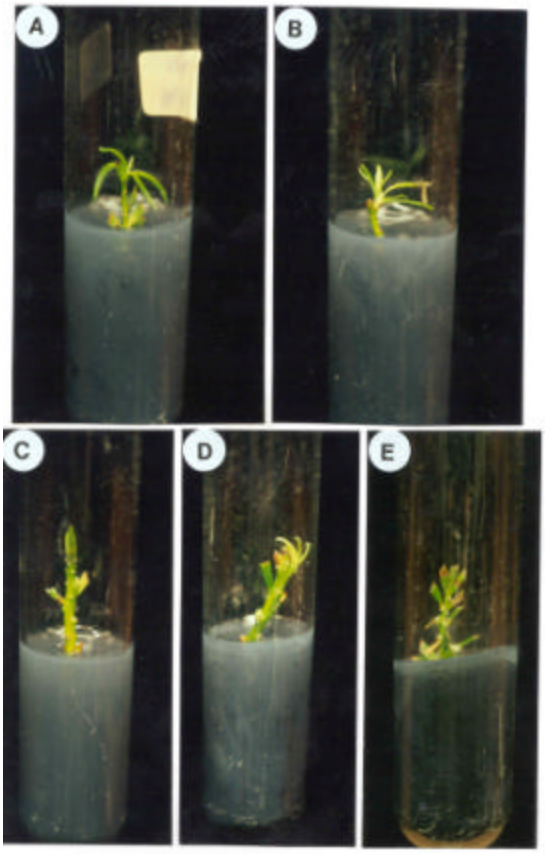


Fig. 5.4 Plant material collected during 1998-1999.

Figs. A - E Initiation of primary sprouting on 1/2 Strength WPM basal medium supplemented with various concentrations of KIN.

A. Sprouting of an apical bud on 1/2 WPM + 0.05 $mg.l^{-1}$ KIN.
B. Sprouting of an axillary bud on 1/2 WPM + 0.05 $mg.l^{-1}$ KIN.
C. Initiation of sprouting of one apical and three axillary buds simultaneously on 1/2 WPM + 0.1 $mg.l^{-1}$ KIN.
D. Initiation of sprouting of one apical and three axillary buds on 1/2 WPM + 0.1 $mg.l^{-1}$ KIN.
E. Absence of sprouting of axillary buds on 1/2 WPM + 0.5 $mg.l^{-1}$ KIN. Each needle base shows light yellow callus.



The results optimized with 1/2 WPM and plant growth regulator combinations were further extrapolated to 1/2 DCR medium in the next set of experiments. TDZ was not tried with 1/2 DCR medium as, although good percentage of sprouting was obtained in 1/2 WPM supplemented with TDZ, the shoots initiated failed to elongate beyond 0.5 cm. (See **Fig. 5.2**). The negative sprouting response obtained with higher concentrations of BAP and KIN (0.5 $mg.l^{-1}$) in 1/2 WPM medium, led us to use only lower concentrations of these cytokinins with 1/2 DCR medium **(Table 5.5 A)**. While 100% sprouting was obtained on 1/2 DCR supplemented with 0.05 and 0.1 $mg.l^{-1}$ BAP, 1/2 DCR supplemented with KIN supported only 46.43-58.89% sprouting (statistically similar) **(Table 5.5 A and Fig. 5.5**). 1/2 DCR medium without plant growth regulators but supplemented with 10% CM gave 100% sprouting, which decreased to 53% and 42% respectively by supplementation with 0.01 and 0.05 $mg.l^{-1}$ BAP. Addition of even such low concentrations of BAP to a medium containing 10% CM might have increased the overall cytokinin availability and its effect on the explants, leading to deterioration in the percentage of sprouting on 1/2 DCR medium containing 10% CM and 0.01 or 0.05 $mg.l^{-1}$ BAP. This once again proved that higher concentrations of cytokinins are not conducive for axillary bud break and sprouting of mature *Taxus* explants.

No sprouting was observed with any of the 2-iP concentrations tried.

Table 5.5 A Effect of Different Plant Growth Regulator Concentrations on % Sprouting from Mature Explants Cultured on 1/2 DCR Medium (Plant material collected from Shimla during 1996-1998).

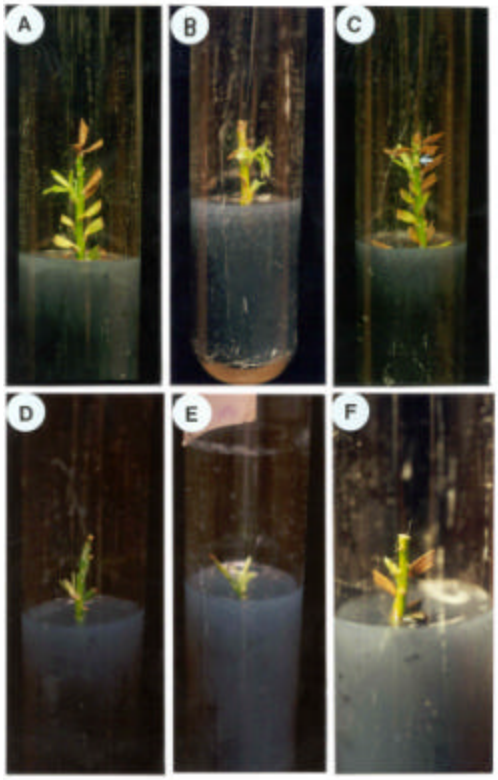
No.	Basal Medium	Plant Growth Regulator	Concentration ($mg.l^{-1}$)	% Sprouting Mean ± S.E.	Shoot Quality/ Elongation
1.	1/2 DCR	Absent	-	50 ± 0^{a}	Good, Shoots elongated well.
2.		TDZ	0.01	Not tried as shoots fail to elongate.	-
3.			0.05		
4.			0.1		
5.			0.5		
6.		BAP	0.01	66.63 ± 0.02^{a}	Very good, Shoots elongated very well.
7.			0.05	100.00 ± 0^{b}	
8.			0.1	100.00 ± 0^{b}	
9.			0.5	Not tried as shoots fail to elongate.	-
10.		KIN	0.01	46.43 ± 3.57^{a}	Good, Shoots elongated well.
11.			0.05	58.89 ± 4.84^{a}	
12.			0.1	54.21 ± 3.00^{a}	
13.			0.5	Not tried as shoots fail to elongate.	-
14.	1/2 DCR With 10% CM	Absent	-	100 ± 0^{a}	Very good, Shoots elongated very well.
15.		BAP	0.01	53.33 ± 13.33^{b}	Good, Shoots elongated well.
16.			0.05	41.67 ± 8.34^{bc}	

$^{a, b, c}$: Treatment means with different letters in the same column are significantly different from each other at 95% confidence level by t-test for 1/2 DCR.

Fig. 5.5 Plant material collected during 1998-1999.

Figs. A – G Initiation of primary sprouting on 1/2 Strength DCR basal medium without plant growth regulators (1/2 DCR – H) and/or supplemented with plant growth regulators.

A. Sprouting of a single axillary bud on 1/2 DCR – H (No plant growth regulators, Control treatment).
B. Sprouting of two axillary buds on 1/2 DCR + 0.01 $mg.l^{-1}$ KIN.
C. Sprouting of two axillary buds on 1/2 DCR + 0.05 $mg.l^{1}$ KIN (Arrow represents a small axillary bud).
D. Initiation of sprouting of a single axillary bud on 1/2 DCR – H.
E. Initiation of sprouting of a single axillary bud on 1/2 DCR + 0.05 $mg.l^{-1}$ BAP.
F. Initiation of sprouting of a single axillary bud on 1/2 DCR + 0.1 $mg.l^{-1}$ BAP.



To see whether a cytokinin and auxin combination would further improve the sprouting percentage of axillary buds, 1/2 WPM and 1/2 DCR media with optimized low concentrations of BAP (0.01 and 0.05 $mg.l^{-1}$) were supplemented with various concentrations of IBA (0.1 and 0.5 $mg.l^{1}$) (**Table 5.6**). Here the stem bits gave rise to only callus and even the axillary buds were completely covered by callus and did not sprout. A combination of an auxin with a cytokinin is therefore not conducive for enhanced axillary budding of mature stem explants of *Taxus* spp.

Table 5.6 Effect of IBA and BAP Combinations on % Callusing in Shoot Bit Explants on 1/2 WPM and 1/2 DCR Medium.

No.	Basal Medium	IBA Concentration ($mg.l^{-1}$)	BAP Concentration ($mg.l^{-1}$)	% Callusing Mean ± S. E	Nature of Callus
1.	1/2 WPM	0.1	0.01	12.5 ± 2.5^{a}	Light yellow, friable callus growing acropetally.
2.		0.1	0.05	$41.87 + 3.75^{b}$	
3.		0.5	0.01	23.86 ± 1.14^{c}	
4.		0.5	0.05	100 ± 0^{d}	
5.	1/2 DCR	0.1	0.01	10.5 ± 0.5^{a}	Light yellowish-brown, friable callus growing acropetally.
6.		0.1	0.05	48.67 ± 3.83^{b}	
7.		0.5	0.01	20.86 ± 2.11^{c}	
8.		0.5	0.05	100 ± 0^{d}	

$^{a, b, c, d}$: Treatment means with different letters are significantly different from each other at 95% confidence level by t-test.

From the foregoing results obtained on sprouting of mature stem explants of *Taxus* spp. collected from Shimla and presented in **Tables 5.2, 5.4, 5.5, 5.5 A** and **5.6**, one may conclude that:

a) Basal media with low total ionic salt strengths and lower ratios of ammonium nitrogen to nitrate nitrogen such as 1/2 WPM and 1/2 DCR are conducive for enhanced axillary budding of mature stem explants of *Taxus* spp.
b) Organic nitrogen in the form of an amino acid like glutamine does not enhance axillary sprouting in *Taxus* spp.
c) Although the ionic strength of 1/2 DCR medium is much lower than 1/2 WPM, (17.649 mM and 23.326 mM respectively), 1/2 DCR-H does not give statistically significant higher sprouting percentage than 1/2 WPM-H (**Tables 5.5 and 5.5 A**).
d) 1/2 WPM supplemented with lower concentrations of BAP (0.05, 0.1 $mg.l^{-1}$) gave higher sprouting percentage as compared to 1/2 WPM supplemented with lower concentrations of KIN (0.05, 0. 1 $mg.l^{-1}$). The results are statistically significant (**Table 5.5**).
e) 1/2 WPM supplemented with lower concentrations of KIN (0.05, 0.1 $mg.l^{-1}$) gave higher sprouting percentage as compared to 1/2 DCR supplemented with lower concentrations of KIN (0.05, 0.1 $mg.l^{-1}$). The results are statistically significant (**Tables 5.5 and 5.5 A**).
f) 1/2 WPM with 0.05 $mg.l^{1}$ BAP with 83.33% sprouting and 1/2 WPM with 0.05 $mg.l^{-1}$ KIN with 75.77% sprouting are the optimal media (**Table 5.5**).
g) 1/2 DCR supplemented with lower concentrations of BAP (0.05, 0.1 $mg.l^{-1}$) gave higher sprouting percentage as compared to 1/2 DCR supplemented with lower concentrations of KIN (0.05, 0.1 $mg.l^{-1}$). The results are statistically significant (**Table 5.5 A**).
h) 1/2 DCR supplemented with lower concentrations of BAP (0.05, 0.1 $mg.l^{-1}$) gave higher sprouting percentage as compared to 1/2 WPM supplemented with lower concentrations of BAP (0.05, 0.1 $mg.l^{-1}$). The results are statistically significant (**Tables 5.5 and 5.5 A**).
i) 1/2 DCR supplemented with lower concentrations of BAP (0.05, 0.1 $mg.l^{-1}$) gave higher sprouting percentage as compared to 1/2 WPM supplemented with lower concentrations of KIN (0.05, 0.1 $mg.l^{-1}$). The results are statistically significant (**Tables 5.5 and 5.5 A**).

j) 1/2 DCR without plant growth regulators (1/2 DCR-H) gave 50% sprouting while 1/2 DCR supplemented with lower concentrations of BAP (0.05, 0.1 $mg.l^{-1}$) gave identical sprouting percentage (100%) and is similar to 1/2 DCR supplemented with 10% CM. Thus apparently the absence of low concentrations of BAP in 1/2 DCR medium can be compensated by addition of CM. This cytokinin-like activity of CM is further proved by the fact that when CM is supplemented with BAP, sprouting percentage decreases, apparently due to higher additive cytokinin activity by CM and BAP together (**Table 5.5 A**).

k) 1/2 DCR with 0.05 $mg.l^{-1}$ BAP or 1/2 DCR with only 10% CM but without additional cytokinins are the best media for primary sprouting of stem explants of *Taxus* spp. with 100% response (**Table 5.5 A**).

l) BAP is a better cytokinin with 1/2 DCR to achieve high sprouting (100%) and good shoot quality as compared to KIN (**Table 5.5 A**).

m) BAP is a better cytokinin with 1/2 WPM to achieve high sprouting but shoot quality and elongation capacity are not as good as shoot quality and elongation ability obtained with KIN (Table 5.5).

n) Hence KIN is a better cytokinin with 1/2 WPM even though sprouting is relatively low as compared to BAP but shoot quality and elongation ability are superior as compared to BAP (Table 5.5).

o) Higher cytokinin concentrations are not conducive for sprouting (**Tables 5.5 and 5.5 A**).

p) TDZ is not conducive for sprouting due to inability of the primary sprouts to elongate (**Table 5.2**).

q) Combination of an auxin with a cytokinin does not give enhanced sprouting but leads to callus formation (**Table 5.6**).

The responding explants developed either two or three primary sprouts in all the media combinations of 1/2 WPM and 1/2 DCR tried (with and without cytokinins) (**Fig. 5.6 C-E**) and even when the apical bud was present, its dominance was overcome by simultaneous sprouting of axillary buds (**Fig. 5.8 D-F**).

5.3.1.2 Repeatability and applicability of the protocol for primary sprouting to *Taxus* plant material collected from different locations (Trees of different ages, with variable macro-and micro-climatic conditions).

The protocol standardized for primary sprouting of mature shoot explants collected from Shimla was further extrapolated to plant material collected from different locations such as Khajjiyar and Almora. The aim was to check for the applicability of the standardized protocol with the plant material collected from Shimla to the plant materials collected from such diverse locations as Khajjiyar and Almora. This is important because it is desirable to have a uniform protocol so that it will have a wider applicability in propagation of plants, irrespective of parameters such as the age of parent plants, different collection locations, differences in natural habitats and different growth patterns due to differences in macro- and micro-climatic conditions at each of these locations.

The protocol standardized with plant material collected from Shimla with respect to basal media and plant growth regulator combinations (1/2 WPM with KIN at 0.01, 0.05, 0.1 and 0.5 $mg.l^{-1}$ and 1/2 DCR with BAP at 0.01, 0.05, 0.1 and 0.5 $mg.l^{-1}$) (C. f. **Section 5.3.1, Tables 5.5, 5.5 A**) were only tried for these experiments. The results obtained on culture of stem bit explants obtained from different locations, on 1/2 WPM-H and 1/2 WPM supplemented with optimized KIN concentrations as well as 1/2 DCR-H and 1/2 DCR supplemented with optimized BAP concentrations are presented in **Table 5.7**. Plant material collected from Khajjiyar gave identical percentage of sprouting (50%) in 1/2 WPM without plant growth regulators and 1/2 WPM supplemented with various KIN concentrations. But percentage of sprouting was significantly superior in 1/2 DCR medium without plant growth regulators (90%). The highest percentage of sprouting (68.57%) was obtained in 1/2 WPM supplemented with 0.5 $mg.l^{-1}$ KIN in plant material collected from Almora and it was statistically similar to 50% sprouting obtained with 1/2 DCR-H. All the other treatments gave statistically identical results.

Table 5.7 Effect of Different Plant Growth Regulator Concentrations on % Sprouting from Mature Explants Cultured on 1/2 WPM and 1/2 DCR Media (Plant material collected from Khajjiyar during 1998 and Almora, 1999).

No.	Basal Medium	Location for Collection	Plant Growth Regulator	Concentration ($mg.l^{-1}$)	% Sprouting Mean ± S.E.
1.	1/2 WPM	Khajjiyar	Nil (Control)	-	50 ± 0^{a}
2.			KIN	0.01	50 ± 10^{a}
3.				0.05	50 ± 10^{a}
4.				0.1	50 ± 20^{a}
5.				0.5	50 ± 10^{a}
6.	1/2 DCR		Nil (Control)	-	90 ± 10^{b}
7.			BAP	0.01	70 ± 5^{a}
8.				0.05	ND
9.				0.1	60 ± 10^{a}
10.				0.5	23.32 ± 10^{c}
11.	1/2 WPM	Almora	Nil (Control)	-	48.1 ± 28.1^{ac}
12.			KIN	0.01	56.85 ± 19.35^{ac}
13.				0.05	45.11 ± 20.11^{ac}
14.				0.1	40 ± 20^{ac}
15.				0.5	68.57 ± 11.43^{a}
16.	1/2 DCR		Nil (Control)	-	50 ± 0^{a}
17.			BAP	0.01	21.88 ± 3.1^{ac}
18.				0.05	$39.29 + 10.72^{ac}$
19.				0.1	48.54 ± 4.79^{ac}
20.				0.5	-

$^{a, b, c}$: Values followed by same letters are not statistically significant at 95% confidence level by t-test.

A compiled comparative study of sprouting response of stem bit explants of *Taxus* spp. collected from different locations (**Table 5.7**) on optimized media standardized with plant material collected from Shimla (**Tables 5.5, 5.5 A**) is presented in **Table 5.8**.

Following conclusions can be arrived at based on **Table 5.8**:

a) The statistically significant, highest percentage of sprouting of 83.33% amongst all the treatments was obtained on 1/2 WPM supplemented with 0.05 $mg.l^{1}$ BAP for plant material collected from Shimla. Similarly the statistically significant high percentage of sprouting of 75.77% amongst all the treatments was obtained on 1/2 WPM supplemented with 0.05 $mg.l^{1}$ KIN for plant material collected from Shimla.
b) 1/2 DCR medium supplemented with 0.05 and 0.1 $mg.l^{-1}$ BAP gave 100% sprouting for plant material collected from Shimla, which is the highest sprouting percentage obtained amongst all the media and collection locations. On the other hand, 1/2 DCR medium supplemented with various concentrations of KIN did not give significantly different results of sprouting as compared to control.
c) 1/2 WPM-H gave 50% sprouting with plant material collected from Khajjiyar which is statistically similar to 52.94% sprouting achieved with plant material collected from Shimla on the identical medium. Although 75.77% sprouting was achieved on 1/2 WPM with 005 $mg.l^{-1}$ KIN for plant material collected from Shimla (significantly higher than 52.94% sprouting achieved with control treatment), the sprouting percentage (50%) did not differ statistically between control and different KIN concentrations in plant material collected from Khajjiyar. 1/2 WPM-H gave 48.1% sprouting with plant material collected from Almora which is statistically different than 52.94% sprouting achieved with plant material collected from Shimla on the identical medium. But here also sprouting percentage did not differ significantly between control and different KIN concentrations and is similar to the observations with plant material collected from Khajjiyar. The only exception was 68.57% sprouting obtained with plant material collected from Almora, on 1/2 WPM supplemented with 0.5 $mg.l^{1}$ KIN that is similar to 52.94% sprouting obtained in 1/2 WPM-H with plant material collected from Shimla.
d) 1/2 DCR-H gave significantly higher sprouting at 90% for plant material collected from Khajjiyar while plant material collected from Shimla showed only 50% sprouting on the same medium. 1/2 DCR supplemented with BAP (especially 0.05

and 0.1 mg.l^{-1}) gave higher sprouting response for plant material collected from Shimla as compared to plant material collected from Khajjiyar. This response of plant material collected from Shimla was comparable to the response obtained on 1/2 DCR-H with plant material collected from Khajjiyar. 1/2 DCR-H gave identical sprouting at 50% for plant material collected from Almora as well as Shimla. 1/2 DCR supplemented with BAP, at all the tested concentrations, gave significantly higher sprouting response for plant material collected from Shimla as compared to plant material collected from Almora.

e) Thus although the general trends such as suitability of low salt media and low cytokinin concentrations for sprouting are identical for all plant materials collected from different locations such as Shimla, Khajjiyar and Almora, the best sprouting response is observed with plant material collected from Shimla on 1/2 DCR medium supplemented with 0.05 and 0.1 mg.l^{1} BAP. Only plant material collected from Khajjiyar gave 90% sprouting on 1/2 DCR-H which is significantly higher than the results obtained with plant material collected from Shimla on the same medium.

5.3.2 Elongation of primary shoots obtained from axillary buds of stem bit explants collected from all the locations

In all the optimized initiation media:

- 1/2 WPM and 1/2 DCR media without plant growth regulators as well as each supplemented individually with 0.01, 0.05 and 0.1 $mg.l^{-1}$ of BAP and KIN for plant material collected from **Shimla**,
- 1/2 WPM without plant growth regulators as well as supplemented individually with 0.01, 0.05 and 0.1 $mg.l^{-1}$ of KIN and 1/2 DCR medium without plant growth regulators as well as supplemented individually with 0.01, 0.05 and 0.1 $mg.l^{-1}$ of BAP for plant material collected from **Khajjiyar**,
- 1/2 WPM without plant growth regulators as well as supplemented individually with 0.01, 0.05 and 0.1 $mg.l^{-1}$ of KIN and 1/2 DCR medium without plant growth regulators as well as supplemented individually with 0.01, 0.05 and 0.1 $mg.l^{-1}$ of BAP for plant material collected from **Almora,**

the primary shoots elongated in their respective media to a length 0.5-1.0 cm during the first two months. For further elongation, such primary shoots were transferred to their respective basal media without plant growth regulators in the third month, where they elongated to a length of 3 cm. **Table 5.9** presents these results for all the three collection locations. These shoots were orthotropic, with short internodes and soft, light green colored needles, thus exhibiting a number of juvenile characteristics. The shoots were healthy without yellowing of needles and hyperhydricity and also withstood the **"Modified Surface Sterilization Protocol"** employed to combat contamination, indicating that the **"Modified Surface Sterilization Protocol"** is not phytotoxic to explants of *Taxus* spp. when cultured under *in vitro* conditions.

Figs. 5.6-5.9 depict the results obtained with various basal media and plant growth regulators for primary shoot elongation. The photographs represent primary sprouts either during the second or the third monthly transfers.

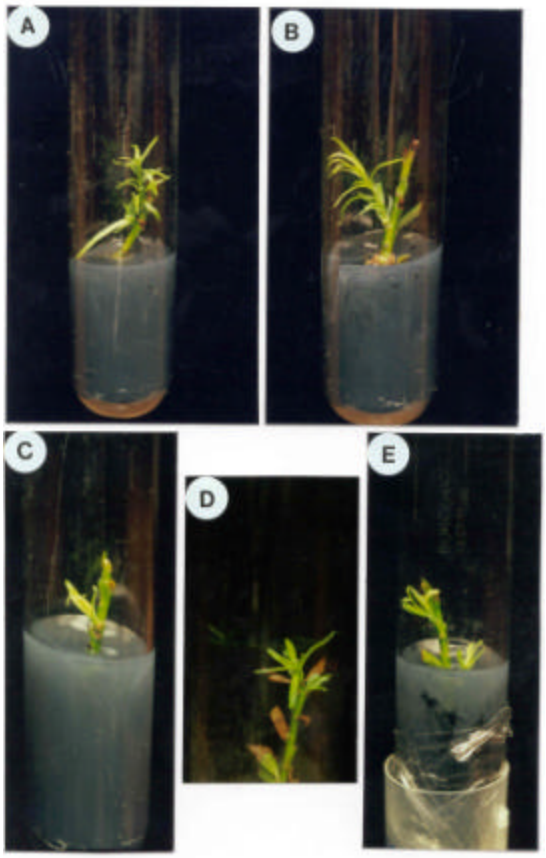


Fig. 5.6

Figs. A – D Elongation of primary sprouts on 1/2 Strength WPM basal medium with or without plant growth regulators during second month of subculture.

A. Elongation of an axillary sprout on 1/2 WPM without plant growth regulators (1/2 WPM – H).
B. Elongation of an axillary sprout on 1/2 WPM –H.
C. Elongation of an axillary sprout on 1/2 WPM containing 0.01 $mg.l^{-1}$ KIN.
D. Elongation of two axillary sprouts on 1/2 WPM containing 0.01 $mg.l^{-1}$ KIN.
E. Elongation of two axillary sprouts on 1/2 WPM containing 0.05 $mg.l^{-1}$ KIN.

Fig. 5.7

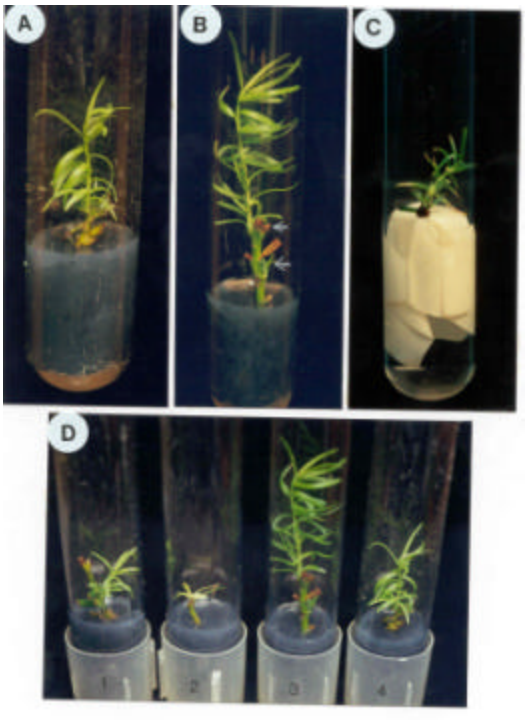
Figs. A – C Elongation of primary sprouts on 1/2 strength WPM basal medium with 0.1 mg.l^{-1} KIN during third month of subculture.

A. An elongated axillary sprout on 1/2 WPM with 0.1 mg.l^{-1} KIN.

B. An elongated axillary sprout and a small axillary sprout (arrow) on 1/2 WPM with 0.1 mg.l^{-1} KIN. Also note the swollen apical buds (arrow *).*

C. A separated and established primary sprout growing on liquid 1/2 WPM – H but with 10% CM and 1 g.l^{-1} CH.

D. A comparison of primary sprouting on 1/2 WPM with or without KIN (Tube no. 1 is control without plant growth regulators (1/2 WPM – H), Tube no. 2 is 1/2 WPM with 0.05 mg.l^{-1} KIN, Tube no. 3 is 1/2 WPM with 0.1 mg.l^{-1} KIN and Tube no. 4 is 1/2 WPM with 0.5 mg.l^{-1} KIN.



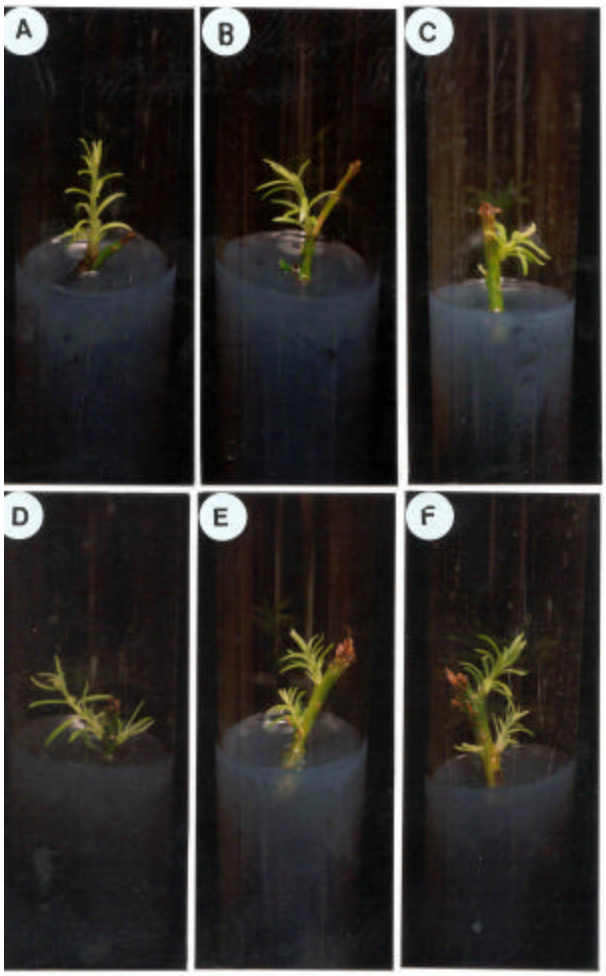


Fig. 5.8

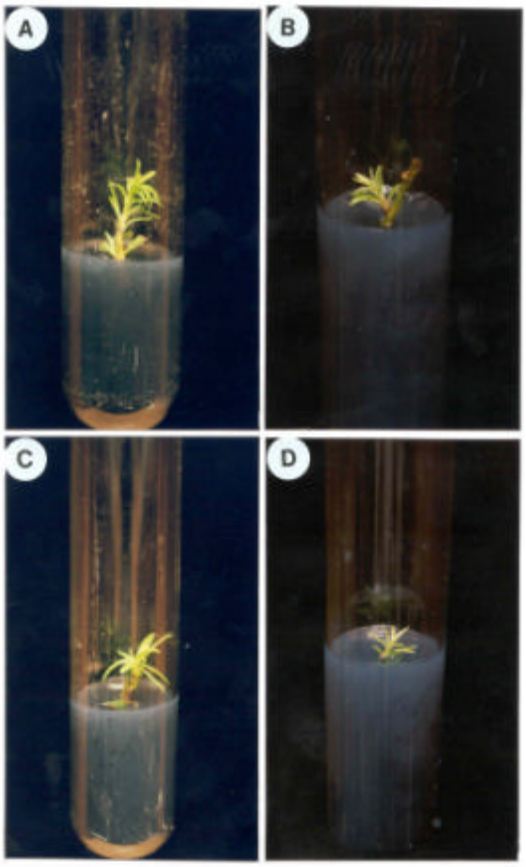
Figs. A – F Elongation of primary sprouts on 1/2 Strength DCR basal medium without plant growth regulators (1/2 DCR – H) during second month of subculture.

A. Elongation of a single apical sprout.
B. Elongation of a single axillary sprout.
C. Elongation of a single axillary sprout.
D. Simultaneous elongation of two axillary sprouts.
E. Simultaneous elongation of two axillary sprouts.
F. Simultaneous elongation of three axillary sprouts.

Fig. 5.9

Figs. A – D Elongation of primary sprouts on 1/2 Strength DCR basal medium supplemented with 0.05 mg.l^{-1} BAP during second month of subculture.

A. Elongation of a single apical sprout.
B. Elongation of a single axillary sprout.
C. Elongation of a single axillary sprout.
D. Separation of a sprout from the mother plant and its independent growth in the identical medium.



5.3.3 Establishment of shoots obtained from axillary buds of stem bit explants collected from all the locations

The elongated primary shoots of a length of 3 cm, growing on 1/2 WPM-H and 1/2 DCR-H media, were cut at the end of third month from the parent explants and transferred to identical media and to media combinations used for growth of primary shoots during the first two monthly transfers (See **Table 5.9**, **Columns D, E and F**). While the primary shoots failed to establish on their transfer from 1/2 WPM-H and 1/2 DCR-H media to the same respective media, the primary shoots established very well and showed sustained growth on their respective basal media with or without plant growth regulator combinations, but each supplemented further with 10% CM and 1g.l^{-1} CH. (**Table 5.9**). 20% of the cytokinin activity of coconut milk has been attributed to a water-soluble compound: G_3A_2-ZR, *14*-O-{3-O-[β-D-galactopyranosyl-(1→2)-α-D-galactopyranosyl-(1→3)-α-L-arabinofuranosyl]-4-O-(α-L-arabinofuranosyl)-β-D-galactopyranosyl}-*trans*-zeatin riboside. Its cytokinin activity might be elicited after its hydrolysis to zeatin in cells or it might be preferentially taken up due to its water solubility (Kobayashi et al., 1995). It has also been reported that reduced nitrogen added as a mixture of amino acids e.g. CH, is extremely growth stimulating (Gupta, 1982). In the basal media without CM and CH, but with plant growth regulators added individually, the shoot tips became necrotic and then died.

Figs. 5.7 C, 5.9 D and 5.10 A depict the results obtained with various basal media, plant growth regulators with CM + CH in shoot establishment The photographs represent established primary sprouts during their fourth month of growth.

Table 5.9 Elongation and Establishment of Primary Shoots Derived by Enhanced Axillary Budding from Mature Stem Explants of *Taxus* spp.

No.	Collection Location	Initiation Medium for Primary Sprouting	Elongation Medium for Primary Shoots		Establishment Medium for Primary Shoots During the Fourth Monthly Transfer	Response
			During First Two Monthly Transfers	During the Third Monthly Transfer		
A	B	C	D	E	F	G
1.	Shimla, Khajjiyar & Almora	1/2 WPM-H	1/2 WPM-H	1/2 WPM-H	1/2 WPM-H	No shoot establishment
2.	Shimla, Khajjiyar & Almora	1/2 WPM-H	1/2 WPM-H	1/2 WPM-H	1/2 WPM-H + 10% CM + 1 $g.l^{-1}$ CH	Successful shoot elongation & establishment
3.	Shimla	1/2 WPM + 0.01, 0.05 and 0.1 $mg.l^{-1}$ BAP individually	1/2 WPM + 0.01, 0.05 and 0.1 $mg.l^{-1}$ BAP individually	1/2 WPM-H	1/2 WPM + 0.01, 0.05 and 0.1 $mg.l^{-1}$ BAP individually	No shoot establishment
4.	Shimla	1/2 WPM + 0.01, 0.05 and 0.1 $mg.l^{-1}$ BAP individually	1/2 WPM + 0.01, 0.05 and 0.1 $mg.l^{-1}$ BAP individually	1/2 WPM-H	1/2 WPM + 0.01, 0.05 and 0.1 $mg.l^{-1}$ BAP individually + 10% CM + 1 $g.l^{-1}$ CH	Successful shoot elongation & establishment
5.	Shimla, Khajjiyar & Almora	1/2 WPM + 0.01, 0.05 and 0.1 $mg.l^{-1}$ KIN individually	1/2 WPM + 0.01, 0.05 and 0.1 $mg.l^{-1}$ KIN individually	1/2 WPM-H	1/2 WPM + 0.01, 0.05 and 0.1 $mg.l^{-1}$ KIN individually	No shoot establishment
6.	Shimla, Khajjiyar & Almora	1/2 WPM + 0.01, 0.05 and 0.1 $mg.l^{-1}$ KIN individually	1/2 WPM + 0.01, 0.05 and 0.1 $mg.l^{-1}$ KIN individually	1/2 WPM-H	1/2 WPM + 0.01, 0.05 and 0.1 $mg.l^{-1}$ KIN individually + 10% CM + 1 $g.l^{-1}$ CH	Successful shoot elongation & establishment

Contd…..

Table 5.9 Contd.

No.	Collection Location	Initiation Medium for Primary Sprouting	Elongation Medium for Primary Sprouting		Establishment Medium for Primary Sprouts During the Fourth Monthly Transfer	Response
			During First Two Monthly Transfers	During the Third Monthly Transfer		
A	B	C	D	E	F	G
7.	Shimla, Khajjiyar & Almora	1/2 DCR-H	1/2 DCR-H	1/2 DCR-H	1/2 DCR-H	No shoot establishment
8.	Shimla, Khajjiyar & Almora	1/2 DCR-H	1/2 DCR-H	1/2 DCR-H	1/2 DCR-H+ 10% CM + 1 $g.l^{-1}$ CH	Successful shoot elongation & establishment
9.	Shimla, Khajjiyar & Almora	1/2 DCR + 0.01, 0.05 and 0.1 $mg.l^{-1}$ BAP individually	1/2 DCR + 0.01, 0.05 and 0.1 $mg.l^{-1}$ BAP individually	1/2 DCR-H	1/2 DCR + 0.01, 0.05 and 0.1 $mg.l^{-1}$ BAP individually	No shoot establishment
10.	Shimla, Khajjiyar & Almora	1/2 DCR + 0.01, 0.05 and 0.1 $mg.l^{-1}$ BAP individually	1/2 DCR + 0.01, 0.05 and 0.1 $mg.l^{-1}$ BAP individually	1/2 DCR-H	1/2 DCR + 0.01, 0.05 and 0.1 $mg.l^{-1}$ BAP individually + 10% CM + 1 $g.l^{-1}$ CH	Successful shoot elongation & establishment
11.	Shimla	1/2 DCR + 0.01, 0.05 and 0.1 $mg.l^{-1}$ KIN individually	1/2 DCR + 0.01, 0.05 and 0.1 $mg.l^{-1}$ KIN individually	1/2 DCR-H	1/2 DCR + 0.01, 0.05 and 0.1 $mg.l^{-1}$ KIN individually	No shoot establishment
12.	Shimla	1/2 DCR + 0.01, 0.05 and 0.1 $mg.l^{-1}$ KIN individually	1/2 DCR + 0.01, 0.05 and 0.1 $mg.l^{-1}$ KIN individually	1/2 DCR-H	1/2 DCR + 0.01, 0.05 and 0.1 $mg.l^{-1}$ KIN individually + 10% CM + 1 $g.l^{-1}$ CH	Successful shoot elongation & establishment
13.	Shimla	1/2 DCR + 10% CM	1/2 DCR + 10% CM	1/2 DCR-H	1/2 DCR-H + 10% CM + 1 $g.l^{-1}$ CH	Successful shoot elongation & establishment

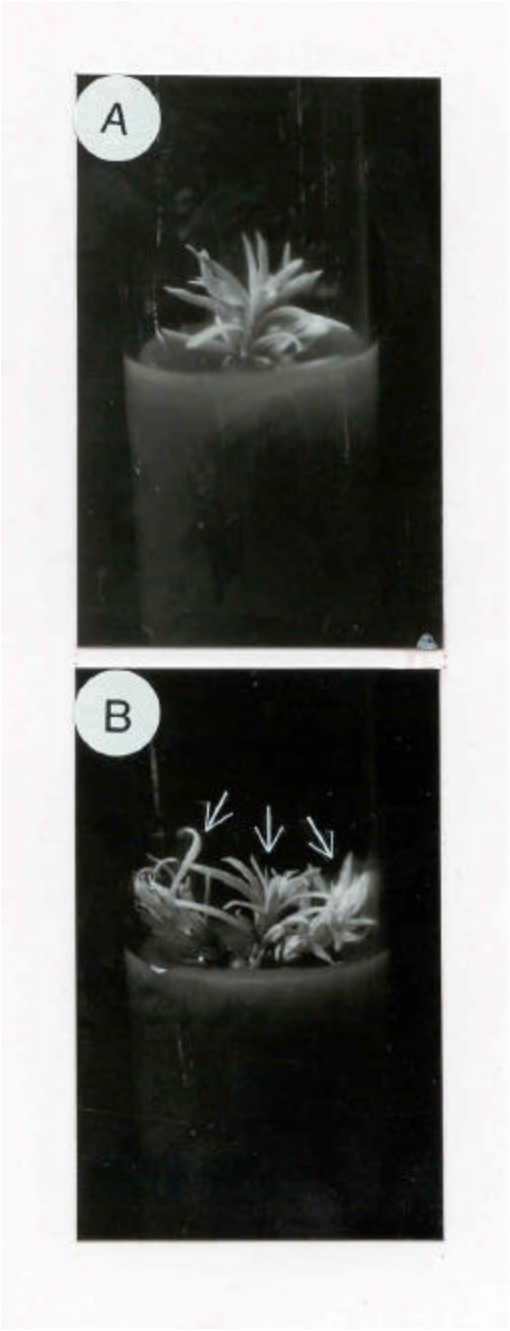


Fig. 5.10 Establishment of primary shoot and initiation of secondary sprouting.

A. Independent establishment and growth of a primary shoot in 1/2 WPM-H supplemented with 10% CM and 1 g.l^{1} CH (during fourth month).

B. The same primary shoot (central arrow) showing two new, *de novo* secondary shoots when subcultured onto 1/2 WPM supplemented with 0.1 mg.l^{-1} KIN + 10% CM + 1g.l^{-1} CH.

5.3.4 Second order sprouting

The established primary sprouts on various media as described in **Table 5.9** were transferred on respective identical media in the fifth month. Second order sprouting (2 new, secondary shoots per each shoot) at a frequency of 25% was observed only on 1/2 WPM supplemented with 0.1 $mg.l^{-1}$ KIN, 10% CM and 1 $g.l^{-1}$ CH. None of the other media used for transfers (C. f. **Table 5.9**) induced second order sprouting from the buds formed *de novo* on primary sprouts *in vitro*. **Fig. 5.10 B** shows second order sprouting.

The remaining cultures of primary shoots that did not develop secondary sprouts did not undergo any further development except for elongation to a length of 7-8 cm in 8 monthly transfers.

During the entire course of this experimentation, 500-600 primary shoots were obtained on various optimal nutrient media, out of which 300 primary shoots could be established and out of them 25 showed development of primary sprouts.

5.3.5 Rooting

Rooting of elongated primary or secondary sprouts has not been achieved. Preliminary experiments were carried out by inoculating the primary sprouts in 1/4 WPM and 1/4 DCR (Major salts of both the media reduced to 1/4 strength) without plant growth regulators and/or supplemented with different concentrations and combinations of IAA, IBA and NAA. But in none of the media rooting was achieved. The great age of the mother trees and the possible recalcitrance of the explants might be reasons for this. Rooting of tissue cultured conifers is considered problematic (Mohammed and Vidaver, 1988). It has also been reported that with repeated trnasfers the degree of rejuvenation and hence the responsiveness of such explants increases (Franclet et al., 1987). The cultures maintained in the laboratory have undergone only 8 transfers and the response may increase with passage of time. Further studies need to be undertaken on this aspect.

5.4 Conclusions

1) **A protocol has been standardized for enhanced axillary budding of mature stem bit explants of *Taxus baccata* ssp. *wallichiana* Zucc. Pilg. Primary and secondary shoots have been obtained at a high frequency within six months.**
2) **Basal media with low total ionic salt strengths and lower ratios of ammonium nitrogen to nitrate nitrogen such as 1/2 WPM and 1/2 DCR are conducive for enhanced axillary budding of mature explants of *Taxus* spp.**
3) **Organic nitrogen in the form of an amino acid like glutamine does not enhance axillary sprouting in *Taxus* spp.**
4) **1/2 WPM supplemented with 0.05 mg.l^{-1} BAP gave 83.33% sprouting while 1/2 WPM supplemented with 0.05 mg.l^{-1} KIN gave 75.77% sprouting in plant material collected from Shimla. The former percentage of sprouting was statistically higher than the latter.**
5) **1/2 DCR medium supplemented with lower concentrations of BAP (0.05, 0.1 mg.l^{-1}) gave 100% sprouting percentage as compared to 58.89% and 54.21% sprouting obtained on 1/2 DCR medium supplemented with lower concentrations of KIN (0.05, 0.1 mg.l^{-1}) with plant material collected from Shimla. The results are statistically significant.**
6) **1/2 DCR medium without plant growth regulators (1/2 DCR-H) gave 50% sprouting while 1/2 DCR supplemented with lower concentrations of BAP (0.05, 0.1 mg.l^{-1}) gave identical sprouting percentage (100%) that is similar to sprouting percentage obtained with 1/2 DCR supplemented with 10% CM. Thus apparently the absence of low concentrations of BAP in 1/2 DCR medium can be compensated by addition of CM.**
7) **BAP is a better cytokinin with 1/2 WPM to achieve high sprouting but shoot quality and elongation capacity is not as good as shoot quality and elongation ability obtained with KIN. So KIN is the cytokinin of choice with 1/2 WPM.**
8) **Higher cytokinin concentrations are not conducive for sprouting.**

9) TDZ is not conducive for sprouting due to inability of the primary sprouts to elongate.

10) Combination of an auxin with a cytokinin is not conducive for enhanced axillary budding of mature stem explants of *Taxus* spp.

11) The standardized protocol of primary sprouting can be extrapolated successfully to plant material collected from Khajjiyar and Almora, despite the plants being of different ages and growing under different macro- and micro-environmental conditions than the plants at Shimla.

12) 1/2 WPM-H gave 50% sprouting with plant material collected from Khajjiyar, statistically similar to 52.94% sprouting achieved with plant material collected from Shimla on an identical medium. Although 75.77% sprouting was achieved on 1/2 WPM with 0.05 mg.l^{-1} KIN with plant material collected from Shimla, sprouting percentage did not differ statistically with KIN concentrations with plant material collected from Khajjiyar.

13) 1/2 DCR-H gave significantly higher sprouting at 90% with plant material collected from Khajjiyar while plant material collected from Shimla had only 50% sprouting on the same medium. 1/2 DCR supplemented with BAP gave higher sprouting response with Shimla material as compared to Khajjiyar material.

14) 1/2 WPM-H gave 48% sprouting with plant material collected from Almora, statistically different than 52.94% sprouting achieved with plant material collected from Shimla on an identical medium. A big standard error of mean was obtained with Almora material hinting at underlying variabilities of the plant material. Although 75.77% sprouting was achieved on 1/2 WPM with 0.05 mg.l^{-1} KIN with plant material collected from Shimla, sprouting percentage did not differ statistically with KIN concentrations with plant material collected from Almora.

15) 1/2 DCR-H gave identical sprouting at 50% with plant material collected from Almora as well as Shimla. 1/2 DCR medium supplemented with BAP gave significantly higher sprouting response with Shimla material as compared to Almora material.

16) **Thus although the general trends such as suitability of low salt media and low cytokinin concentrations for sprouting are identical in plant material collected from Shimla, Khajjiyar and Almora, the best sprouting response is observed with plant material collected from Shimla. Only plant material collected from Khajjiyar gave 90% sprouting on 1/2 DCR-H which is significantly higher than the results of sprouting obtained with plant material collected from Shimla.**

17) **Elongation of primary sprouts into primary shoots of length 3 cm was possible on respective basal media without plant growth regulators in the cultures established from plants from all the locations in three months time.**

18) **Independent establishment of primary shoots was possible on respective basal media supplemented with 10% CM and 1 g.l^{-1} CH in the fourth month.**

19) **Secondary sprouting of buds developed *in vitro* on primary shoots was achieved on 1/2 WPM supplemented with 0.1 mg.l^{-1} KIN, 10% CM and 1 g.l^{-1} CH at a frequency of 25% during the fifth month.**

20) **"Modified Surface Sterilization Protocol" developed was not phytotoxic to the explants as primary and secondary sprouts were healthy and gave good *in vitro* response.**

PART B: *IN VITRO* STUDIES WITH EMBRYO EXPLANTS OF *TAXUS* SPP.

5.5 Introduction

5.5.1 Dormancy

Seed dormancy is defined as the absence of germination of an intact, viable seed under germination favoring conditions within a specified time lapse (Hilhorst, 1995). It is controlled both at the metabolic and the genomic levels. It is an adaptive trait that optimizes the distribution of germination over a period of time in a population of seeds (Bewley, 1997). Usually two types of dormancy are recognized, based on timing rather than the exogenous or endogenous dormancy-controlling factors (Hilhorst, 1995):

1. Primary dormancy: Related to seed development and maturation. Such seeds when dispersed from parent plant are in a dormant state.
2. Secondary dormancy: Occurs after seed dispersal and is subjected to annual dormancy cycles in the seed bank and is reversible.

Both developmental arrest of growth and primary dormancy have been associated with the presence of *in situ* ABA and sensitivity to ABA during seed development (Hilhorst, 1995; Bewley, 1997). After harvesting and cleaning, most seeds require drying to an appropriate seed moisture content prior to cold storage, to stabilize the dormant state. Based on this, the seeds are grouped in two classes (Chao and Lin, 1996):

I. Orthodox Seeds – Tolerant of desiccation. Their longevity increases with decreasing storage temperature and moisture contents.

II. Recalcitrant Seeds – Desiccation sensitive. High moisture content is required for maintaining seed viability.

In general, dry storage at elevated temperatures (**'After-ripening'**) is very effective in breaking primary dormancy as ABA is degraded at higher temperatures. **'Pre-chilling'** is another way to remove ABA by leakage. Endogenous ABA contents are also lowered by red light irradiation and exogenous or endogenous GAs. Thus the "Hormone Balance Theory" still appears to be valid, in that ABA action during development or as a remnant in the mature seed may be antagonized by GA which is synthesized upon

germination. This process is further aided by low temperatures. The role of GA is probably restricted to the induction of endosperm-weakening enzymes (Bewley, 1997).

5.5.2 Germination

By definition, germination incorporates those events that commence with the uptake of water by the quiescent dry seed and terminate with the elongation of the embryo axis (Bewley, 1997). Visible germination occurs after penetration of the structures surrounding the embryo by the radicle.

Uptake of water by a mature dry seed is triphasic where a rapid initial uptake (Phase I) is followed by a plateau phase (Phase II) and a further increase in water uptake after germination, when the embryonic axes elongate (Phase III) (Bewley and Black, 1994). Upon imbibition, metabolic activity is rapidly resumed with turnover or replacement of necessary components. At this stage, the seeds are perceptible for external stimuli like light, chilling, alternating temperatures, chemical and hormonal treatments. The stimuli may act at the level of transcription. Most of the receptors for these stimuli are believed to be membrane-bound. The primary events in the release from dormancy are the reception of the stimulus by the embryo and the immediate signal transduction chain that leads to the secondary events involving metabolic and hormonal changes (Bewley, 1997).

5.5.3 Dormancy and Germination of *Taxus* spp. Seeds

Plants belonging to *Taxus* spp. commonly produce many more ovules than seeds. But ovule attrition occurs due to: inadequate pollination, resource limitation, genetic load, loss of seeds to predators and pathogens and physical damage from abiotic factors like frost. Sine these are understory plants, light availability due to overstory canopy becomes critical. Successful sexual reproduction occurs only beneath overstory gaps where sufficient light levels are available (DiFazio et al., 1998). *Taxus* spp. plants are dioecious and the numbers of male plants are limited, affecting the success of pollination. Pollination is also affected by wind, weather conditions, microclimate, genetic load and maternal effects (DiFazio et al., 1998). The mature seeds of *Taxus* are enclosed in an edible red aril and hence undergo heavy predation. Seed production in the Himalayan Yew shows a periodical effect. During some years, more seeds are produced while at

other times; very low seed production is observed (Dr. O. Singh, Personal Communication). All these factors contribute towards very low production of functional seeds, which can be collected in a growing season.

Storage of *Taxus* spp. in germplasm banks *ex situ* may help to preserve the threatened diversity of this important genus. The high level of dormancy, the small size of embryo and inadequate supply of seeds have hampered the efforts to gain knowledge about storage characteristics of *Taxus* seeds and also the large scale use of seeds and embryos for *in vitro* experiments to exploit the characters of this juvenile tissue. All these facts have limited the number of experiments which could be carried out on embryos during the given period of time of the present thesis.

The development of *Taxus* seeds is asynchronous and a single female tree shows seeds in all stages of developments. Fully mature seeds of *Taxus* spp. survive almost complete desiccation and if adequately dried and stored at 14% relative humidity (RH), they survive sub-freezing temperatures without any lethal injuries, for a long period of time Here longevity is projected to increase with reductions in temperatures This is further aided by extremely high lipid contents (71%) of the seeds. This suggests that these seeds exhibit orthodox rather than recalcitrant or intermediate storage characteristics. Immature embryos can be partially dried to avoid desiccation damage and then stored at 5°-6°C. For short-term storage, *Taxus* seeds can be placed under prolonged stratification period where catabolism is limited and dormancy is eventually broken. Here seed viability can be maintained for a few years (Walters-Vertucci et al., 1996).

Naturally, *Taxus* seeds have a dormancy requirement of at least two long freezing seasons before germination can occur. Vernalization at low temperatures can help to overcome this dormancy (Hartzell, 1991). Germination *ex vitro* has been reported to require alternating temperatures and cold stratification for 8.5 months to achieve 50% germination (Chang and Yang, 1996).

Embryo culture *in vitro* is the sterile isolation and growth of an immature or mature embryo, with the aim of obtaining a complete, viable plant (Pierik, 1987). It can be a useful tool to overcome seed dormancy and stratification problems as well as to abbreviate the breeding cycle. In case of endangered spp. like *Taxus* it can serve the additional function of providing the planting material for replenishing the dwindling

natural populations of trees while maintaining the natural genetic variation present in the species. It can also serve as a physiological tool in identifying the various factors involved in dormancy and germination of these plants. The success of embryo culture depends on a number of factors such as genotype, developmental stage of embryo at isolation, growth conditions of mother plant, composition of nutrient media, oxygen availability, light and temperature (Pierik, 1987).

A number of studies have been carried out in embryo culture of *Taxus* spp. as presented earlier in **Table 5.1**. But till now there are no reports on precocious germination of embryos of the Himalayan Yew. The present chapter describes studies carried out to overcome the natural dormancy of the Yew seeds by removing the embryos from the seeds and by culturing them on various nutrient media under a range of incubation conditions.

The objectives of the present work were as follows:

1. To standardize a protocol for high frequency precocious germination and seedling development from excised embryos of immature and mature seeds of *Taxus* spp.
2. To extrapolate the protocol developed to seeds collected from different locations.
3. To initiate and maintain fast-growing haploid callus cultures derived from endosperm of the seeds and
4. To analyze for the possible production of **Taxanes** therefrom.

5.6 Material and Methods

5.6.1 Collection and storage of seeds

The seeds were collected during the months of September and October from the locations mentioned earlier in **Chapter 4, Section 4.2.1**. At the time of collection the seeds were in various developmental stages (**Fig. 5.11 A**). The immature seeds without any aril development were stored at 4°C in zip-locked polythene bags until use. Wherever aril development was observed (**Fig. 5.11 B**), care was taken to remove the aril. Such seeds were then immersed in water and only the filled seeds (those sinking to the bottom in water in a glass beaker) were stored at 4°C in zip-locked polythene bags until use, after drying them on filter papers at room temperature for one day.

5.6.2 Excision and inoculation of embryos and endosperm

After the entire seeds were surface sterilized according to the **"Modified Surface Sterilization Protocol"** (**Chapter 3, Flow Chart 3.3**), they were imbibed overnight in sterile distilled water on an orbital shaker ("Steelmate", India) at 90 rpm at 25 $\pm$ 2°C.

On the next day, the imbibed seeds were rinsed with sterile water and the embryos were excised by cutting the seeds longitudinally. The embryos were rinsed in sterile water to remove adhering tissues of endosperm and inoculated in various media.

In immature seeds, the endosperm was either liquid or amorphous and hence could not be inoculated while in mature seeds the endosperm was relatively compact and solid and could be easily removed and inoculated for *in vitro* experiments.

5.6.3 Media and culture conditions

5.6.3.1 Precocious embryo germination and seedling development

The following media without plant growth regulators were used for embryo germination experiments and the details of their compositions were as given in **Chapter 2, Table 2.1;** ⟨DCR medium, B5 medium, B5 medium with SH vitamins (B5SH medium), DCR medium with SH vitamins (DCRSH medium), WPM medium and PR medium with half strength major salts of B5 + full strength minor salts of B5 + full

strength vitamins of SH medium (Chee, 1995). All these media contained 3% sucrose and 0.3% phytagel and the details of media preparation were as described in **Chapter 2, Section 2.4**.

For the establishment and further growth of the seedlings media identical to germination media but supplemented with 10% CM were used.

Two excised embryos were inoculated per each culture tube or five embryos were inoculated per each dish of 55 mm diameter. For further growth of the seedlings they were transferred to culture tubes individually one month after germination. The cultures were incubated either in total darkness or in light (19-38 $\mu Em^{-2}s^{-1}$) with 16 h photoperiod, at 25 + 2°C.

5.6.3.2 Adventitious bud induction

Following media were used for experiments on adventitious bud induction: B5SH or DCRSH at pH 5.8, supplemented individually with either 1.5 or 2.5 or 3.5 $mg.l^{-1}$ BAP. These media without plant growth regulators served as controls. All these media contained 3% sucrose and 0.3% phytagel. Subcultures were done on their respective induction media without plant growth regulators.

Two excised embryos were inoculated per each culture tube and the cultures were incubated in light (19-38 $\mu Em^{-2}s^{-1}$) with 16 h photoperiod, at 25 $\pm$ 2°C.

5.6.3.3 For endosperm culture

Six endosperm halves were inoculated in each of the 55 mm sterile plastic dishes containing 10 ml of DCR or B5 medium with 2% (w/v) sucrose, 0.3 % (w/v) phytagel and supplemented with 2, 4, 6, 8 or 10 $mg.l^{1}$ of Picloram (P). Some of the subculture media contained 0.5% AC to prevent phenolic browning.

Endosperm cultures were always incubated in total darkness at 25 $\pm$ 2°C.

5.6.4 Growth analysis of endosperm-derived callus cultures

Growth analyses of callus cultures derived from endosperm, by means of fresh and dry weights, were performed as described earlier in Chapter 4, Section 4.2.5.

5.6.5 TLC and HPLC analysis of endosperm-derived callus cultures and media containing them

All the procedures related to extraction of callus tissues, their primary screening by TLC and quantitation of Taxane contents in promising callus lines by HPLC and the results obtained were described in detail in Chapter 4, Sections 4.2.6 and 4.3.2.

5.6.6 Statistical analysis

In optimization experiments of nutrient media, each medium and plant growth regulator combination (experimental unit) consisted of 5 plastic dishes (replicate), each dish containing either 5 embryos or endosperm halves. The experiments were repeated over three successive years for each of the trees. The treatments were analyzed by one-way ANOVA and the individual treatment means were compared by Student's *t*-test (Wardlaw, 1985).

5.7 Results and Discussion

5.7.1 Precocious germination and seedling development

Initial experiments on precocious embryo germination were performed with seeds collected from the Khajjiyar region of Himachal Pradesh. The seeds were either light green in color with liquid endosperm or dark green in color with solid endosperm. In the former stage of development, embryo was extremely small and could not be dissected out easily. But in the latter developmental stage, the embryo was white or light yellow, 2 mm long, torpedo shaped with a distinct suspensor (**Fig. 5.11 C**) and could be easily separated from the solid endosperm. The two halves of endosperm also could be inoculated separately.

Embryos excised from the seeds and inoculated immediately after collection on DCR medium without plant growth regulators failed to germinate. Hence the seeds were stored at 4°C for one month and then the excised embryos were inoculated on the same medium. This gave high values of germination on DCR medium (**Table 5.11**). The pre-chilling probably helped in the inactivation of ABA in the embryos, which has been implicated in the dormancy of embryos of *Taxus* spp. (Le Page-Degivry, 1968, 1973a, b). It has also been suggested that at the time of seed-shedding, the embryos are not fully developed (Has been confirmed in the present study by the torpedo shape of the excised embryos) and hence need to undergo two years growth (**'After-ripening'**) before they could germinate (Wareing and Phillips, 1990; Hartzell, 1991). Thus *Taxus* spp. exhibit **'Primary Dormancy'**.

In the present case, the pre-chilled embryos were inoculated in DCR medium without plant growth regulators at pH 5.8 based on the preliminary experiments where DCR medium was proven to be superior to achieve precocious germination in *Taxus* embryos by Flores and Sgrignoli (1991). The embryos were either inoculated in culture tubes with cotton plugs or in plastic dishes sealed hermetically to determine the effect of culture vessel on the percentage of germination. The cultures were incubated either in darkness or in light to test the effect of light on germination. The obtained results are presented in **Table 5.10**. It is clear from this table that the percentage of precocious germination (**Precocious germination was scored as swelling of embryo followed by radicle emergence and greening of cotyledons**) is not significantly different amongst

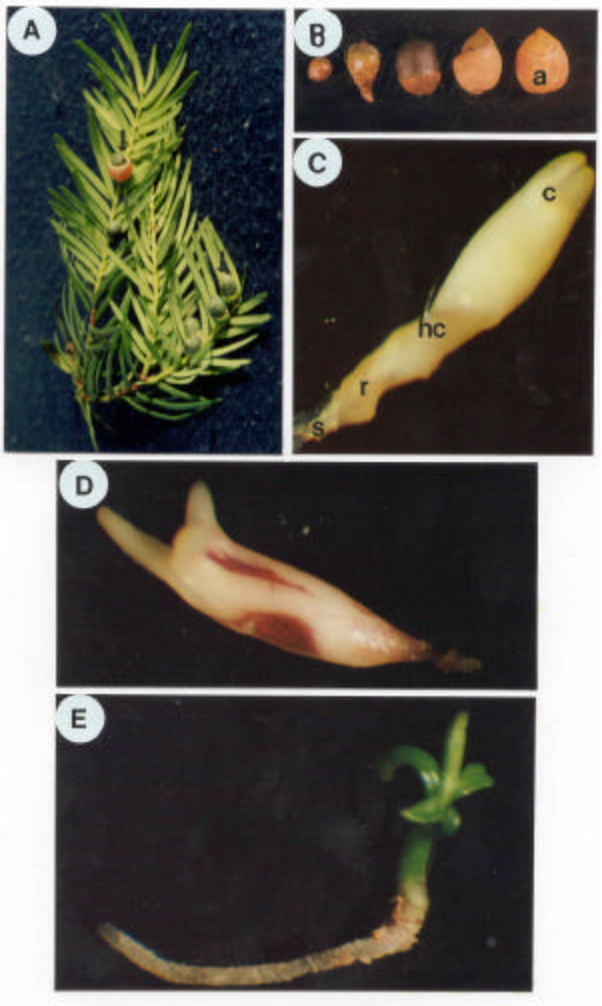


Fig. 5.11 Seeds, Embryos and Seedlings of *Taxus baccata* ssp. *wallichiana* Zucc. Pilg.

A. A twig of female plant showing asynchronous development of seeds. Note the presence of different stages of seed development on the same twig on the abaxial side (From green aril to red aril).

B. Seeds arranged in increasing order of maturity from left to right. The last three stages of seed development yielded germinable embryos. (a: aril).

C. A freshly dissected embryo (Magnification: 100x). c: cotyledons, hc: hypocotyl, r: root, s: suspensor.

D. A newly germinated embryo. Note the greening and opening of cotyledons, elongation of root pole and typical red colored spots in the region of hypocotyl (Magnification: 40x).

E. A 35 day old normal seedling (Magnification: 10x).

the different treatments at 95% confidence level by t-test. But the embryo development was normal and vigorous with fully expanded cotyledons in culture tubes under light (**Fig. 5.12 C**) and hence for all further experiments, DCR medium, in culture tubes at pH 5.8 and incubation of embryos under a photoperiod of 16 h were used. Germination of embryos occurred in 15-20 days in light (**Fig. 5.11 D, E**). But in dark, although root emergence occurred with high frequency, further development of cotyledons and seedlings was highly irregular and many of the embryos produced callus (**Fig. 5.12 A, B and Fig. 5.13 B, C**). These results are in agreement with an earlier study (Flores et al., 1993). In the present study, along with a high germination rate (75-100%), seedling development rate was also very high (**Table 5.10**). All the germinated embryos, under optimal conditions, gave rise to seedlings that were completely normal and vigorously growing (**Fig. 5. 12 C-F and Fig. 5.13 A**). Such a high rate of germination and seedling development has not been reported earlier. The germination percentages in earlier studies ranged between 27-70% and percentage of seedling development was still lower depending upon the species and culture conditions (Flores and Sgrignoli, 1991; Flores et al., 1993, Chee, 1994). However Zhiri et al. (1994) and Chang and Yang (1996) have reported germination and seedling development in the range of 95-100%.

Table 5.10 % Germination and Seedling Development of Khajjiyar Seeds on DCR Medium without Plant Growth Regulators at pH 5.8 in Plastic Dishes and Culture Tubes and in Darkness and Light.

No.	Seed Pre - treatment	Culture Conditions		% Germination & Seedling Development. Mean $\pm$ S. E.
		Culture Vessel	Light Conditions	
1.	No pre-chilling.	Culture tubes	16 h photoperiod.	0 ± 0^{a}
2.	Pre-chilling at 4°C for one month.	Plastic dishes	16 h photoperiod.	75 ± 25^{b}
3.		Plastic dishes	Darkness.	90 ± 10^{b}
4.		Culture tubes	16 h photoperiod.	90.91 ± 9.09^{b}
5.		Culture tubes	Darkness.	100 ± 0^{b}

[a, b] : Values followed by the same letters are not significantly different from each other at 95% confidence level by t-test.

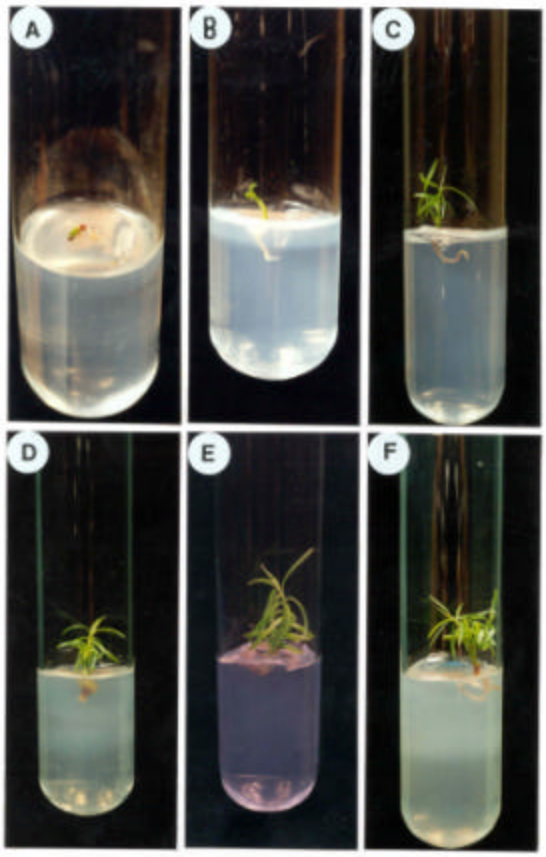


Fig. 5.12 Effects of various culture conditions on embryo germination (35 day oldseedlings).

A. and B. Abnormal embryo germination under dark incubation on DCR medium, pH=5.8.

C. Normal embryo germination under light incubation on DCR medium, pH=5.8. Note the well-developed root and 6-8 new leaves.

D. Normal embryo germination under light incubation on DCR medium, pH=5.8. Note the callus on root and 6-8 new leaves and short internodes.

E. A seedling after 50 days of growth on DCR medium, pH=5.8 and under light.

F. A seedling after 50 days of growth on DCR medium, pH=5.8 and under light. Note the spontaneous development of two shoots.

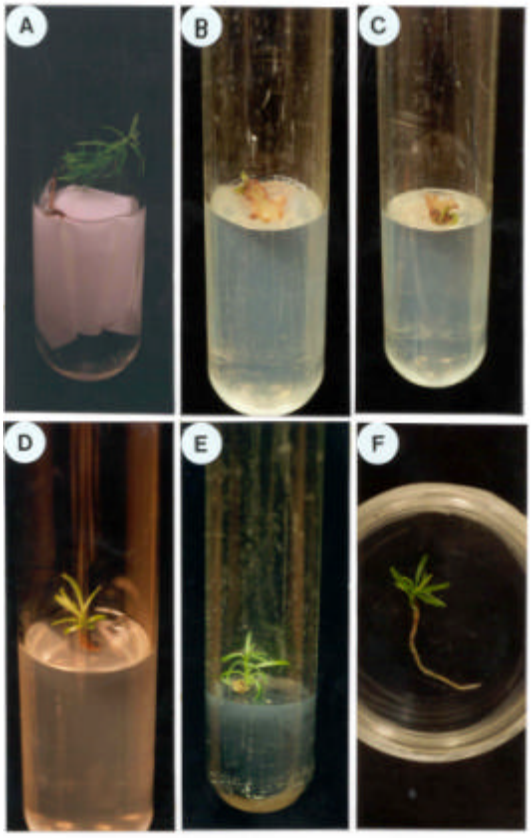


Fig. 5.13 Effects of various media and culture conditions on embryo germination (35 day old seedlings) except Fig. A.

A. A 60 day old seedling growing on liquid DCR medium with pH= 5.8 and under light.
B. Spontaneous callus formation on embryo incubated in dark on DCR medium with pH=5.8.
C. Spontaneous callus formation on embryo incubated in dark on DCR medium with pH=5.8.
D. Embryo germination on PR medium under light.
E. Embryo germination on WPM under light. Note the callus at root pole.
F. Embryo germination on B5 medium under light. Note the normal development of seedling.

In the next set of experiments, DCR medium without plant growth regulaotrs at pH 5.8 was used for culturing embryos dissected out from seeds collected from various locations. The results are depicted in **Table 5.11** .

Table 5.11 % Germination and Seedling Development of *Taxus* spp. Seeds Collected from Different Locations (Seeds were cultured in culture tubes with DCR medium + 3% sucrose + 0.3% phytagel, pH=5.8, 16 h photoperiod).

No.	Location & Developmental Stage of Seeds	% Germination & Seedling Development. Mean ± S. E.
1.	Almora, Fully mature seeds, half covered with red arils.	44.55 ± 2.42^{a}
2.	Khajjiyar, Dark green seeds with small arils	90.91 ± 9.1^{b}
3.	Shimla, Fully mature seeds half covered with red arils.	50 ± 0^{a}
4.	Darjeeling, Fully mature seeds half covered with red arils.	50 ± 0^{a}

$^{a, b}$: Values followed by the same letters are not significantly different from each other at 95% confidence level by t-test.

Embryos dissected out from seeds from Khajjiyar appeared to be superior than other locations, giving 91% germination as well as seedling development. The earlier developmental stage of the seeds (presence of small arils and smaller size of embryos) also must have contributed to this effect. But unfortunately, sufficient numbers of seeds were not available for further experiments due to political unrest and insurgency activities in that area and hence maximum number of experiments were done with embryos excised from fully mature seeds half covered with red arils collected from Almora region although the germination percentage was lower in these seeds than the seeds collected from other locations. Problems were also encountered for seed collection from Shimla and Darjeeling. Embryos excised from seeds collected from Almora region gave a lower percentage of germination as well as seedling development. But still the values obtained for germination percentages are higher than those reported earlier in literature by use of chilling treatment. No freezing treatment was used and simple basal medium without

plant growth regulators and organic additives were used in the present case as opposed to the literature reports (c. f. **Table 5.1)**.

Flores and Sgrignoli (1991) could not achieve germination from embryos excised from mature seeds. In a later publication, Flores et al. (1993) reported upto 70% germination from embryos excised from fully mature seeds after a prolonged freezing treatment and the seedlings obtained were even lower in number. Chee (1994) obtained 58-64% germination from embryos excised from mature seeds of various *Taxus* spp. on B5 medium with a seedling development percentage between 11-17%. However 100% germination and seedling development was reported after one week long leaching of mature embryos in running water by Zhiri et al. (1994). Leaching in running water probably leached out the ABA from the seeds, allowing germination to be completed on modified MS medium supplemented with CH, yeast extract, ascorbic acid and AC. 95% germination and seedling development were also reported by Chang and Yang (1996) by use of 1/2 MS medium with PVP. Here also PVP probably helped in binding the inhibitors leached out from the embryos during culture period.

Different basal media without plant growth regulators were tried in the next ser of experiments with embryos excised from mature seeds from Almora to enhance the percentage of embryo germination and the results are presented in **Table 5.12**.

B5 medium was found to be the best medium with 91% germination followed by WPM and DCR at 55% and 46% respectively. The differences were statistically significant. Although WPM gave high percentage of germination, the root pole of embryos showed callus development (**Fig. 5.13 E**) which later on spread over the entire seedling and eventually the seedling died. Hence this medium was not used for further experiments. For further experiments on adventitious bud induction, B5 and DCR basal media were therefore used (**Fig. 5.13 D-F**).

Table 5.12 % Germination and Seedling Development of Embryos Excised from Seeds of *Taxus* spp. (Almora) on Different Basal Media Compositions without Plant Growth Regulators (Media supplemented with 3% sucrose, 0.3% phytagel, pH=5.8 were used in culture tubes and were incubated at 25 ± 2°C and 16 h photoperiod).

No.	Basal Medium	% Germination & Seedling Development. Mean ± S. E.
1.	DCR	45.55 ± 2.42^{a}
2.	B5	91.66 ± 8.33^{b}
3.	B5SH	25.00 ± 0.00^{c}
4.	DCRSH	30.00 ± 20.00^{ac}
5.	PR	37.5 ± 12.5^{ac}
6.	WPM	55.00 ± 5.00^{a}

$^{a, b, c}$: Values followed by the same letters are not significantly different from each other at 95% confidence level by t-test.

The germinated seedlings were transferred to their respective germination media (solid or liquid) supplemented with 10% CM for further growth for two months. In this medium, the seedlings developed 15-20 leaves and a well developed root system (**Fig. 5.13 A**). Unfortunately, the seedlings could not be acclimatized and transferred to pots under the hot conditions of Pune but 100 full-grown seedlings were obtained on the optimal media such as B5 and DCR without plant growth regulators.

5.7.2 Adventitious bud induction experiments

Chee (1996) reported formation of adventitious buds on embryo explants of *T. brevifolia* in modified B5 medium supplemented with various concentrations of BAP. Similar experiments were carried out in our laboratory with embryos from Almora seeds. Since DCR and B5 media supported 45.55% and 91.66% precocious germination and seedling development respectively in embryos excised from Almora seeds, B5 and DCR basal media were modified by supplementation with SH vitamins, 3% sucrose 0.3% phytagel with various BAP concentrations. However, in none of the experiments, adventitious bud production was observed. The embryos either germinated normally or

produced friable, whitish brown or yellow callus, which survived only for three subcultures (3 months). Nodular callus was not obtained in any of the concentrations of BAP. The germination was also not normal and the root pole showed callus development in most of the seedlings. The results are presented in Table 5.13 and Fig. 5.14.

Table 5.13 Response of Embryos Derived from Seeds Collected from Almora to Various Media and BAP combinations.

No.	Basal Medium	Amount of BAP ($mg.l^{-1}$)	% Germination* Mean ± S. E.	% Callusing* Mean ± S. E.	Nature of Callus
1.	B5SH	0	33.33 + 8.33	37.5 + 12.5	Friable, white callus.
2.		1.5	43.75 ± 6.25	52.78 ± 12.1	Friable, white callus.
3.		2.5	33.33 ± 0	38.88 ± 11.1	Friable, whitish brown callus.
4.		3.5	41.67 ± 8.34	33.33 ± 16.7	Nodular, green or friable brown callus.
5.	DCRSH	0	30.0 ± 20.0	22.5 ± 2.5	Friable, yellow callus.
6.		1.5	48.33 ± 15.9	42.5 ± 2.5	Friable, yellow callus.
7.		2.5	40.0 ± 0.0	50.0 ± 0.0	Friable, yellow callus.
8.		3.5	40.0 ± 10.0	12.5 ± 0.5	Friable, yellow callus.

* All the differences are statistically insignificant at 95% confidence level by t-test.

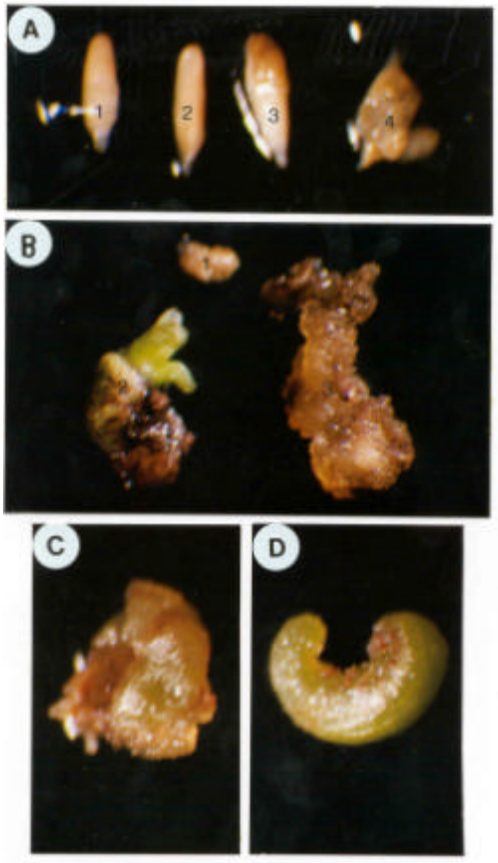


Fig. 5.14 Adventitious bud induction experiments.

A. Effect of DCRSH medium supplemented with various concentrations of BAP. No. 1: DCRSH – H, No. 2: DCRSH + 1.5 $mg.l^{-1}$ BAP, No. 3: DCRSH + 2.5 $mg.l^{-1}$ BAP and No.4: DCRSH + 3.5 $mg.l^{-1}$ BAP showing yellowish-brown friable callus (Magnification: 100x).

B. Effect of B5SH medium supplemented with various concentrations of BAP. No. 1: B5SH + 1.5 $mg.l^{-1}$ BAP, No. 2: B5SH + 2.5 $mg.l^{-1}$ BAP showing whitish-brown callus at hypocotyl and root pole and No. 3: B5SH + 3.5 $mg.l^{-1}$ BAP showing brown friable callus all over the embryo (Magnification: 40x).

C. Friable yellow callus all over the embryo on DCRSH with 2.5 $mg.l^{-1}$ BAP (Magnification: 40x).

D. Nodular callus all over the embryo on B5SH with 3.5 $mg.l^{-1}$ BAP (Magnification: 40x).

5.7 .3 Endosperm Culture

In case of Gymnosperms, no **'triple fusion'** is observed during fertilization and the haploid female gametophyte (a pre-fertilization product) itself takes over the role of nutrition played by polyploidic Angiospermic endosperm formed by 'triple fusion' during fertilization. Hence Gymnospermic endosperm is haploid. Culture of endosperm was attempted in order to raise haploid callus lines and to check for the presence of Taxane class of compounds in these lines.

A number of callus lines were raised from endosperm halves derived from seeds from Almora on various media and growth regulator combinations that have proved to be superior for embryo culture and callus cultures of stems and needles. As the auxin P was found to be superior for obtaining good diploid callus growth (**Chapter 4, Section 4.3.1**) and Taxane content therefrom (**Chapter 4, Section 4.3.2**), it was combined with B5 DCR media (Optimum for culture of embryos) (**C. f. Chapter 5, Section 5.7.1**) for culturing endosperm explants. All the inoculated endosperm halves produced varying amounts of callus depending on the medium and the percentage response was 100%. However, the callus morphology and growth rates varied in different media combinations. The results obtained are presented in **Table 5.14** and **Figs. 5.15 and 5.16**.

Table 5.14 Callus Production on Various Media and Growth Regulator Combinations by Endosperm Halves from Seeds Collected from Almora.

No.	Basal Medium	Plant Growth Regulator	Concentration ($mg.l^{-1}$)	Amount of Callus	Nature of Callus
1.	B5	Picloram (P)	2	++	White, friable, moist, fast-growing callus.
2.			4	++	White, friable, moist, fast-growing callus with globular cells.
3.			6	++	Yellowish-white, friable, moist, fast-growing callus with globular cells.
4.			8	+++	Light yellow, friable, fast-growing callus.
5.			10	+++	Light yellow, friable, fast-growing callus.

Contd…..

Table 5.14 Contd.

No.	Basal Medium	Plant Growth Regulator	Concentration ($mg.l^{-1}$)	Amount of Callus	Nature of Callus
6.	DCR	Picloram (P)	2	+++	White, friable, moist, fast-growing callus, becoming green and then yellow with age.
7.			4	+++	White, friable, moist, fast-growing callus, becoming green and then yellow with age.
8.			6	+++	Yellow, friable, fast-growing callus with globular cells.
9.			8	++++	Yellow, friable, fast-growing callus becoming brown with age.
10.			10	++++	Yellow, friable, fast-growing callus becoming brown with age.

* \+ : Fresh wt. of callus: 150-200 mg
++ : Fresh wt. of callus: 200-250 mg
+++ : Fresh wt. of callus: 250-300 mg
++++: Fresh wt. of callus: >300 mg

Fig. 5.15 Callus formation from endosperm explants.

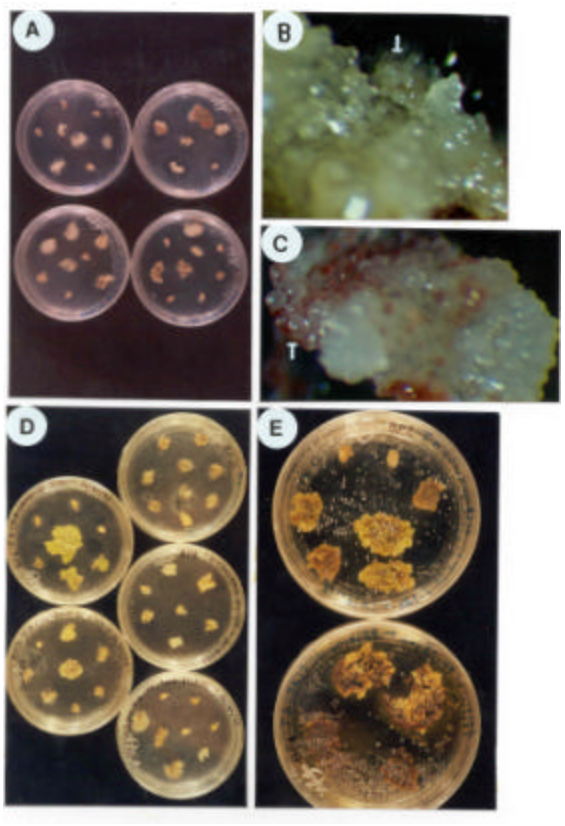
A. Initiation of callus formation (20 days) on endosperm halves on B5 medium supplemented with various concentrations of Picloram (P) (4, 6, 8, 10 mg.l^{1} individually). Note the moist and white colored callus.

B. A magnified view (40x) of endosperm callus grown on B5 medium supplemented with 4 mg.l^{1} P. Note a number of globular structures (Arrow).

C. A magnified view (40x) of endosperm callus grown on B5 medium supplemented with 6 mg.l^{1} P. Note a number of globular structures (Arrow).

D. Same callus as shown in Fig. A but after 50 days of growth on B5 medium supplemented with various concentrations of Picloram (P) (2, 4, 6, 8, 10 mg.l^{-1} individually). Note the moist and white colored callus has changed into friable and yellow colored callus.

E. 70 days old callus grown on B5 medium supplemented with 2 and 4 mg.l^{-1} P. The callus has become dry, friable and brown in color.



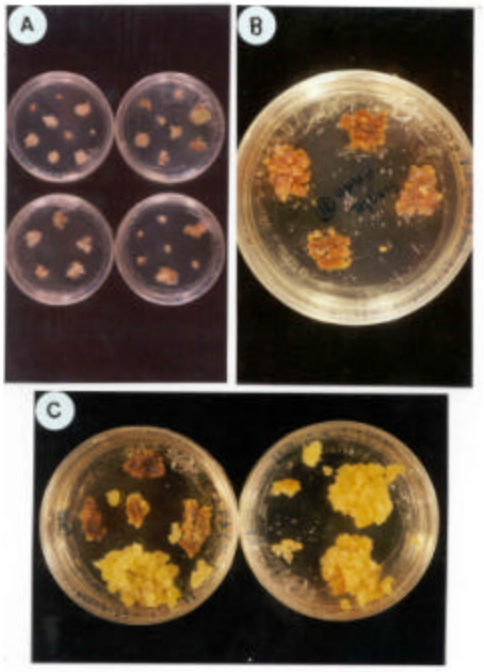


Fig. 5.16 Callus formation from endosperm explants.

A. Initiation of callus formation (20 days) on endosperm halves on DCR medium supplemented with various concentrations of Picloram (P) (2, 4, 8, 10 $mg.l^{-1}$ individually). Note the moist and white colored callus interspersed with green colored callus.
B. 50 day old callus grown on DCR medium with 8 $mg.l^{-1}$ P. Note the browning of callus with age.
C. Green callus initiated on DCR medium with 2 $mg.l^{-1}$ P did not maintain its color and turned yellow with subcultures.

Earlier workers reported the inability to induce callus from female gametophytes/endosperms of various *Taxus* spp. and the callus induced in some instances was reported to be very slow-growing (Tuleke, 1959; David and Plastira, 1974, 1976). Our observations on the other hand demonstrate **the production of fast growing haploid calli and to our knowledge this is the first report of success in development of haploid endosperm cultures from *Taxus* spp.**

B5 medium supplemented with 8 and 10 $mg.l^{-1}$ of P supported fast growing, friable callus with FW of callus 250-300 mg. DCR medium supplemented with 8 and 10 $mg.l^{-1}$ of P also supported faster growing, friable callus with FW of callus more than 300 mg. The se calli were analyzed by TLC for presence of **Taxanes**.

5.7.4 Analysis of Endosperm Cultures (Callus and Culture Medium) for Taxane Production.

Out of 10 endosperm-derived callus lines screened by TLC, considerable amounts of **Taxanes** were detected in five callus lines. All the five **Taxane** positive callus lines were found to grow on DCR medium supplemented with varying concentrations of P. These were then further analyzed quantitatively by HPLC. Similarly the solid nutrient media of these callus lines were also analyzed by HPLC for presence of **Taxanes**. The details are presented in **Chapter 4, Section 4.3** and **Tables 4.9 and 4.10**.

5.8 Conclusions

1) **Precocious embryo germination and seedling development has been achieved at a frequency of 90% from immature seeds collected from Khajjiyar on DCR basal medium at pH 5.8, in light and without plant growth regulators.**
2) **Precocious embryo germination and seedling development was found to vary with developmental age of seeds (as judged by aril development and embryo size) and the location of collection.**
3) **Precocious embryo germination and seedling development has been achieved at a frequency of 91% from mature seeds collected from Almora on B5 basal medium without plant growth regulators.**
4) **Fast growing, haploid callus lines have been established from solid endosperm of seeds collected from Almora on the optimal B5 and DCR media supplemented with various concentrations of Picloram (P) and higher concentrations of P were conducive for faster growth of calli.**
5) **Considerable amount of Taxane production has been reported here for the first time from endosperm-derived callus lines (For details please refer to Chapter 4, Section 4.3.2 and Table 4.9.**

CHAPTER 6

Direct Regeneration Of Shoots from Various Explants of *Withania* spp. and Analysis of Withaferin-A Production Therefrom.

6.1 Introduction

6.1.1 Botany of *Withania somnifera* (L.) Dunal (Sivarajan and Balachandran, 1994).

The Indian sub-continent has two species of *Withania* namely, *W. somnifera* and *W. coagulance*. *W. coagulance* is found in the North-Western India and neighboring regions. *W. somnifera* is an erect, grayish, tomentose, undershrub (30-75 cm high) with long tuberous roots and is distributed throughout the drier sub-tropical regions of India. Leaves are alternate or sub-opposite, broadly ovate, sub-acute, entire, 5-10 x 2.5-7 cm. Flowers are small, greenish, axillary, solitary or in few-flowered cymes and bisexual. Calyx is gamosepalous with 5 lobes, accrescent and inflated in a fruit. Corolla is campanulate, greenish-yellow with 5 lobes. 5 included stamens are present. Ovary is ovoid/globose, glabrous, many ovuled. Style is filiform and stigma is 2-lobed. Fruit is a globose berry, orange-red when ripe and enclosed in the enlarged calyx. Seeds are many, discoid, yellow and reniform (**Fig. 6.1**).

The chromosome number is 2n = 48.

6.1.2 Clinical/Pharmacological Studies in *Withania Somnifera* (L.) Dun.

Withania somnifera (L.) Dun. (Ashwagandha) is an important Solanaceous medicinal plant. Overall the drug from *Withania* spp. is believed to impart long life, youthful vigor and good intellectual powers (Sivarajan and Balachandran, 1994) In Ayurveda, roots of *Withania* spp. are prescribed for gynecological disorders, bronchitis, arthritis, rheumatism, inflammation, fevers, skin diseases etc. The clinical activity of roots is due to a number of alkaloids like **Somniferine**, **Withasomnine** etc. The total alkaloidal content of roots of Indian chemotype varies between 0.13-0.31% (Majumdar, 1955; Malhotra et al., 1961; Anonymous, 1976; Asthana and Raina, 1989; Nigam and Kandalkar, 1995). The leaves contain a number of withanolides like **Withaferin-A** and exhibit anti-bacterial (Against a number of Gram-positive bacteria), anti-fungal and anti-tumor properties (Kurup, 1956; Kupchan et al., 1965; Uma Devi et al., 1992; Uma Devi et al., 1993).

Withaferin-A acts as a tumor inhibitor due to its ability to arrest dividing cells at metaphase. It has shown growth inhibitory activity against a number of cancerous cell lines such as KB, Sarcoma 180 as well as Ehrlich ascites carcinoma and mammary

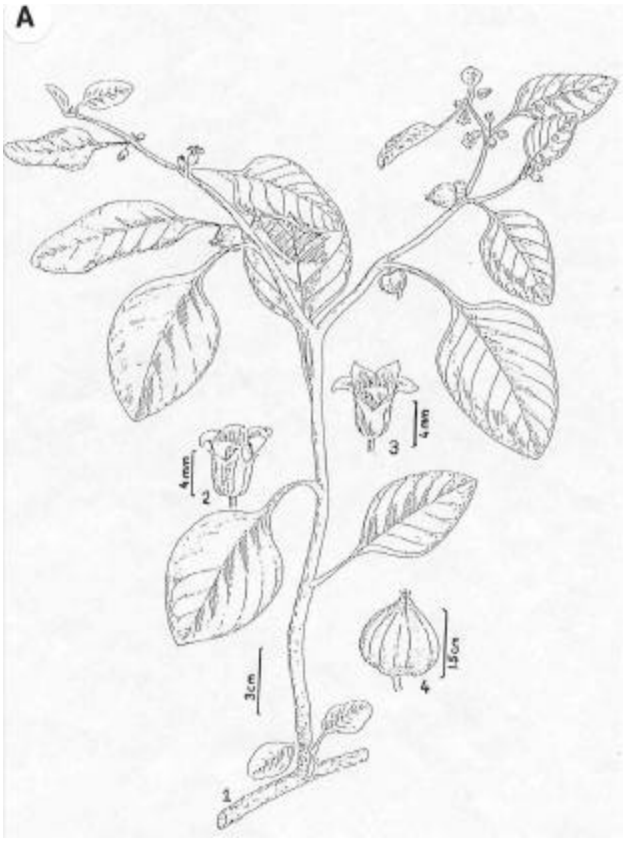


Fig. 6.1 The habit and morphology of *Withania somnifera* (L.) Dun. (Sivarajan and Balachandran, 1994).

1. A twig.
2. A flower.
3. A flower.
4. A fruit enclosed in the accrescent calyx.

carcinoma (Asthana and Raina, 1989; Uma Devi et al., 1992). It also exerts radiosensitizing effects on the tumors and carcinomas (Uma Devi et al., 1993; Uma Devi et al., 1995). Besides **Withaferin-A**, **Withanolide-E** and **Withanolide-D** also exhibit anti-tumor activities. These compounds act as mitotic poisons, arresting the division of tumor cells at metaphase and also inhibit RNA and protein synthesis (Budhiraja and Sudhir, 1987). The essential requirements in a withanolide for antitumor activity are considered to be epoxide and enone functions in the AB rings and an unsaturated lactone in the side chain (Ray and Gupta, 1994).

Withaferin-A and **Withaferin-E** also have immunosuppressive as well as immunostimulating properties. **Withaferin E** has insect anti-feedant properties (Budhiraja and Sudhir, 1987 and references therein). Antifungal activity also has been attributed to **Withanolide-I** and **Withanolide-K** from *W. coagulance* (Choudhary et al, 1995).

6.1.3 Alkaloids in *Withania somnifera* (L.) Dun.

A wide variation (0.13-0.31%) in alkaloid contents of *Withania somnifera* roots has been found. Most of this variation is genotypic. Following alkaloids have been described in this plant (**Table 6.1**). Out of these, **Withanine** constitutes 38% of the total alkaloids (Nigam and Kandalkar, 1995).

Table 6.1 Alkaloids in *Withania somnifera* (L.) Dun.

Ser. No.	Alkaloid Name	Ser. No.	Alkaloid Name
1.	Nicotine.	5.	Withanine
2.	Somniferine	6.	Withananine.
3.	Somniferinine	7.	Withananinine
4.	Somnine	8.	Pseudo-withanine

6.1.4 Withasteroids in *Withania somnifera* (L.) Dun.

Withasteroids are a group of naturally occurring C-28 steroidal lactones built on an intact or rearranged ergostane framework where C-22 and C-26 are appropriately oxidized to form a δ-lactone ring. They have a nine-carbon side chain with a six membered ring lactone. This basic structure is referred to as the **"Withanolide Skeleton"** (**Fig. 6.2 A**). **Withaferin-A**, the first withanolide to be studied in detail, was first isolated from *Withania somnifera* (L.) Dun. (Kurup, 1965) and its structure was first determined by Lavie et al. (1965). See **Fig. 6.2 B**. Afterwards many compounds (around 50) with similar structural features were isolated from leaves of a number of Solanaceous plants (Budhiraja and Sudhir, 1987). These are poly-oxygenated compounds and the parent plants are believed to have an enzyme system capable of oxidizing all carbon atoms in the steroid nucleus.

Withanolides are one of the nine and the most abundant groups of the broad parental group of Withasteroids. Withanolides can be grouped into two classes:
(1) With normal 17β- oriented chain. (132 compounds known)
(2) With the unusual 17α- oriented chain. (36 compounds known)

Withasteroids are mainly synthesized by some members of Solanaceae as well as by some members of Taccaceae, Leguminosae and even by some marine organisms. Lockley et al. (1974) have shown by incorporation of radioactive substrates in *Withania somnifera* (L.) Dun. that 24-methylene-cholesterol rather than its double bond isomer, is the true precursor of withanolides.

Plants that are morphologically indistinguishable but differ in their chemical constituents are termed as "Chemotypes". Although there is evidence that there are more than one chemotype of *Withania* spp. in India (Chakraborty et al., 1974), the exact number of chemotypes has not yet been studied systematically. Abraham et al. (1968) have identified two chemotypes in Indian varieties of *Withania somnifera*. The major constituents of the Indian Chemotypes are **Withanone** and 20-H **Withanolides**, many of which contain a 5α-hydroxy-6α,7α-epoxy grouping. The ability of different chemotypes to produce withanolides with different nuclear substitution patterns is genetically controlled and the hybrids derived after crossing of different parents yield withanolides either with new nuclear substitutions depending on the genotype of the parents or entirely

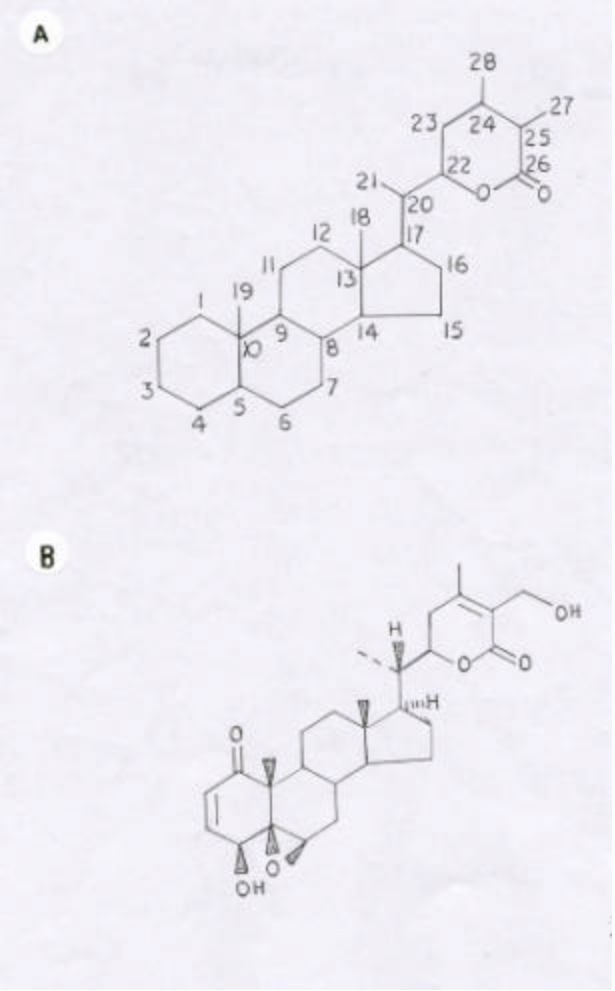


Fig. 6.2 Withanolides detected in *Withania somnifera* (L.) Dun. (Adapted from Kun du et al., 1976).

A. The basic, parent 'Withanolide' Steroidal Lactone.
B. Structure of 'Withaferin - A'.

new withanolides not found in the parents (Kirson et al., 1977; Eastwood et al., 1980; Nittala and Lavie, 1981; Bessalle et al., 1987). Thus it appears that it might be possible to develop hybrids with the ability to yield tailor-made withasteroids to meet specific needs.

Withaferin-A is isolated conjointly with **Dihydrowithaferin-A** (Kundu et al., 1976) from leaves of *Withania somnifera*. It also contains 27-desoxyWithaferin-A and 27-desoxy-14α-hydroxyWithaferin-A. The yield of **Withaferin-A** is 0.18% on air-dry basis in Indian plants while South African varieties contain 0.86% of the compound (Asthana and Raina, 1989). Gupta et al. (1996) have reported **Withaferin-A** content of 1.6% in leaves of *Withania somnifera* by TLC densitometry. Besides these, the three chemotypes of Indian *Withania somnifera* contain a large variety of different withanolides.

6.1.5 *In Vitro* Studies in *Withania Somnifera* (L.) Dun.

The natural wild populations of *Withania somnifera* contain a tongue-paralyzing constituent in their roots and hence cannot be used for ayurvedic treatments. However the improved, cultivated varieties do not have such an active ingredient. Also they have different morphology and therapeutic actions although both contain the same alkaloids (Kaul, 1957). However since the cultivated plants show many different chemotypes (Kirson et al., 1971; Glotter et al., 1973), production of a large number of plants of superior selected chemotypes without any seasonal constraints is desirable. The natural populations of *Withania* spp. are not easily amenable for vegetative propagation and seed raised plants need 4-6 years to reach maturity, when they can be harvested for their natural, active components. These difficulties can be overcome by the use of *in vitro* technologies. Direct regeneration of shoots without any intermediate callus phase can yield clonal plants for large-scale propagation. Genetic transformation using *Agrobacterium rhizogenes* for hairy root formation for production of novel metabolites (Flores and Medina-Bolivar, 1995) is an attractive possibility.

Table 6.2 summarizes the *in vitro* studies carried out in *Withania* spp. so far.

Table 6.2 Establishment of Differentiated/Un-differentiated and Transformed Cultures from *Withania somnifera* (L.) Dun.

Regeneration Studies						
No.	**Explants**	**Medium**	**Plant Growth Regulators & Additives**	**Culture Response**	**Amount of Withasteroids Produced**	**Reference**
1.	Anthers with uni-nucleate microspores	MS	BAP	Andro-genesis→ Haploid Callus	-	Vishnoi et al. (1979)
2.	Germinating seeds and shoot tips	MS	BAP, 2,4-D & IBA	4-10 shoots in 15% seeds & 120 shoots in 80% shoot tips	-	Sen & Sharma (1991)
3.	Axillary meristems	MS	BAP & CM, KIN & 2,4-D	Callus & multiple shoots	No active compound in callus. Withanolide-E: 67 µg/g dw; Withanolide-D: Traces; Withanone: 90-486 µg/g dw; Withanolide-I: 13-172 µg/g dw; Withanolide-G: 19-192 µg/g dw in multiple shoots.	Roja et al. (1991)

Contd…..

Table 6.2 Contd.

No.	Explants	Medium	Plant Growth Regulators & Additives	Culture Response	Amount of Withasteroids Produced	Reference
4.	*In vitro* leaves	MS	IAA & BAP	16 *de novo* multiple shoots in 57% leaf bits	-	Kulkarni et al. (1996a)
5.	Seeds, *in vitro* leaves	MS	IAA & BAP, BAP, TDZ	2-13 shoots from seeds, 60% leaves→ multiple shoots	-	Kulkarni et al. (1996b)
6.	Nodes, Internodes, Hypocotyls	MS	BAP	2-5 shoots per node, more than 25 shoots per internode, 12 shoots per hypocotyl	-	Kulkarni et al. (1999)
7.	Leaves, Shoots, Hypocotyls and Roots	MS	Callus on 2,4-D & KIN. Shoot regeneration on BAP	Plantlet regeneration from callus	-	Rani & Grover (1999)

Contd…..

Table 6.2 Contd.

Transformation Studies						
No.	**Explants**	**Medium**	**Plant Growth Regulators & Additives**	**Culture Response**	**Amount of Withasteroids Produced**	**Reference**
1.	Leaves	MS	-	Hairy roots with *A rhizogenes*	Traces of **Withaferin-A**, Withaferin-E	Banerjee et al. (1994)
2.	Leaves	MS	-	Hairy roots with *A rhizogenes*	Traces of Withanolide-D, Absence of **Withaferin-A**.	Ray et al. (1996)
3.	Leaves	MS	-	Crown galls, rooty terratomas &shooty terratomas with *A tumefaciens*	0.07-0.1% dry wt. **Withaferin-A** in shooty terratomas & 0.04-0.05 % dry wt. **Withaferin-A** in non-transformed shoots.	Ray & Jha (1999)

It is clear from the above literarture review that not much work has been done with respect to tissue culture studies in *Withania* spp. When the present work on morphogenesis in *Withania somnifera* (L.) Dun. was started in 1994, only three reports of tissue culture of *Withania* spp. were available, which made use of pre-existing meristems (nodes, seeds, embryos). The regeneration efficiency reported was also very low. In addition, there were no reports on transformation studies. Also not much work has been done on secondary metabolite production from these cultures. Hence it was felt necessary to develop regeneration protocols from *de novo* meristems, with higher frequencies of regeneration. Transformation studies with *Agrobacterium rhizogenes* were also attempted (Please refer to **Annexure** for details).

The objectives of the present work in *Withania somnifera* (L.) Dun. were as follows:

1. Use of pre-existing meristems limits the number of explants that could be used in micropropagation studies. Hence efforts were made to explore the possibility of regeneration of shoots from different explants such as nodes, internodes, hypocotyls, leaves and embryos by *de novo* formation of shoot buds *in vitro*.
2. Acclimatization of all the regenerants, derived from different explants and their transfer to green house.
3. Assessment of differentiated and undifferentiated cultures for **Withanolide** production and to check for possible correlation between tissue organization and secondary metabolite production, if any.
4. Preliminary experiments on standardization of raising liquid cultures from normal roots and analysis of cultures for **Withanolide** formation, especially **Withaferin-A**.
5. Preliminary experiments on standardization of raising liquid cultures from hairy roots obtained with various explants of *Withania somnifera* by infection with *Agrobacterium rhizogenes* and analysis of cultures for **Withanolide** formation, especially **Withaferin-A**.

The preliminary experiments on raising liquid cultures from normal and hairy roots (Please see **Annexure** for details) were carried out as pilot experiments to study for the feasibility of their scale-up in large scale either in shake flasks or bioreactors for production of **Withaferin-A**. However scaling up of cultures, if any, based on the obtained results is not in the scope of studies aimed in this thesis.

6.2 Materials and Methods

6.2.1 Collection of plant material

The seeds of the cultivated variety: 'WS-20' of *Withania somnifera* were collected from the nurseries in and around Pune (**Fig. 6.3 A**). These were maintained at room temperature since they have a long viability period of three years.

6.2.2 Seed germination and seedling development

Seeds were used to raise *in vitro* seedlings of *Withania somnifera* (L.) Dun. Seeds were surface sterilized as per the **"Routine Surface Sterilization Protocol"** indicated in **Chapter 3, Section 3.2.2.2**. Five seeds were inoculated per each glass culture tube containing 20 ml of nutrient medium and these were maintained at a light intensity of 38 $\mu Em^{-2}s^{-1}$ provided by cool white fluorescent tubelights ("Phillips", India) for 24 hours (Continuous light) at a temperature of 25 $\pm$ 2°C. Germination occurred in 15 days on MS basal medium (Murashige and Skoog, 1962) without plant growth regulators but supplemented with 2% sucrose and 0.7% agar. This medium is hereinafter referred as **Basal Medium (BM)**. The medium composition and its method of preparation were as described earlier in **Chapter 2, Section 2.4** and **Table 2.1**. The germinated seedlings were also incubated under conditions described above.

6.2.3 Preparation and incubation of explants for shoot bud induction

One month after seed germination, small intact seedlings were transferred to screw capped bottles containing 50 ml BM to achieve further growth of seedlings for another month. These two-month old seedlings of height 8-10 cm (**Fig. 6.3 B**) were the source of explants of leaves, nodes, internodes and hypocotyls. After inoculation, all these explants were maintained at a light intensity of 38 $\mu Em^{-2}s^{-1}$ provided by cool white fluorescent tubelights ("Phillips", India) for 24 hours (Continuous light) at a temperature of 25 $\pm$ 2°C.

Very young or very mature leaves from the seedlings were discarded while a leaf of an optimum size (length: 15-30 mm and width: 20-25 mm at the broadest) was cut into 3-5 pieces of size 1 cm x 1 cm with a portion of the midrib. To ensure that all the leaf pieces had a portion of the midrib, each leaf was cut length-wise through the midrib.

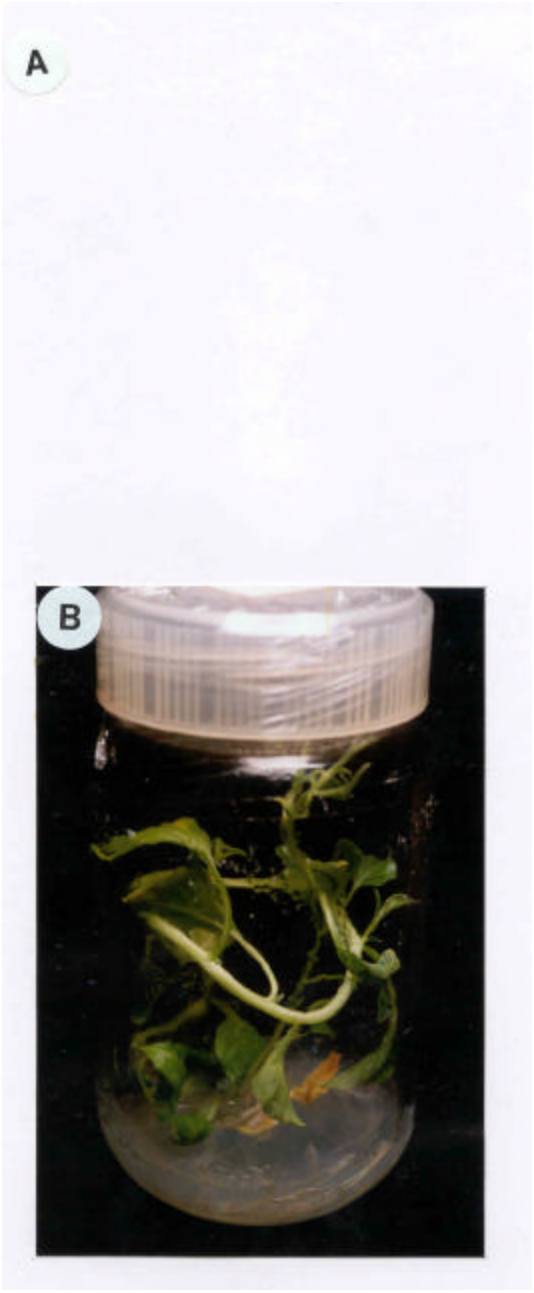


Fig. 6.3

A. Seeds of *Withania somnifera* (L.) Dun.
B. A two-month old, *in vitro* seed-derived seedling of *Withania somnifera* (L.) Dun.

These two halves were then cut in transverse and were inoculated, either in the culture tubes (20 ml nutrient medium) or in sterile plastic dishes with 55 mm diameter (12 ml nutrient medium) with either the adaxial or abaxial surface in contact with BM fortified with various concentrations and combinations of IAA (0.5, 1.0, 1.5, 2.0 $mg.l^{-1}$) and BAP (1.0, 2.0, 3.0 $mg.l^{-1}$) or NAA (0.5, 1.0, 1.5, 2.0 $mg.l^{-1}$) and BAP (1.0, 2.0, 3.0 $mg.l^{-1}$).

Three nodes (3-5 mm long and 2-4 mm wide) and three internodes (3-5 mm long and 2-4 mm wide) were inoculated independently per tube containing BM supplemented with BAP (0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 5.0 $mg.l^{-1}$) and TDZ (0.05, 0.1, 0.2, 0.3 $mg.l^{-1}$) individually. For each concentration and combination (if any) of plant growth regulators, ten tubes of each type of explant were inoculated. Since the internodal explants showed presence of yellow colored associate bacterial contaminants, they were treated with **"Modified Surface Sterilization Protocol"** described earlier in **Chapter 3, Flow Chart 3.3** and then used for culture.

Five hypocotyls (3-5 mm long and 2-4 mm wide) were inoculated in each bottle containing BM with BAP (0.1, 0.2, 0.3, 0.4, 0.5 $mg.l^{-1}$) and for each concentration of BAP, five bottles were inoculated. Response of hypocotyl explants on higher concentrations of BAP (1.0, 5.0 $mg.l^{-1}$) and on any of the TDZ concentrations (0.05, 0.1, 0.2, 0.3 $mg.l^{-1}$) was not determined because from a full grown two month old seedling only three hypocotyl explants could be derived.

The embryos (4 mm long) were dissected out by cutting open the imbibed and swollen seeds and inoculated in sterile disposable plastic dishes containing 12 ml BM in each dish supplemented with either BAP (0.1, 0.2, 0.3, 0.4, 0.5,1.0, 5.0 $mg.l^{-1}$) or TDZ (0.05, 0.1, 0.2, 0.3 $mg.l^{-1}$) individually. Each dish contained eight embryos and for each BAP and TDZ concentration, five dishes were inoculated.

All the plant growth regulators used in above experiments were added to nutrient media before autoclaving as described earlier in **Chapter 2, Section 2.4**.

6.2.4 Elongation and rooting of multiple shoots

Multiple telescopic shoots formed on BM supplemented with various IAA and BAP combinations from leaf explants and on BAP concentrations from node, internode and hypocotyl explants were separated and transferred to BM with 0.01 mg.l^{-1} BAP for elongation as well as rooting (One step protocol). Multiple shoots formed on TDZ containing media from node and embryo explants could not be elongated on the above medium but they could be elongated as well as rooted on MS basal medium with 1/2 strength major salts (1/2 BM) without plant growth regulators (One step protocol).

6.2.5 Acclimatization

Twenty plantlets, 6 cm long (5 cm long shoot portion and 1 cm long root portion), regenerated from each type of explant with well formed roots were carefully removed from the culture vessels and washed by gently shaking in a beaker with glass distilled water to remove the gelling agent and medium sticking to the surface. The plantlets were then dipped in 1% (w/v) aqueous solution of the systemic fungicide Bavistin ("BASF", India) for 10-15 min and then washed with a fine spray of water. Treated plantlets were transferred to autoclaved clay pots filled with a mixture of sand : soil or soil : 'Soilritemix' ("KEL Perlite", India) in 1:1 proportion. These were kept in moist chambers prepared by covering plastic tubs with glass sheets to prevent the rapid loss of humidity in tropical environments. They were kept in the hardening room at 27 $\pm$ 1^{o}C for two weeks. The hardened plants were sprayed with a sterile solution of major salts of MS medium at 1/10 strength, twice a week, for a period of two weeks. Such hardened plants were transferred to a green house with ambient temperature (28-30^{o}C), diffuse sunlight and 70% humidity maintained by overhead sprinklers.

6.2.6 Statistical analysis

Each tube or bottle or plastic dish with three or five or eight explants respectively was taken as one replicate. Each experiment consisted of either ten (in case of tubes) or five (in case of bottles and plastic dishes) such replicates for each treatment and each experiment was repeated at least twice. Shoot buds longer than 0.5 mm with distinctly visible apical meristems were only counted in each treatment. These were then compared

by one way ANOVA and the individual treatment means were compared by Student's t - test (Wardlaw 1985).

6.2.7 Histology

Explants at different stages of differentiation were fixed in formalin : acetic acid : distilled ethyl alcohol (1:1:20) for 48-72 h depending upon the thickness of the explants. The fixative was decanted and the explants were dehydrated in tertiary butanol series (Sharma and Sharma, 1980). Dehydrated explants were then embedded in paraffin wax ("E Merck", India, melting point 58-60^{o}C). Serial sections of thickness 10 µM were cut using a rotory microtome ("Reichert Jung 2050, Supercut", Germany). The sections were heat-fixed to the slides and passed through xylene-alcohol series (Sharma and Sharma, 1980). Staining was done with Heidenhein's haematoxylin (Heidenhein, 1892; see Purvis et al., 1964) with or without counterstaining with 1% Eosin. Slides were mounted in DPX mountant (Purvis et al., 1964).

6.2.8 Extraction of *in vitro* grown tissues for analysis of Withafe rin-A by HPLC

Following steps were carried out (Modified from Roja et al., 1991):

1. Two month old seedlings, multiple shoots derived from nodes on the optimal medium (MS with 0.5 mg.l^{-1} BAP) and callus formed on the same medium at the basal cut end of the explant were selected for extraction.
2. 200 mg dry weight (DW) of each type of tissue was taken for analysis. (The tissues were dried in an oven at 60°C, till constant weight was obtained).
3. The tissues were ground in a pre-cooled mortar and pestle with liquid nitrogen and extracted overnight in methanol (5 times w/v) on a rotary shaker at 26°C and 100 rpm.
4. The procedure was repeated three times and the methanolic extracts were pooled together.
5. The extracts were filtered through Whatman No.1 filter paper and diluted with deionised water (1 methanol : 4 water).
6. The resultant solution was extracted with 3 volumes of chloroform.
7. Chloroform layer was separated from other layers through a separating funnel.
8. The chloroform extract was dried in Eppendorf tubes in a Centrivap Concentrator ("Labconco", USA).

9. The residue was redissolved in 1 ml methanol, filtered through a 40 µM nylon filter and 20 µL was used for HPLC analysis.
10. The standard of **Withaferin-A**, kindly provided by Dr. M. I. Choudhary from HEJ Research Institute, Pakistan was also dissolved in methanol at a strength of 0.5 $mg.l^{-1}$ and 20 µL was used for HPLC analysis.

6.2.9 Analytical HPLC and calculation of Withaferin-A in tissue extracts

All routine procedures of analytical HPLC were followed (Melander and Horvath, 1980; Scott, 1996). However the actual procedure used in HPLC analysis is a modified procedure of the protocol of Roja et al. (1991).

HPLC was carried out on a Merck-Hitachi instrument with a series D-7000 interface with HSM Manager, L-7100 pump, L-7420 diode array detector, a rheodyne injector with a 20 µL injection loop. RP-18 ("Merck", India) column of the dimension 250 x 4.6 mm with particle size of 5µm was used. The column was operated in the **reverse-phase** mode. The data were recorded at 254 nm (Roja et al., 1991) on a computer and the chromatogram data reports were printed on HP Deskjet 200 printer ("Hewlett Packard", India). Photographs of selected runs were recorded with color films. External standard method of quantitation was used.

All the solvents used were of HPLC (Lichrosolv) grade ("Merck", India). The in-house purified Milli-Q water was used ("Millipore Corporation", USA). All the solvents and samples were filtered through suitable 0.22 µM pore size filters ("Millipore Corporation", USA). Degassing of solvents was done under vacuum (equivalent to 20-25" Hg). The mobile phase used for the analysis of **Withaferin-A** consisted of Methanol (57%) and Water (43%). The flow rate for the isocratic HPLC run (57% methanol and 43% water) was kept at 1.0 ml/min. and the run was continued for 40 min for complete resolution and detection of the withanolides. All the samples were filtered as described in the preceding sections and 20 µL was injected. The standard solution of **Withaferin-A** was prepared in HPLC grade methanol at the strength of 0.5 mg/ml. It was also filtered before HPLC analysis and 20 µL was injected. Injection volume of 20 µL was kept constant during the experimentation. Spiking of the samples was accomplished by

addition of 10 µL of authentic standard such that approximately 2.5 µg of the standard was present in the spiked injection.

After every injection (either of standard or sample) the HPLC system was allowed to re-equilibriate with the mobile phase till the baseline again became horizontal (10 min.). Since there was no significant back-pressure development, it was not necessary to develop complicated regeneration procedures for the column.

Plotting of graphs and calculations of amounts of **Withaferin-A** in the tissues by the external standard method of quantification were done using routine protocols as described in the "Instruction Manual for HSM Manager" as well as using the formula given by Scott (1996):

$$C_{p(s)} = \frac{a_{p(s)}}{a_{p(st)}} \times C_{p(st)}$$

where, $C_{p(s)}$ is the concentration of solute in the mixture.

$a_{p(s)}$ is the area of the peak for the solute (p) in the sample chromatogram.

$a_{p(st)}$ is the area of peak for solute (p) in the reference chromatogram.

$C_{p(st)}$ is the concentration of the standard in the reference solution.

The linearity of detector response for standard of **Withaferin-A** was observed by injecting solutions of standards ranging in concentration from 1.25-10 µg/ml (ElSohly et al., 1995) and plotting the standard curve (**Fig. 6.10**). Identification of **Withaferin-A** in callus and tissue samples was confirmed by retention time, co-chromatogrphy with the standard and peak purity by wavelength (Fett-neto et al., 1993a, b).

HPLC injections of callus and tissue samples extract as well as the standards were done at least in duplicates and the injections were repeated on different days to check for the repeatability of the experimental procedure. Day to day variations were also checked with the standard solutions.

6.3 Results and Discussion

The commercial cultivation of *Withania* spp. is associated with two major problems namely, the plant to plant variation in alkaloid and withanolide yield and quality as well as the long gestation period (4-6 years) between planting and harvesting. Agronomically the plants are propagated through seeds since the plants do not have the natural ability for vegetative propagation and the variability generated by sexual recombination is very high. To overcome these constraints, biotechnological tools like plant tissue culture can be gainfully employed where cultures can be started from chemically superior mother plants and on-line harvesting of secondary metabolites can be done from such differentiated cultures.

Although Skoog and Miller (1957) have shown that organ formation results from quantitative interactions between auxins and cytokinins, many systems show organ formation in the presence of a single growth regulator like cytokinin depending upon the amount and balance of endogenous growth regulators (Buising et al., 1994). Here direct formation of shoots without an intervening callus phase from a variety of explants is reported in the presence of an auxin plus cytokinin and also in the presence of a single cytokinin.

For all the experiments, MS was chosen as the nutrient medium because earlier reports described its usefulness in *Withania somnifera* for *in vitro* experiments (Roja et al., 1991; Sen and Sharma, 1991). Leaf explants were inoculated on BM supplemented with different combinations of IAA and BAP as well as NAA and BAP while node, internode, hypocotyl and embryo explants were inoculated on BM supplemented with various concentrations of either BAP or TDZ individually. The threshold concentrations of these growth regulators required for optimum organogenetic induction differed with different explant types (**Tables 6.4, 6.5, 6.8 and 6.9**).

6.3.1 Regeneration of plants from leaf explants

6.3.1.1 Size and source of explants

When leaf pieces from very young (smaller than 10 mm) or very old (senescent) leaves were used as explants, callusing was observed in all the treatments mentioned in **Tables 6.4, 6.5 and 6.6** and there was no shoot bud formation in any of the treatments.

But when explants were chosen from leaves of an intermediate size (length: 15-30 mm and breadth: 20-25 mm), direct regeneration of shoots was observed (**Tables 6.4 and 6.5**). Hence for further experimentation, leaves of of an intermediate size (length: 15-30 mm and breadth: 20-25 mm) were used.

6.3.1.2 Effect of BAP, IAA and NAA

When leaf pieces were prepared (as described earlier in **Section 6.2.3**) from leaves of optimum size and were inoculated on BM supplemented with only BAP (0.1-5.0 $mg.l^{-1}$) or only NAA (0.1-5.0 $mg.l^{-1}$), or only IAA (0.1-5.0 $mg.l^{-1}$), all the explants formed callus irrespective of the plant growth regulator (**Fig. 6.4 D-F**). On BM alone, the explants did not form callus but eventually became necrotic (**Table 6.3**). Callus formed on BM supplemented with NAA was dry and friable in nature but it never gave rise to roots. Callus formed on BM supplemented with IAA was moist on BM with 0.1 $mg.l^{1}$ IAA while higher IAA concentrations gave rise to dry and friable callus from cut ends of leaf explant and formed roots from midribs and lateral veins of the leaf explant. Callus formed on BM supplemented with BAP was moist and green. At 5.0 $mg.l^{-1}$ BAP, green, nodular callus was produced but it never gave rise to shoot buds or shoots. All the three growth regulators on BM supplemented with 5.0 $mg.l^{-1}$ concentration formed 1.5-2.5 g FW of callus.

Table 6.3 Callus Formation from Leaf Explants on BM with NAA, IAA and BAP Individually (Under continuous light at 38 $\mu Em^{-2}s^{-2}$).

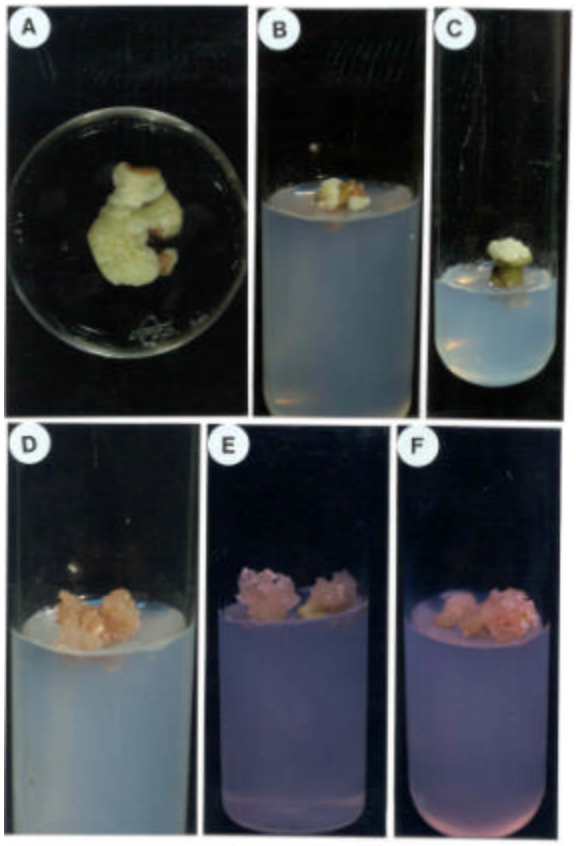
Plant Growth Regulator	**Concentration ($mg.l^{-1}$)**	Amount of Callus*	**Callus Type**
Absent	-	-	-
NAA	0.1	+	Dry, white, friable callus turning brown with age.
	0.5	++	Dry, white, friable callus turning brown with age.
	1.0	++	Faint yellow, friable callus at cut ends of leaf explants.
	5.0	+++	White callus on veins and yellow-brown callus at cut edges of the leaf explants.
IAA	0.1	+	Moist, white callus
	0.5	++	Yellowish brown callus from cut ends and veins of leaf explants. One-two roots only from midrib of the leaf explants.
	1.0	++	Cut ends of leaf explants with dry, white callus while leaf midrib and other lateral veins show many fat roots with many root hairs.
	5.0	+++	Cut ends of leaf explants with dry, white callus while leaf midrib and other lateral veins show many fat roots with many root hairs.
BAP	0.1	+	Green, moist callus at cut ends of leaf explant.
	0.5	++	Green, moist callus at cut ends of leaf explant.
	1.0	++	Green, moist callus at cut ends of leaf explant.
	5.0	+++	Light green, fat, nodular callus all over the leaf explant.

*+ : Fresh weight of callus: 0.5-1.0 g, ++ : Fresh weight of callus: 1.0-1.5 g,
+++ : Fresh weight of callus: 1.5-2.5 g - : Absence of callus

Fig. 6.4

A – F Callus formation from nodal and leaf bit explants.

A. Non-organogenetic callus from leaf bit explant on MS medium supplemented with 1.5 mg.l^{-1} NAA and 1.0 mg.l^{-1} BAP.
B. Non-organogenetic callus from leaf bit explant on MS medium supplemented with 1.0 mg.l^{-1} NAA and 1.0 mg.l^{-1} BAP.
C. Non-organogenetic callus from nodal explant on MS medium supplemented with only 2.0 mg.l^{-1} NAA.
D. Non-organogenetic callus from leaf bit explant on MS medium supplemented with 0.1 mg.l^{-1} IAA.
E. Non-organogenetic callus from leaf bit explant on MS medium supplemented with 0.5 mg.l^{-1} IAA.
F. Non-organogenetic callus from leaf bit explant on MS medium supplemented with 0.5 mg.l^{-1} IAA.



Since presence of a single auxin or a single cytokinin in the medium gave rise only to callus, an auxin and a cytokinin were combined together in the next set of experiments. Since BM with IAA alone gave roots and BM with BAP alone formed nodular callus, these two plant growth regulators were first combined together. Both of them were used at lower concentrations to reduce chances of callus formation. In another experiment, BAP was combined with NAA at the same concentrations as optimized for IAA. Both of them were used at lower concentrations to reduce chances of callus formation.

A combination of IAA (0.5, 1.0, 1.5, 2.0 $mg.l^{-1}$) and BAP (1.0 $mg.l^{-1}$) supported shoot bud formation (**Table 6.4**). This is consistent with the earlier studies reported for Dicotyledonary plants (Tran Thanh Van, 1981; Thorpe and Kumar, 1993). The formation of shoot buds occurred either on the adaxial or on the abaxial surface of the leaf explant, whichever was in contact with the medium. The percentage frequency of leaf explants forming shoot buds was 35-63.3% (**Table 6.4**) irrespective of the orientation of the explant on the medium (adaxial or abaxial surface in contact with the medium).

Although shoot bud formation was observed in all the concentrations of BAP and IAA tested, the number of buds produced per explant differed.

The percentage response of explants forming shoot buds increased with increase in concentration of IAA in BM with 1.0 $mg.l^{1}$ BAP, the optimum being 1.5 $mg.l^{-1}$ IAA (**Table 6.4**), (**Fig. 6.5 A**). The number of shoots formed per explant was also maximum at 1.5 $mg.l^{1}$ IAA. The frequency of explants forming shoot buds decreased at 2.0 $mg.l^{1}$ IAA. This may be due to high endogenous auxin levels which lead to a decrease in the percentage response of explants to form shoot buds with an increase in the auxin concentration beyond a threshold value. Similarly, very young leaves failed to support adventitious shoot bud formation probably because they are the sites actively synthesizing auxins (Moore, 1989).

Table 6.4 Effect of Increasing Concentrations of IAA on Formation of Shoots after 45 Days of Culture on BM + 1.0 mg.l^{-1} BAP irrespective of Orientation of Leaf Explant.

BAP (mg.l^{-1})	**IAA (mg.l^{-1})**	**% Response of Leaf Explants Forming Shoot Buds**	No. of Shoots per Leaf Explant (longer than 2 mm) **Mean ± S.D**
0	0	-	-
1.0	0.5	53.3	3.16 ± 1.46
	1.0	35.0	3.00 ± 0.50
	1.5	**63.3**	**9.33 ± 3.16**
	2.0	43.3	5.50 ± 1.07

-: No response.

In the next set of experiments, BAP concentrations were varied from 1.0 to 3.0 mg.l^{-1}, keeping IAA constant at 1.5 mg.l^{-1}, for evaluating the percentage response of explants to form shoot buds and the number of elongated shoots per explant. The percentage frequency of explants forming shoot buds even though varied with increase in BAP concentration, the number of elongated shoots per explant was optimum at 2.0 mg.l^{-1} BAP (**Table 6.5**), (**Fig. 6.5 B**). Thus, the number of shoot buds produced per explant could greatly be increased by manipulating the balance of the growth regulators in BM (Thorpe, 1980).

Table 6.5 Effect of Increasing BAP Concentrations on Adventitious Shoot Production from Leaf Explants After 45 Days of Culture on BM + 1.5 mg.l^{-1} IAA.

IAA (mg.l^{-1})	BAP (mg.l^{-1})	% Response of Leaf Explants Forming Shoot Buds	No. of Shoots per Explant (longer than 2 mm) Mean ± S.D
0	0	-	-
1.5	1.0	63.30	9.33 ± 3.16
	2.0	**56.65**	**16.00 ± 2.82**
	3.0	70.0	13.37 ± 1.66

-: No response.

Sixteen shoots with visibly distinct apical meristems could be produced from a single leaf piece in the optimal medium (**Table 6.5; Fig. 6.5 C, D**). Besides, there were a number of smaller, continuously proliferating shoot buds. With repeated subculture on the same medium at intervals of 3 weeks, each leaf piece can produce 400 shoots within a year. Since each leaf has been explanted into 35 pieces of size 1 cm^2, on an average, 1600 plantlets can be produced from one leaf within a year after elongation and rooting.

The leaf explants, on the contrary, on BM with BAP (1.0, 2.0, 3.0 mg. l^{-1}) and NAA (0.5, 1.0, 1.5, 2.0 mg.l^{-1}) formed only non-organogenetic callus (**Table 6.6**) (**Fig. 6.4 A, B**) or callus with thick white roots, suggesting the preferential nature of auxin to induce shoot buds in combination with BAP.

Fig. 6.5

A – F Direct shoot regeneration from leaf bit explants.

A. Initiation of shoot buds on MS medium supplemented with 1.5 mg.l^{-1} IAA and 1.0 mg.l^{-1} BAP.
B. Initiation of shoot buds on MS medium supplemented with 1.5 mg.l^{-1} IAA and 2.0 mg.l^{-1} BAP.
C. Elongation of shoot buds on MS medium supplemented with 1.5 mg.l^{-1} IAA and 2.0 mg.l^{-1} BAP.
D. Elongation of shoot buds on MS medium supplemented with 1.5 mg.l^{-1} IAA and 2.0 mg.l^{-1} BAP.
E. Further elongation of shoots on MS medium with 0.01 mg.l^{-1} BAP.
F. Rooting of shoots on MS medium with 0.01 mg.l^{-1} BAP.

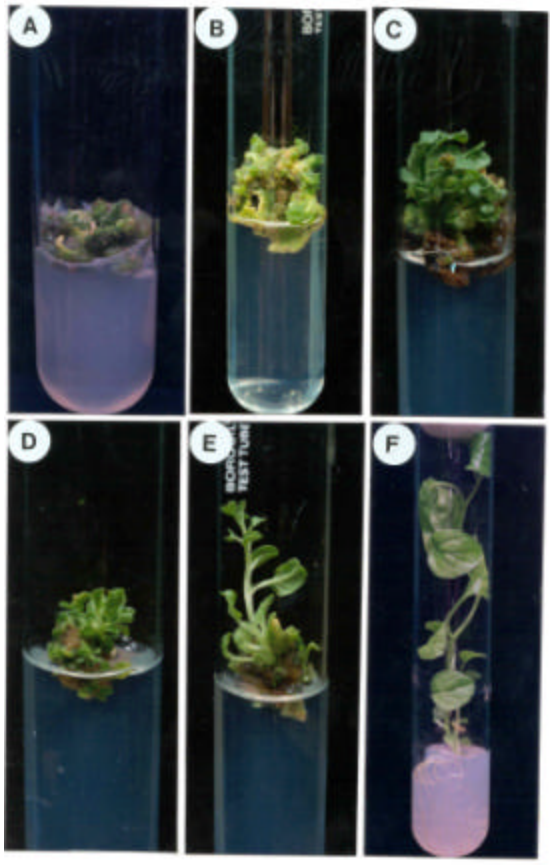


Table 6.6 Effect of BM + NAA and BAP on Callus Formation from Leaf Explants

Plant Growth Regulators ($mg.l^{-1}$)	Amount of Callus*	Callus Type
Nil	-	-
0.5 NAA + 1.0 BAP	++	Whitish-greenish callus with thick, white roots, all over the leaf piece.
1.0 NAA + 1.0 BAP	++	Whitish-greenish callus with thick, white roots, all over the leaf piece.
1.5 NAA + 1.0 BAP	++	Dry, whitish-greenish callus, all over the leaf piece.
2.0 NAA + 1.0 BAP	+++	Some areas of leaf explant show green, nodular callus while other areas show whitish-greenish callus with thick, white roots.
1.5 NAA + 2.0 BAP	++	Friable, whitish-greenish callus, all over the leaf piece.
1.5 NAA + 3.0 BAP	+++	Friable, light green to dark green callus, all over the leaf piece.

*+ : Fresh weight of callus: 0.5-1.0 g.
++ : Fresh weight of callus: 1.0-1.5 g.
+++ : Fresh weight of callus: 1.5-2.5 g.
- : Absence of callus formation.

6.3.1.3 Effect of the culture vessel

The percentage response of explants forming shoot buds was 55.5% when leaf pieces were cultured in the culture tubes on BM with 1.5 $mg.l^{-1}$ IAA and 2.0 $mg.l^{-1}$ BAP, while plastic dishes supported only callus formation (**Table 6.7**). The positive and negative effects of culture vessel have been reported earlier (Wright et al., 1987; McClelland and Smith, 1990). It has been suggested that organogenesis is influenced by the amount of ethylene and carbon dioxide present in the culture vessel. In this case, probably the higher amount of ethylene in the culture tubes has helped in the organogenetic response of the leaf explants. Ethylene is involved in the regulation of cell division during the induction phase while carbon dioxide is required to modify the biosynthesis and action of ethylene (Kumar et al., 1987). It has also been shown that

culture vessels with dosures allowing free gas exchange (cotton plugs in this case) are more conducive for regeneration than hermetically sealed culture vessels (plastic dishes sealed with parafilm in this case) (De Proft et al., 1985; Van Aartrijk et al., 1985).

Table 6.7 Regenerative Response of Leaf Explants on BM with 1.5 mg.l^{-1} IAA and 2.0 mg.l^{-1} BAP in Different Culture Vessels.

Culture Vessel	Replicates	% Response of Explants Forming Shoot Buds	Results
Plastic Dishes (Diameter=55 mm)	60	0	Only green and white callus formation.
Glass Culture Tubes (2.5 x 15 cm)	60	55.5	Shoot buds and shoot formation.

6.3.1.4 Elongation and Rooting of Shoots

All the shoot buds produced on the leaf explants in all the combinations of IAA and BAP were able to elongate to a length of 1 cm on the induction medium itself. Such 1 cm tall shoots were separated individually from the leaf explants and transferred to BM with 0.01 mg.l^{-1} BAP for elongation and rooting. 10 shoots derived from leaf explants on each of the six IAA + BAP combinations (**Tables 6.4 and 6.5**) were transferred to the elongation and rooting medium (**Table 6.10**). Elongation as well as rooting of shoots occurred at a frequency of 100% (One step protocol), (**Fig. 6.5 E, F**) on one and the same medium simultaneously during the first month itself. (Also see **Table 6.10)**. Thus starting from shoot bud induction, plantlets can be obtained in two months.

6.3.2 Regeneration of plants from node, internode and hypocotyl explants on BM supplemented with only BAP.

The node, internode and hypocotyl explants were prepared and inoculated on various BM and plant growth regulator combinations as described earlier in **Section 6.2.3**. Since percentage response of leaf explants in forming shoot buds was practically zero in plastic dishes (**Table 6.7**), these were not used in further experiments with nodes, internodes and hypocotyls. BAP and TDZ are the preferred auxins for induction of multiple shoots from pre-existing meristems and earlier authors had also reported positive effect of BAP for *in vitro* cultures of *Withania* spp. (Vishnoi et al., 1979; Sen and Sharma, 1991; Roja et al., 1991). Hence in the present case, BM was supplemented with either BAP or TDZ individually.

6.3.2.1 Regeneration of plants from nodes

On BM without plant growth regulators, only a single shoot from the pre-existing axillary bud of the node elongated (**Fig. 6.6 A**).

On BM supplemented with lower concentrations of BAP (0.1, 0.2, 0.3, 0.4 $mg.l^{-1}$), a few multiple shoots were produced only from the pre-existing axillary buds (**Table 6.8**), (**Fig. 6.6 B**). On BM supplemented with 0.5 and 5.0 $mg.l^{-1}$ BAP, multiple shoots were produced from the pre-existing meristems of axillary buds (PEM), as well as from the portions of nodes below the axillary buds and embedded inside the medium. Ten to twelve direct adventitious shoot buds were induced from these *de novo* meristems (DNM) (**Table 6.8**), (**Fig. 6.6 C**). The number of shoots produced from both PEM and DNM with 0.5, 1.0 and 5.0 $mg.l^{-1}$ BAP were not significantly different from each other as determined by ANOVA. A total of 16 shoot buds derived from both PEM and DNM of each node at 0.5 $mg.l^{-1}$ BAP and a total of 14 shoot buds derived from both PEM and DNM of each node at 5.0 $mg.l^{-1}$ BAP were elongated and rooted after one passage on BM supplemented with 0.01 $mg.l^{-1}$ BAP (**Table 6.10**). In 2% of nodes inoculated in BM supplemented with 0.5 $mg.l^{-1}$ BAP, the cut end gave rise to green colored callus. This was separated from the explants and subcultured separately in identical media for eventual use in extraction and HPLC analysis of **Withaferin-A**.

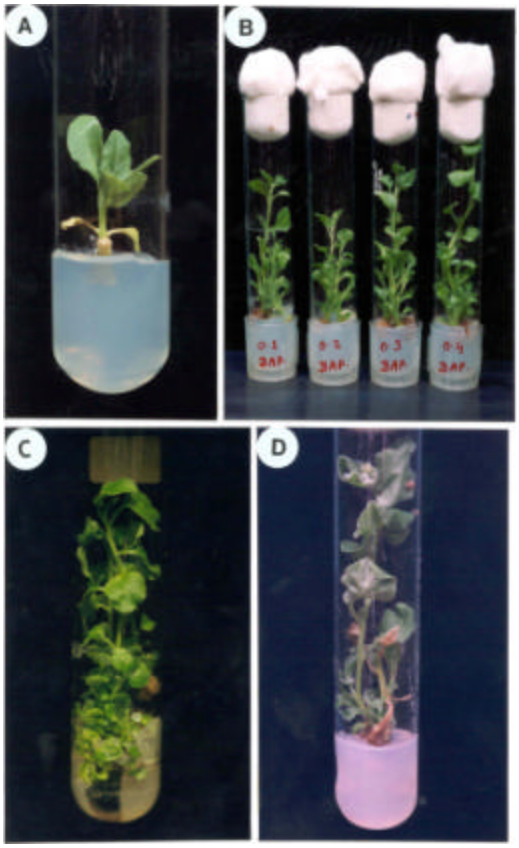


Fig. 6.6

A. – D Response of nodal explants to various plant growth regulators.

A. Elongation of a single axillary shoot on MS medium without plant growth regulators.
B. Formation of multiple shoots on MS media supplemented with various BAP concentrations (From left to right, 0.1, 0.2, 0.3 and 0.4 mg.l^{-1} BAP).
C. Formation of multiple shoots from PEM (above the medium) and DNM (inside the medium) on MS media supplemented with 0.5 mg.l^{-1} BAP.
D. Flowering of shoots developed on MS medium supplemented with 1.0 mg.l^{-1} BAP.

De novo shoot bud formation was not observed on BM with 1.0 mg l^{1} BAP, the reason for which is unclear. However, all the multiple shoots produced from the pre-existing axillary buds flowered (**Fig. 6.6 D**).

The axillary buds on both the PEM and DNM types of newly induced shoots on 0.5 and 5.0 mg.l^{-1} BAP (**Table 6.8**) sprouted simultaneously overcoming the natural apical dominance and effectively increasing the overall number of shoots which could be separated, elongated and rooted with 100% efficiency. But only the newly induced shoots and not the precociously sprouting axillary buds were taken into account for counting the number of shoots produced in these BAP concentrations.

Unlike the leaf explants, once the first round of shoots was cut from the nodal explant, it was not possible to get another round of shoot induction from the same nodal explant.

6.3.2.2 Regeneration of plants from internodes

On lower concentrations of BAP (0.1-0.5 mg.l^{-1}), internodes did not respond to form shoots. Very high numbers (25-40) of *de novo* shoot buds were induced from internodes only at higher BAP concentrations (1.0, 5.0 mg.l^{-1} BAP) (**Table 6.8**), (**Fig. 6.7 A, B**). Since the shoot buds were crowded, their exact number could not be counted even under magnification. The complete surface of the internodes was covered with well-developed shoot buds with distinct apical meristems. These shoot buds could be easily elongated and rooted as mentioned earlier in **Section 6.2.4** (**Fig. 6.7 D**).

6.3.2.3 Regeneration of plants from hypocotyls

Twelve shoots per hypocotyl explant piece were induced on BM supplemented with 0.5 mg l^{1} BAP (**Table 6.8**) and the explants continued to form shoots even when they were transferred to a medium without plant growth regulators (**Fig. 6.8 A, C**). This effect was more pronounced in screw capped bottles that were large and had more amount of head space volume (**Fig. 6.8 B**). During each monthly subculture on BM without plant growth regulators, twelve shoots could be recovered upto the fourth subculture resulting in 48 shoots from a single hypocotyl explant piece during a four month period and 144 shoots in one year. Since one hypocotyl gives rise to 3 pieces of hypocotyl explant, a single hypocotyl would produce 432 shoots in an year. BM supplemented with lower

concentrations of BAP (0.1, 0.2, 0.3 and 0.4 mg.l^{1}) failed to induce any shoot regeneration in hypocotyl explants while higher BAP concentrations were not tried. These shoot buds could be easily elongated and rooted as mentioned earlier in **Section 6.2.4**. Also see **Table 6.10** and **Fig. 6.8 D**.

6.3.2.4 Regeneration of plants from embryos

The embryos did not form shoot buds on any of the BAP concentrations tried (0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 5.0 mg.l^{-1}).

Table 6.8 Effect of BAP Concentrations on the Number of Shoots Longer than 0.5 mm (Mean ± S. E.) Produced from Nodes, Internodes and Hypocotyls.

BAP (mg.l^{-1})	**Explant**			
	Node		**Internode**	**Hypocotyl**
	PEM*	**DNM****	**DNM****	**DNM****
0.0 (Control)	1.00 ± 0	-	-	-
0.1	1.94 ± 0.14^{b}	-	-	-
0.2	1.71 ± 0.46^{b}	-	-	-
0.3	1.55 ± 0.33^{a}	-	-	-
0.4	1.65 ± 0.40^{b}	-	-	-
0.5	4.53 + 1.11^{c}	12.5^{a}	-	12.88 + 2.36
1.0	4.58 ± 1.12^{c}	-	More than 25^{a}	nd
5.0	4.07 ± 0.82^{c}	10.22^{a}	More than 40^{b}	nd

* PEM : Pre-existing meristems.
** DNM : *de novo* meristems.
\- : No response.
nd : Not determined.
$^{a, b, c}$: Treatment means with different letters in a column are significantly different at the 95% confidence level by t-test.

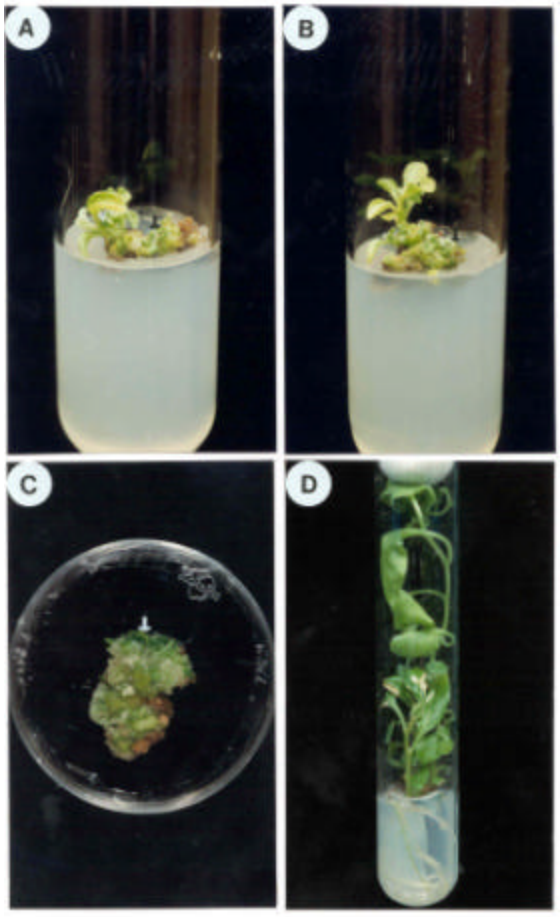


Fig. 6.7

A. – D Response of internodal and embryo explants to various plant growth regulators.

A. Direct initiation of shoot buds on internode explants grown on MS media supplemented with 1.0 $mg.l^{-1}$ BAP. The black arrow indicates small shoot buds.

B. Direct initiation of shoot buds on internode explants grown on MS media supplemented with 5.0 $mg.l^{-1}$ BAP. The black arrow indicates small shoot buds.

C. Direct initiation of shoot buds on embryo explants grown on MS media supplemented with 0.3 $mg.l^{-1}$ TDZ. The white arrow indicates small shoot buds.

D. Elongation and rooting of shoots derived from internodal explants on MS medium supplemented with 0.01 $mg.l^{-1}$ BAP.

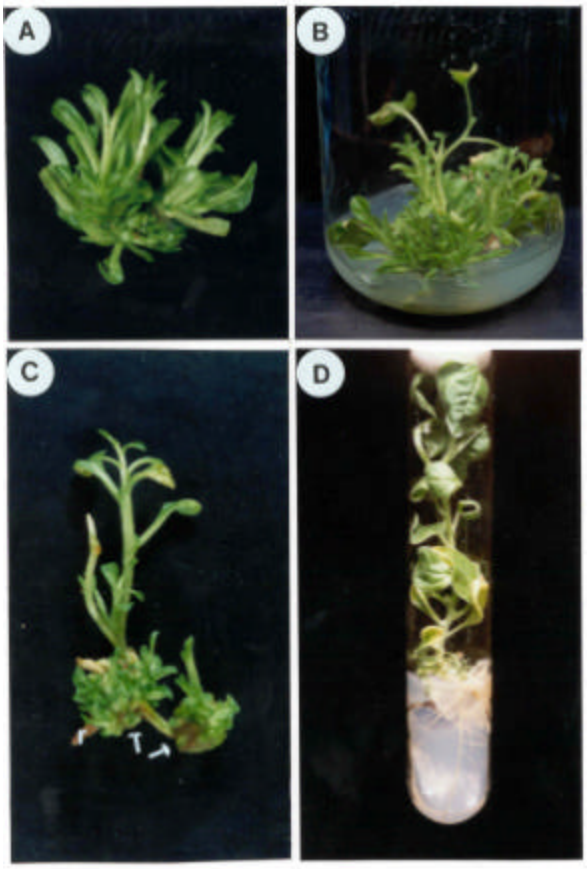


Fig. 6.8

A. – D. Response of hypocotyl explants to 0.5 mg.l^{-1} BAP incorporated in MS medium.

A. Multiple shoot induction from hypocotyl explants in culture tubes.
B. Further growth of multiple shoots derived from hypocotyl explants and cultured in glass bottles.
C. Multiple shoot induction from hypocotyl explants. Arrows indicate the actual portion of hypocotyl, r = root portion.
D. Elongation and rooting of shoots derived from hypocotyl explants on MS medium supplemented with 0.01 mg.l^{-1} BAP.

6.3.3 Histology

6.3.3.1 Shoot regeneration from leaves

The shoot buds were observed all over the surface of the explant. The buds arose from the mid-vein as well as the lateral veins and the cut edges of the leaf explant, indicating their induction from the pre-existing meristems as well as from the newly formed meristematic cells near the cut edges of the leaf pieces. This was also proven by histological observations (**Fig. 6.9 A**). **Fig. 6.9 B** shows a newly developed shoot bud with an apical meristem and two leaf primordia with distinct vasculature.

6.3.3.2 Shoot regeneration from DNM of nodes

When the portions of nodes embedded inside the medium were analyzed by histology, it could be seen that *de novo* shoot bud initiation occurred from meristematic activity located below the epidermis (**Fig. 6.9 C**).

6.3.3.3 Shoot regeneration from DNM of internodes

Histologically it could be seen that meristematic patches developed from the sub-epidermal regions of the explant and subsequently the meristematic activity spread over the entire cortical region (**Fig. 6.9 D**).

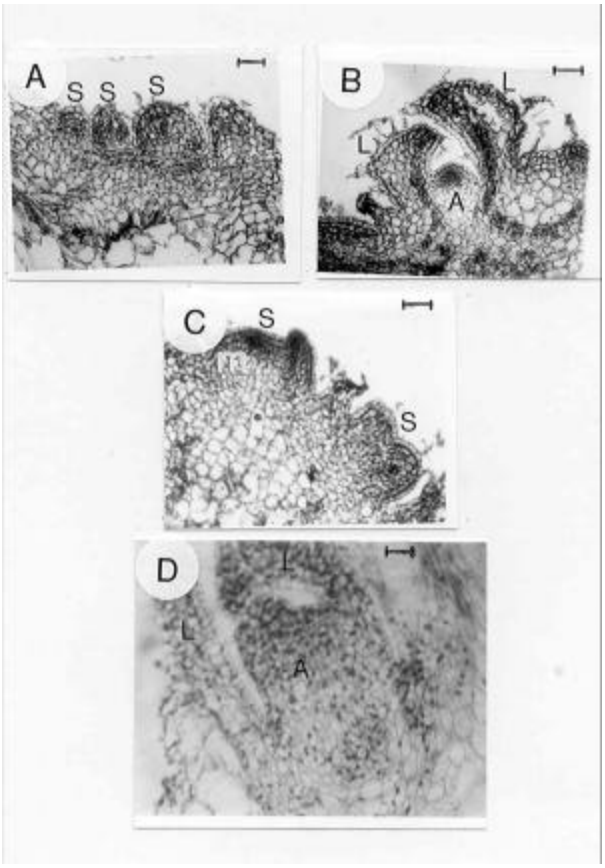


Fig. 6.9 Histological analysis of shoot bud formation from different explants.

A. Initiation of shoot buds (S) from sub-epidermal region of leaf explant. Bar: 165µ.
B. A well-developed shoot from leaf explant showing an apex (A), bound by two-well developed leaves (L) with distinct vascular trace. Bar: 165µ.
C. Initiation of shoot buds (S) from *de novo* meristems (DNM) developed sub-epidermally from nodal explants. Bar: 165µ.
D. A well-developed shoot from internodal explant showing an apex (A), bound by two-well developed leaves (L). Bar: 120µ.

6.3.4 Regeneration of plants from node, internode and embryo explants on BM supplemented with only TDZ.

The node, internode and hypocotyl explants were prepared and inoculated on various BM and plant growth regulator combinations as described earlier in **Section 6.2.3,** in culture tubes only as plastic dishes were not used for the reasons described earlier in **Section 6.3.2**. Only the small embryonal explants were inoculated in plastic dishes

6.3.4.1 Regeneration of plants from nodes

One or two shoots with a stunted pattern of development were induced from the pre-existing meristem on BM supplemented with 0.05 and 0.1 mg.l^{-1} TDZ. Rest of the explant was covered with callus (**Table 6.9**). Maximum number of 24 shoots per node were obtained from the pre-existing meristems (PEM) of axillary buds on BM supplemented with 0.2 mg.l^{-1} TDZ. Unlike BAP, no precocious sprouting of axillary buds was observed and the shoots were shorter in length. In addition a distinct ring of *de novo* meristem (DNM) derived adventitious shoot buds (ten to twelve) formed below and surrounding the pre-existing axillary bud on media with 0.2 and 0.3 mg.l^{-1} TDZ (**Table 6.9**), (**Fig. 6.10 A**). The number of shoots first increased with an increase in TDZ concentrations and then decreased (**Table 6.9**). This method will prove to be of a great advantage in large-scale clonal propagation of selected elite plants.

The shoots derived from PEM as well as DNM could be elongated and rooted only after four passages on BM with 1/2 strength of major salts (1/2 BM) without BAP (**Table 6.10**) (**Fig. 6.10 B**). The high carry-over effect of TDZ has been well documented in the literature (Meyer and Van Staden, 1988; Lu, 1993).

Table 6.9 Effect of TDZ Concentrations on Number of Shoots Longer than 0.5 mm (Mean ± S. E.) Produced from Nodes, Internodes and Embryos.

TDZ ($mg.l^{-1}$)	Explant						
	Node			Internode		Embryo	
	Number of Shoots PEM*	DNM**	Callus Type	Number of Shoots	Callus Type	Number of Shoots	Callus Type
Absent	-	-	-	-	-	-	
0.05	1.00 ± 0.0[b]	-	Yellowish-green nodular callus. No visible shoot buds	-	Whitish-green callus above the medium. Green, nodular callus inside the medium. No expressed shoot buds.	-	Slightly swollen embryos but no further growth.
0.1	2.0 ± 0.41[b]	-	-	-	Yellow, soft callus and green, nodular callus. No expressed shoot buds.	-	Swollen embryos with little green callus.
0.2	24.13 ± 1.28[c]	10.1[a]	-	-	White and brown, friable callus. Also green, nodular callus. No expressed shoot buds.	10 ± 0.54	Much swollen embryos with many adven-titious shoot buds.
0.3	1.00 + 0.0[b]	12.3[a]	Yellowish-green nodular callus inside the medium.	-	Greenish yellow, soft callus.	Innumera-ble, difficult to count and elongate.	Much swollen embryos with many adven-titious shoot buds.

* PEM : Pre-existing meristems
** DNM : *de novo* meristems
\- : No response
nd : Not determined
[a, b, c] : Treatment means with different letters are significantly different at the 95% confidence level.

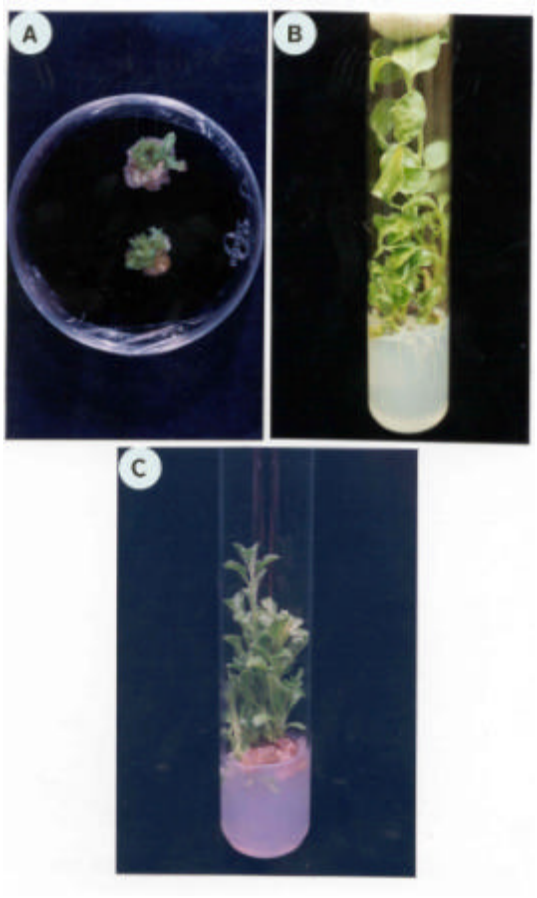


Fig. 6.10

A. A ring of *de novo* shoot buds formed on nodes grown on MS medium supplemented with 0.2 mg.l^{1} TDZ.

B. Simultaneous elongation and rooting of shoots derived from nods growing on 0.2 mg.l^{-1} TDZ supplemented MS medium, on 1/2 MS medium without plant growth regulators.

C. A multiple shoot culture derived from nodes grown on MS medium supplemented with 0.5 mg.l^{1} BAP and used for extraction of Withanolides for HPLC analysis.

6.3.4.2 Regeneration of plants from internodes

The internodes did not form shoot buds or shoots on any of the TDZ concentrations tried (0.05, 0.1, 0.2, 0.3 mg.l^{1}). At 0.05 mg.l^{1}TDZ, friable callus was formed while higher concentrations (0.1, 0.2 mg.l^{-1}) induced typical, nodular, green callus embedded in the medium which did not differentiate into shoot buds (**Table 6.9**).

6.3.4.3 Regeneration of plants from hypocotyls

The response of hypocotyls for shoot bud formation was not determined for any of the TDZ concentrations (0.01 - 0.3 mg.l^{-1}) because enough explants were not available for experimentation.

6.3.4.4 Regeneration from embryos

The embryos directly gave rise to shoot buds on media containing high concentrations of TDZ. On media with 0.2 mg.l^{-1} TDZ, ten shoot buds were produced while on 0.3 mg.l^{-1} TDZ, innumerable shoot buds were produced which could not be individually counted (**Fig. 6.7 C**). But these shoot buds demonstrated a carry over effect of high TDZ concentrations and had to go through at least four passages on 1/2 BM (Macro salts of MS medium reduced to 1/2 strength) without plant growth regulators before they could be elongated and rooted. Only 50% of the induced shoot buds developed into full length shoots (**Table 6.10**).

Comparison of **Tables 6.8 and 6.9** indicates that TDZ is a better cytokinin than BAP for multiple shoot induction from nodal explants since it is able to induce far more number of shoots than BAP, at much lower concentrations. The high stability of TDZ due to its resistance to cytokinin oxidase (Mok et al., 1987) might be a reason for its efficacy. Another possible reason could be its auxin-like and cytokinin-like activities (Visser et al., 1992). TDZ might be impinging upon the endogenous auxins by their modified biosynthesis and/or their protection *in vivo*.

The direct induction of shoot buds on internodes in the presence of only a cytokinin without the need of an auxin is not an often encountered phenomenon since the internodes do not have pre-formed meristems. This result may be attributed to the high intrinsic auxin levels in the internodes of this plant; so that by supplementing them with

high concentrations of BAP it is possible to balance both the growth regulators so that the explant becomes *competent* for organogenesis (Christianson and Warnick, 1985). TDZ might not be able to induce organogenesis in internodes because of its auxin as well as cytokinin like activities which might be disrupting the delicate balance necessary for shoot bud formation.

In the present study, since shoot regeneration from node, internode, hypocotyl and embryo explants could be achieved on MS medium supplemented with either BAP or TDZ, further experiments by supplementation of these cytokinins with auxins were not carried out.

6.3.5 Acclimatization

Twenty regenerants were randomly selected from each type of explant during subculture numbers 1, 3, 6, 9, 12 (subcultures of 3-4 weeks duration during the year) and hardened at different periods of time taking care that they were derived from different explants of different parent plants while the remaining sterile shoots were used for further regeneration studies and initial experiments in plant transformation. Except for the embryo-derived plantlets, regenerants from all the other explants (leaf bits, nodes, internodes, hypocotyls) could be hardened with 100% success rate (**Table 6.10**). All the regenerants exhibited normal morphology with respect to leaf shape and size and stem morphology.

Table 6.10 Elongation, Rooting and Acclimatization of Shoots Derived from Various Explants.

Explants	Plant Growth Regulator in Induction Medium	Elongation and Rooting Medium	% Elongation and Rooting	Number of Passages*	% Acclimatization
Leaves	IAA + BAP	BM + 0.01 BAP	100	One	100
Node	BAP	BM + 0.01 BAP	100	On low BAP - One On high BAP - Two	100 100
	TDZ	1/2 BM	100	Four	100
Internode	BAP	BM + 0.01 BAP	100	Two	100
	TDZ	-	-	-	-
Hypocotyl	BAP	BM + 0.01 BAP	100	One	100
	TDZ	nd	nd	nd	nd
Embryo	BAP	-	-	-	-
	TDZ	1/2 BM	50	Four	0

- : No response
nd : Not determined
* : Passage duration is one month.

6.3.6 HPLC analysis of Withaferin-A standard

To detect the presence of **Withaferin-A**, normal, *in vitro* seedlings (Control), multiple shoot cultures induced on MS medium supplemented with 0.5 mg.l^{-1} BAP and the callus formed on the same medium in 2% nodal explants (C. f. **Section 6.3.2.1**) were extracted and analyzed by HPLC. **Fig. 6.11 A** shows the chromatogram of standard (A kind gift from Dr. M. I. Choudhary of HEJ Res. Inst., Pakistan) of **Withaferin-A** (10 µg injected in 20 µL) obtained under conditions described earlier in **Section 6.2.9**. It can be seen that there are two well defined and baseline resolved peaks (12 min. and 15 min.) in the standard itself. Hence the tissue extracts were compared with both of these peaks. A standard curve plotted for various concentrations of **Withaferin-A** (1.25-10 µg per 20 µL) is shown in **Figs. 6.11 B, C**. It shows that the detector response is linear for the concentrations of less than 10 µg.

6.3.7 Extraction and HPLC analysis of *in vitro* tissues

For extractions methanol was the preferred solvent as described above. No additional extractions with hexane or other apolar solvents was necessary to remove lipophilic compounds as the tissues were very juvenile (Theodoridis et al., 1998). This was proven during the HPLC analysis because there was no significant increase in back-pressure of the column and the chromatograms sometimes showed only an initial broad peak of polar impurities which did not interfere with the **Withaferin-A** analysis.

HPLC is the most appropriate technique for an efficient separation of crude plant extracts and can be coupled with different spectroscopic detection methods (Hostettmann and Wolfender, 1997). In the present endeavor, HPLC coupled with UV photodiode array detection was used. The UV spectra of natural compounds give useful information about the type of constituents and their structures (Hostettmann and Wolfender, 1997).

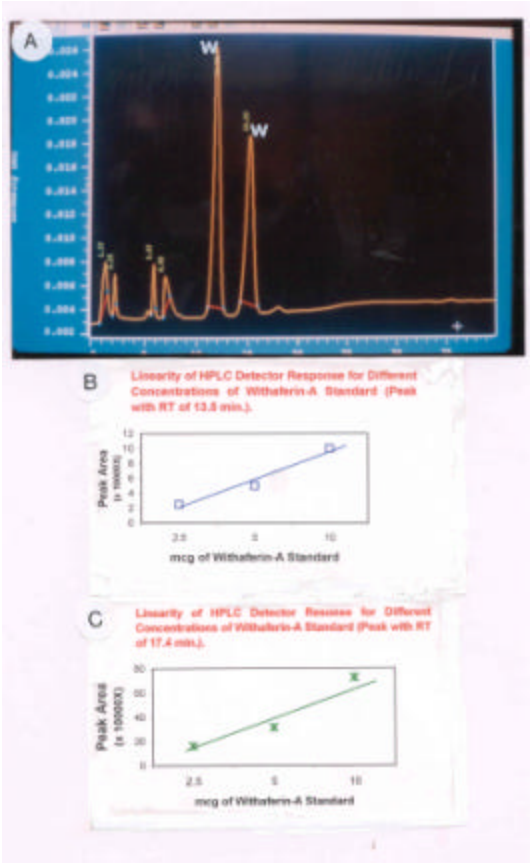
While carrying out the HPLC analysis of tissue cultures of *Withania* spp., everyday at least two independent injections of the samples were performed and an average of the peak areas was calculated. The standard error of the mean (S. E.) obtained was always less than 8 over the analysis period. The sample injections from the same sample preparation were repeated on consecutive days and again the S. E. for the peak areas was less than 6.

Fig. 6.11

A. HPLC chromatogram of Withaferin-A standard. It shows two distinct, baseline-resolved peaks at retention times of 11.3 min. and 15.2 min.

B. A graph showing linearity of HPLC UV detector response for various concentrations of Withaferin-A standard, for the peak eluting at retention time of 13.5 min. The graph is plotted as peak area versus mcg of Withaferin-A standard. Note that the detector response is linear for all the concentration tried of Withaferin-A standard for the peak a 11.3 min.

C. A graph showing linearity of HPLC UV detector response for various concentrations of Withaferin-A standard, for the peak eluting at retention time of 17.4 min. The graph is plotted as peak area versus mcg of Withaferin-A standard. Note that the detector response is linear for all the concentration tried of Withaferin-A standard for the peak a 15.2 min.



Shoots of two-month old seedlings (source of explants for all the experiments) had **Withaferin-A** (**Table 6.11**) (**Fig. 6.12 A**). The multiple shoot cultures derived from nodes grown on agar solidified MS medium with 0.5 $mg.l^{-1}$ BAP were also able to synthesize **Withaferin-A** (**Fig. 6.12 B**). The amount was at least ten times more than that present in the seedling shoots (**Table 6.11**). However callus cultures derived from the same nodal explants on identical medium did not have **Withaferin-A** (**Table 6.11**). This corroborates the earlier observations made by Roja et al. (1991) that tissue organization into organs is necessary for **Withanolide** production. Roja et al. (1991) reported production of **Withanolide-I** in the range of 13-172 mg/g dw of shoots, **Withanolide-G** in the range of 10-317 mg/g dw of shoots, **Withanone** in the range of 90-486 mg/g dw of shoots and traces of **Withanolide-D** except **Withaferin-A** (the major anti-tumor compound).

But in the present study, **Withaferin-A** production has been observed and it is produced in the range of 3.903-4.000 mg/g dw of shoots of *in vitro* grown seedlings and in the range of 55.1667-51.1833 mg/g dw of *in vitro* shoots, even though **Withaferin-A** in leaves of *in vivo* plants has been reported to be in the range of 0.16% DW (Gupta et al., 1996) or 0.2-0.3% dw (Abraham et al., 1968). The values reported here are far higher than the values reported by others and in addition, are even higher than those reported for *Agrobacterium tumefaciens* transformed tissues (Ray and Jha, 1999). The range of **Withaferin-A** produced in shooty terratomas was in the range of 0.7-1.0 mg/g dw of shoots while the untransformed shoots contained 0.4-0.5 mg **Withaferin-A**/g dw of shoots. Since the chemotype of *Withania* spp. and the *in vitro* shoots raised from this chemotype used in the present study are far superior in the production of **Withaferin-A**, the knowhow generated in this study could be further used for scale up studies in a bioreactor for large scale production of **Withaferin-A**, even without resorting to hairy root cultures.

Although in case of **Withanolides**, no particular specialized structures have been identified for accumulation, the biosynthetic capacity may be localized in specific cell types in the leaves and a regulatory hierarchy may be involved in which morphology is a dominant factor (Robins, 199). Leaves are believed to be the sites of withanolide synthesis since the steroids are derived from mevalonic acid (Ray and Gupta, 1994) and probably the withanolides are transported across the entire shoot system. Similar

observations have been reported for other systems like *Mentha* spp. and *Ruta* spp. In *Nothapodytes foetida*, the amount of Camptothecin and 9-methoxy-camptothecin was higher in differentiated cultures than in callus cultures (Roja and Heble, 1994). Also refer to **Chapter 1, Section B** of the present thesis for more examples.

Further studies are necessary for finding the relationships between withanolide production and plant growth regulators employed for getting multiple shoot cultures. It is also necessary to undertake detailed studies to elucidate the reason for production of withanolides in un-transformed seedling root cultures where the necessary biochemical machinery for steroidal lactone production is believed to be absent (See **Annexure** for details).

Table 6.11 A Comparison of Withaferin-A Production in Different Types of Tissue Cultures of *Withania somnifera*.

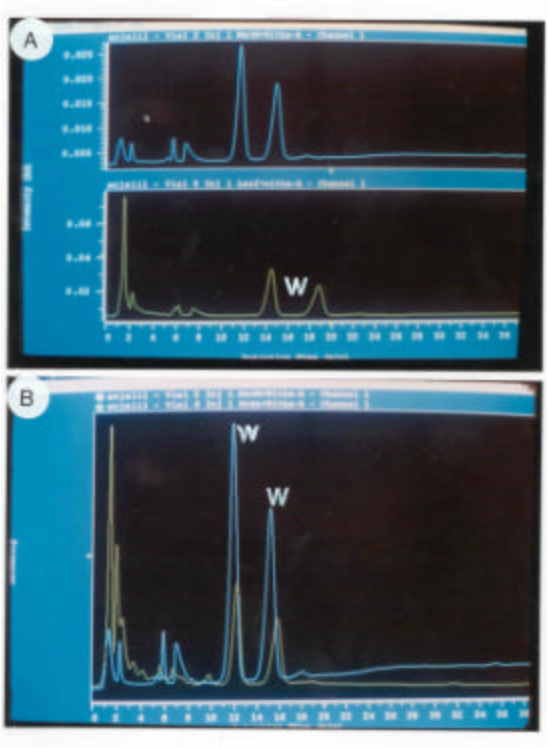
No.	Type of *in vitro* Tissues	Amount of Withanolides (mg/g dry wt. of Tissues)	
		Peak with retention time of 12 min.	Peak with retention time of 15 min.
1.	Shoots from two-month old seedlings, germinated *in vitro*.	3903.5	4000
2.	Multiple shoot cultures derived *in vitro* from nodes of two-month old seedlings on MS medium supplemented with 0.5 mg.l^{-1} BAP.	55166.7	51183.3
3.	Callus cultures derived *in vitro* from nodes of two-month old seedlings on MS medium supplemented with 0.5 mg.l^{-1} BAP.	-	< 2

-: Not detected/Absent.

Fig. 6.12

A. HPLC chromatogram showing presence of Withaferin-A in 2 month old seedling-derived shoot tissues of *Withania somnifera* (L.) Dun. The upper frame shows the chromatogram for the standard of Withaferin-A while the lower frame represents the spiked extract of seedling-derived shoot tissues with the standard. The peaks named as W represent those of Withaferin-A.

B. HPLC chromatogram showing presence of Withaferin-A in 4 month old multiple shoot cultures derived from nodal explants of *Withania somnifera* (L.) Dun., cultured on MS medium supplemented with 0.5 $mg.l^{-1}$ BAP. The blue colored peaks in the chromatogram are for the standard of Withaferin-A while the green colored peaks represent the spiked extract of multiple shoot culture-derived shoot tissues with the standard. The peaks named as W represent those of Withaferin-A.



6.4 Conclusions

1) **Direct regeneration of plants has been achieved from a number of explants like leaves, nodes, internodes, hypocotyls and embryos. Regenerated shoots have been obtained in 70% of leaf explants on an optimal medium while all the remaining explants gave 100% response for shoot formation on media standardized during the course of the present work.**
2) **By the protocol standardized in this study, 1600 and 432 plantlets per year per explant could be produced from leaves and hypocotyl explants respectively.**
3) ***De novo* regeneration of 24 shoots from a single node explant could be obtained with TDZ. TDZ was found to be the best plant growth regulator among the tested plant growth regulators.**
4) **All the regenerants had a high success rate of acclimatization and hardening.**
5) **High amounts of Withaferin-A were detected in both the seedlings and *in vitro* regenerated shoots from nodes. *In vitro* grown seedlings had 10 times more Withaferin-A than the reported values in literature for *in vivo* tissues of *Withania somnifera* (L.) Dun. *In vitro* regenerated shoots growing on MS medium supplemented with 0.5 mg.l^{-1} BAP had 100 times more Withaferin-A than *in vivo* tissues and to our knowledge such high amounts of Withaferin-A reported here are for the first time in literature.**

The work presented in this chapter has been published in two international papers:

a) **Kulkarni A. A., Thengane S. R. and Krishnamurthy K. V. (1996) Direct *in vitro* regeneration of leaf explants of *Withania somnifera* (L.) Dunal. Plant Science 119: 163-168.**
b) **Kulkarni A. A., Thengane S. R. and Krishnamurthy K. V. (2000) Direct Shoot Regeneration from Node, Internode, Hypocotyl and Embryo Explants of *Withania somnifera* (L.) Dun. Accepted for publication in Plant Cell Tissue and Organ Culture.**

ANNEXURE

Induction of Hairy Root Cultures in *Withania* spp. by Infection with *Agrobacterium rhizogenes*.

A.1 Introduction

Withania somnifera (L.) Dunal is widely prescribed in the Ayurveda because of the pharmacological properties due to a number of **Tropane Alkaloids** present in the roots of the plant (Anonymous, 1976). It is called as 'Indian Ginseng' and shows comparable properties to 'Chinese Ginseng' (*Panax ginseng*) (Ray and Gupta, 1994). Lavie's group (1965) elucidated the structure of **Withaferin-A** (A Steroidal Lactone) in leaves of this plant, which is mostly valued for its anti-cancerous properties. The anti-tumor activity of this compound was further demonstrated by many researchers (Kupchan et al., 1969; Uma Devi et al., 1992). The yields of **Withaferin-A** in intact plants of *Withania* spp. (Israeli Chemotype) are 0.2-0.3% of DW of leaves (Abraham et al., 1968). Gupta et al. (1996) have done quantitative analysis of Indian chemotypes of *Withania somnifera* by TLC densitometry and observed that **Withaferin-A** was totally absent in roots, stems, seeds and persistent calyx of fruits of intact plants except in leaves (1.6%). Surprisingly the alkaloids were detected in all the above mentioned plant parts and leaves had the highest content. This is in contrast to the general belief that the **Tropane Alkaloids** are restricted to the roots of *Withania* spp.

Up till now, attempts have been made to analyze callus, shoot and transformed tissue cultures of *Withania somnifera* for the presence of antitumor **Withanolides** (**Table 6.2**). These studies have indicated that Withanolide production is related to morphogenesis of cultures and the regenerated shoots have more content of Withanolides than the unorganized callus cultures (Roja et al., 1991; Results presented in **Chapter 6** of the present thesis).

As discussed earlier in **Chapter 1**, hairy roots from many species have been shown to synthesize a number of novel compounds absent in the parent plant. In addition many of the compounds present in the parent plant are synthesized in higher quantities by the hairy roots. Hairy root cultures were induced in *Withania somnifera* (Banerjee et al., 1994) by *Agrobacterium rhizogenes* strains A4, LBA 9402 and LBA 9360 in an attempt to further increase the quantities of **Withaferin-A**, normally present in shoots of intact plants. Its presence was detected in the hairy roots as well as in the growth medium. But quantitative estimation of **Withaferin-A**, the baseline separation of its peak and data on peak purity, co-chromatography with standard of **Withaferin-A** were not reported and

thus the data is not comprehensive and conclusive. In another report, Ray et al. (1996) reported presence of **Withanolide-D** and absence of **Withaferin-A** in hairy roots of *Withania somnifera*. In a recent publication, even though Ray and Jha (1999) have reported formation of galls, rooty and shooty terratomas and hairy roots from leaves of *Withania somnifera* with a number of *Agrobacterium tumefaciens* strains, presence of **Withaferin-A** in hairy roots and rooty terratomas was not detected. On the other hand shooty terratomas contained both these compounds.

In view of the above, the present study made a preliminary attempt to genetically transform leaf and nodal explants of *Withania somnifera* with different strains of *Agrobacterium rhizogenes* in order:

1. To induce hairy roots on different explants.
2. To check for possible production of Withanolides by these hairy roots since their site of synthesis is leaves and not the roots.
3. To estimate the contents of Withanolides, especially Withaferin-A, quantitatively in the *in vivo* and *in vitro* generated hairy roots. The results obtained are further compared with Withaferin-A contents of the untransformed seedling roots and shoots.

A.2 Materials and Methods

A.2.1 Strains and growth of *Agrobacterium rhizogenes*

Five wild strains of *Agrobacterium rhizogenes* were procured from "American *Type Culture* Collection", USA in the form of freeze dried cultures. These were ATCC 15834, ATCC 11325, ATCC 13332, ATCC 13333 and ATCC 25818. These were rehydrated and revived according to the supplier's instructions in Medium 3 (**Table A.1**). The wild strain A4 was a kind gift from Dr. M. R. Heble ("BARC", Mumbai, India). All these strains were grown routinely from single colonies in liquid YEB medium (Dietze et al., 1995) and in liquid AB medium to reduce the clumping during growth (White and Nester, 1980). The compositions of the media used are described in **Table A.1.** Glass distilled water was used for media preparations and pH was adjusted at 7.0 using 0.1 N KOH or 0.1 N HCl. Autoclaving was done at 121°C and at a pressure of 1.1 $kg.cm^{-2}$ for 20 min. When needed, 2% bacto-agar ("Difco Laboratories", USA) was added to the respective media and 20 ml of this medium were poured in each of the plastic dishes (85 mm diameter, "Laxbro", India).

Table A.1 Composition of Bacterial Culture Media Used (mg l^{-1}).

Chemicals	YEB Medium (Dietze et al., 1995)	Medium 3 (ATCC sheet of instructions)	AB Medium (White and Nester, 1980)
Beef Extract	5000	1000	-
Yeast Extract	1000	-	-
Peptone	5000	1000	-
$MgSO_4$, 7 H_2O	490	-	31
NaCl	-	500	-
KH_2PO_4	-	-	3000
Na_2HPO_4, 2HO	-	-	1000
NH_4Cl	-	-	1000
KCl	-	-	150
$FeSO_4$, 7 H_2O	-	-	2.5
$CaCl_2$, 2 H_2O	-	-	13
Biotin	-	-	0.2
Sucrose	5000	-	5000
Agar (For solid media only).	20000	20000	20000

A.2.2 Growth and maintenance of microbial cultures

Short-term storage of all types of pure bacterial cultures was achieved by maintaining them in the form of single colonies on plates of respective solid media (**Table A.1**) kept at 4°C. When needed for experimentation, all these strains were grown routinely from single colonies on respective liquid media, in dark and at 200 rpm in an incubator shaker ("Steelmate", India) at 25 $\pm$ 2°C. The cultures in the log phase of growth (Usually grown for 24-48 hours) were used for all the experiments (O. D. = 1 at wavelength of 600-650 nm) (Senior et al., 1995). Long term storage was achieved by making glycerol stocks of each of the pure strains and storing them at a temperature of –70°C (Maniatis et al., 1982).

A.2.3 Plant material

Two months old the seedlings grown as described in **Chapter 6, Sections 6.2.2 and 6.2.3** served as the source material for obtaining nodes and leaf explants used in co-cultivation experiments.

A.2.4 Co-cultivation experiments

Leaves were cut into pieces of size 1 cm x 1 cm while nodes were cut into 3-5 mm long pieces. These were then dipped in *Agrobacterium rhizogenes* bacterial cell suspension (5 ml) in the log phase of its growth, for 15 min. The explants were then blotted on sterile filter papers and transferred to plastic dishes (55 mm diameter, each with 10 ml medium) containing solid MS medium without plant growth regulators for co-cultivation for 48-72 hours in total darkness, to prevent drying of bacteria under illumination during the 'Window of Competence' for transformation of wounded tissues (Binns and Thomashow, 1988). At the end of co-cultivation period, the explants were washed in sterile distilled water and transferred to dishes of solid MS medium without plant growth regulators but supplemented with 500 $mg.l^{-1}$ Claforan (Sterile Cefotaxime sodium U.S.P., "Roussel", India) and incubated for 15 days in total darkness at 25 $\pm$ 2°C. The explants with emerging roots or calli were then transferred to fresh solid MS medium without plant growth regulators but supplemented with 250 $mg.l^{-1}$ Claforan and incubated for another 15 days in darkness at 25 $\pm$ 2°C.

A. 2.5 Culture of hairy roots

One month from the beginning of co-cultivation of explants, the root tips of the hairy roots formed were excised (terminal 2-3 cm long portion) and transferred to liquid MS medium without plant growth regulators but supplemented with 100 mg.l^{1} Claforan (30 ml medium in each 100 ml Erlenmeyer flask) and incubated in darkness on a magnetic shaker ("Infors AG", Germany) at 110 rpm and 25 $\pm$ 2^{o}C. After 7 days in this medium, the root tips of hairy roots were further subcultured in liquid MS medium without plant growth regulators but with 50 mg.l^{-1} Claforan and grown for another 7 days. The hairy roots after this period were routinely subcultured in liquid MS medium without plant growth regulators and the antibiotic and the established hairy roots were maintained by subculturing regularly at 7-day intervals. During each of these subcultures, 300 mg of hairy roots (fresh weight) were added as fresh inocula to 30 ml of liquid medium. The absence of Agrobacteria as contaminants was confirmed by subculturing the hairy roots for another two subcultures of one-week duration each. For confirmation of the absence of Agrobacteria along with the roots, three representative growing root tips from each flask, chosen at random, were squashed and smeared on plates of solid YEB medium. The plates were incubated in dark at 25 $\pm$ 2°C for four days and the absence of bacterial growth was confirmed. Such hairy root lines (3 months old) were then analyzed for their **Withanolide** content by HPLC.

A 2.6 Culture of seedling roots

Seedling root tips (Apical 2-3 cm long, actively growing portion) selected from 2-month old seedlings were cultured either in 1/2 MS (Murashige and Skoog, 1962) medium (Strength of major elements reduced to half strength) without plant growth regulators (Control treatment) or in 1/2 MS medium supplemented with 0.4 mg.l^{-1} each of IAA and IBA. 300-mg fresh weight of roots was added to 30 ml of liquid medium in each 100-ml capacity Erlenmeyer flask. The flasks were incubated in darkness on a magnetic shaker at 110 rpm and at 25 $\pm$ 2°C. Subcultures were done every week. At the end of the growth period of 3 months the roots were harvested for **Withanolide** analysis.

A 2.7 Withanolide analysis by HPLC

The tissue extraction, HPLC analysis and calculations of **Withaferin-A** contents were done as described earlier in **Chapter 6, Sections 62.8 and 6.2.9** .

A 2.8 Statistical analysis

Each plastic dish with five node or leaf bit explants was taken as one replicate for hairy root induction experiments. Each experiment involving each strain of *Agrobacterium rhizogenes* co-cultivated with either of the explants, consisted of five such replicates and each experiment was repeated thrice. Hairy roots longer than 0.5 mm with distinctly visible apical meristems were only counted in each treatment. The number of hairy roots induced were then compared by one way ANOVA and the post-hoc comparisons were made by Student's 't – test' (Wardlaw 1985). The hairy roots derived from either nodal or leaf bit explants with each of the successful *A. rhizogenes* strains were cultured in three replicate Erlenmeyer flasks of 100 ml capacity in liquid medium. For analysis of withanolides, one of these replicate flasks of hairy roots was harvested on a particular day for extraction and subsequent analysis by HPLC. To check for the reproducibility of the extraction and HPLC procedure, another of the two remaining replicate flasks was extracted and analyzed by HPLC on a different day. Dry weight measurements were carried out at the time of these extractions. For this the hairy roots from a flask were blotted dry on a filter paper and kept in an oven at 60ºC for 16-24 hours to achieve constant dry weight.

The seedling roots were also cultured in triplicate in Erlenmeyer flasks of 100-ml capacity, for each of the media combinations. All the other procedures followed were the same as those for the hairy roots.

A.3 Results and Discussion

A.3.1 Development and establishment of hairy roots

The hairy roots emerged within 15 days from both nodal and leaf bit explants with *Agrobacterium rhizogenes* strains: A4, ATCC 15834, ATCC 15332 and ATCC 25818. Various morphologies of hairy roots induced are shown in **Figs. A.1 - A.3**. When the explants were cultured on MS medium without plant growth regulators and without *Agrobacterium* co-cultivation, no roots were produced (**Fig. A.2 A, Fig. A.3 A**). It has been observed that the success of transformation depends on following factors (Godwin et al., 1992):

〈 Bacterial strains and their ability to attach to plant cells.

〈 Host genotype and explant physiology with respect to presence of competent cells, release of wound-induced substances/signal molecules.

〈 Co-cultivation conditions.

As seen from **Table A.2**, it is clear that the transformation ability of different *Agrobacterium rhizogenes* strains is different. The best results in terms of percentage of hairy root formation and the number of hairy roots produced were observed with strains A4 and ATCC 15834. The hairy roots produced by these strains were thick, long with many root hairs and were fast growing (**Fig. A.1 A, B and Fig. A.2 B, C and Fig. A.3 E**) while the hairy roots produced with strain ATCC 25818 were thin, long, with a few root hairs and slow growing (**Fig. A.3 B**). No hairy roots were produced after infection with strain ATCC 15832 (**Fig. A.1 C and Fig. A.3 D**). Little amount of white/brown, friable, rooty callus was produced after infection with strain ATCC 11325 (**Fig. A.3 C with leaves as explants**). Similar differences in hairy root formation have been reported earlier for *Withania somnifera* with *Agrobacterium rhizogenes* (Banerjee et al., 1994) and *Agrobacterium tumefaciens* (Ray and Jha, 1999). Such differences might be explained by the higher capacity of Agropine strains to induce hairy roots due to presence of both TL and TR DNA as described earlier in **Chapter 1**. In addition, these different root morphologies observed among clones of the same cultivar may highlight the randomness of processing and integration of Ri plasmid T-DNA in plant DNA (Bhadra et al., 1993).

Leaf explants infected with bacterial strains ATCC 11325 and ATCC 15832 were unable to form hairy roots but gave rise to callus. On the other hand, no response was

observed in nodal explants. This again proves the interdependence and interaction between bacterial strains and the explant types. The Mannopine strains with only TL DNA are clearly not able to induce hairy roots in *Withania somnifera*.

Both the earlier reports of hairy root formation in *Withania somnifera* had used leaves as explants. **Here for the first time success with nodal explants for formation of hairy roots has been described.**

It was also observed that the hairy roots derived from *A. rhizogenes* strains A4 and ATCC 15834 could adapt to liquid cultures without undergoing any callusing but the hairy roots derived from strain ATCC 25818 were not amenable for liquid culture and soon died. These were not therefore analyzed by HPLC for **Withanolide** content, especially **Withaferin-A** content. Bhadra et al. (1993) had also observed that not all the hairy root lines developed on solid media in *Catharanthus roseus* were amenable for liquid culture. In general, thin, regularly branched root clones are suitable for liquid cultures and for subsequent scale-up in bioreactors. Hence proper selection of plant genotype and its combination with *A. rhizogenes* strain needs to be optimized in every case.

Table A.2 Effect of Different *Agrobacterium rhizogenes* Strains on Percentage of Hairy Root Formation on Leaf and Node Explants of *Withania somnifera* (L.) Dun.*

Explant	Bacterial Strain	% Response for Hairy Root Formation Mean ± S. E.	No. of Hairy Roots Induced Mean ± S. E.	Type of Callus Produced
Nodes	A4	30 + 20[a]	4.33 + 2.4[a]	Yellowish-white callus.
	ATCC 15834	32.5 ± 7.5[a]	7.0 ± 3.0[a]	Yellowish-white callus with green patches.
	ATCC 25818	Overgrown bacteria; no hairy roots	-	-
	ATCC 11325	-	-	-
	ATCC 15832	-	-	-
Leaves	A4	62.5 ± 10.5[b]	8.2 ± 5.6[a]	Whitish-brown callus.
	ATCC 15834	35 + 2.5[a]	17.8 + 10.9[a]	Whitish-brown callus.
	ATCC 25818	10 ± 3.7[a]	1.5 ± 0.5[a]	Scanty brown callus.
	ATCC 11325	-	-	Scanty whitish-brown callus.
	ATCC 15832	-	-	Whitish-brown callus.

* **Control** : The leaf and nodal explants were cultured without bacterial co-cultivation on MS medium without hormones, where no root formation was observed.

[a, b] : Values followed by different letters are significantly different from each other at 95% confidence level by t-test.

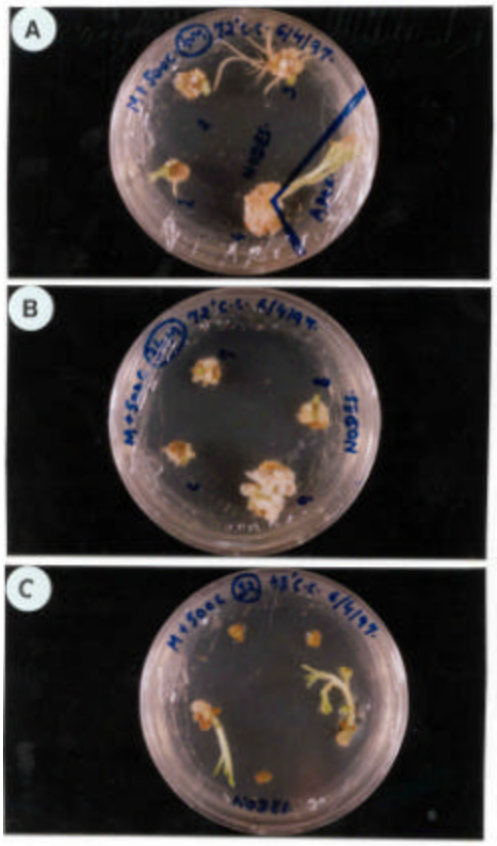
Fig. A.1

A - C Hairy root induction by various strains of *Agrobacterium rhizogenes* in nodal explants of *Withania somnifera* (L.) Dun.

A. Hairy root formation from nodes after 72 hours of co-cultivation with *Agrobacterium rhizogenes* strain A4. The apical bud forms white friable callus and no roots.

B. Stunted hairy roots and white, rooty callus formation from nodes after 72 hours co-cultivation with *Agrobacterium rhizogenes* strain ATCC 15834.

C. No hairy root formation was obtained from nodes after 48 hours co-cultivation with *Agrobacterium rhizogenes* strain ATCC 15332. The axillary bud showed normal elongation and at the lower end of nodes brown callus formation was observed.



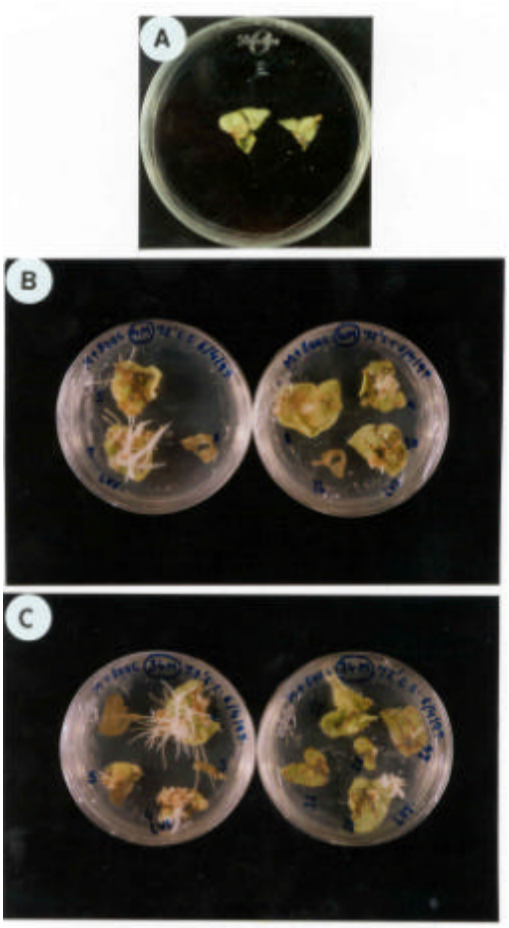


Fig. A.2

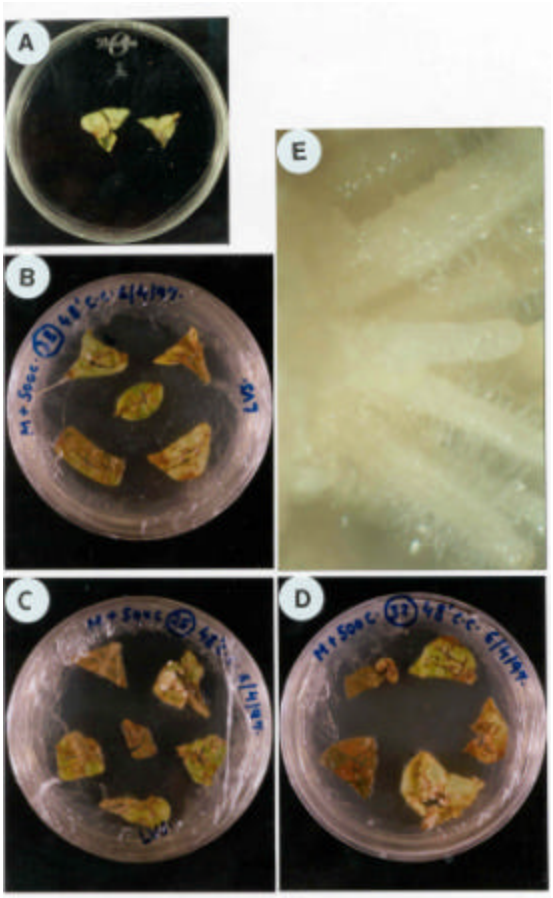
B - C Hairy root induction by various strains of *Agrobacterium rhizogenes* in leaf bit explants of *Withania somnifera* (L.) Dun.

A. Untreated leaf bits in MS medium without plant growth regulators (Control). They did not show any response on this medium.
B. Typical, profuse hairy root formation and yellowish-white callus from leaf bit explants after 72 hours co-cultivation with *Agrobacterium rhizogenes* strain A4.
C. Typical, profuse hairy root formation from leaf bit explants after 72 hours co-cultivation with *Agrobacterium rhizogenes* strain ATCC 15834.

Fig. A.3

B - C Hairy root induction by various strains of *Agrobacterium rhizogenes* in leaf bit explants of *Withania somnifera* (L.) Dun.

A. Untreated leaf bits in MS medium without plant growth regulators (Control). They did not show any response on this medium.
B. Scanty hairy root formation from leaf bit explants after 48 hours co-cultivation with *Agrobacterium rhizogenes* strain ATCC 25818.
C. Absence of hairy roots and formation of a little bit of white, rooty callus from leaf bit explants after 48 hours co-cultivation with *Agrobacterium rhizogenes* strain ATCC 11325.
D. Absence of hairy roots and formation of a little bit of white, rooty callus from leaf bit explants after 48 hours co-cultivation with *Agrobacterium rhizogenes* strain ATCC 13333.
E. A microscopic photograph (Magnification: 100x) of typical hairy root morphology induced by *Agrobacterium rhizogenes* strains A4 and ATCC 15834.



A.3.2 Development and establishment of liquid cultures of seedling roots

The seedling root cultures of *Withania somnifera* were easy to establish and maintain. In medium without plant growth regulators, the roots were thin, long, white and with few laterals. In medium supplemented with IAA and IBA, the roots were thick, light brown and very fast growing with many laterals. At the end of three-month growth period, the fresh weight of roots in this medium reached to 6 g. In all, 10 different seedling root lines were established starting from different parent seedling. These were consecutively numbered as R1 to R10. All of these lines were analyzed by HPLC for their withanolide contents

A.3.3 Withanolide analysis of seedling and hairy roots by HPLC

By following the HPLC protocol described earlier in **Chapter 6**, the following results on **Withaferin-A** analysis with seedling and hairy roots have been obtained and are presented in **Table A.3**.

Since the standard of **Withaferin-A** demonstrated two peaks of equal intensity during HPLC, both these were used for comparison with root extracts. The hairy roots chosen for HPLC analysis were derived from leaf explants as a larger number of hairy roots could be harvested for analysis (See **Table A.2**).

Table A.3 A Comparison of Withaferin-A Production in Seedling and Hairy Root Cultures of *Withania somnifera*.

No.	Type of *in vitro* Tissues (All the seedling derived and hairy root lines were three months old at the time of extraction and analysis).	Amount of Withanolides: Withaferin-A. (mg/g dry wt. of Tissues)	
		Peak with retention time of 12 min.	Peak with retention time of 15 min.
1	Seedling roots (Control) derived from two-month old seedlings and grown in 1/2 MS without plant growth regulators.	-	-
2.	R2 line of seedling roots grown in 1/2 MS with 0.4 mg.l^{-1} each of IAA and IBA.	676.8	239.25
3.	R10 line of seedling roots grown in 1/2 MS with 0.4 mg.l^{-1} each of IAA and IBA.	< 2	-
4.	R1, R3-R9 lines of seedling roots grown in 1/2 MS with 0.4 mg.l^{1} each of IAA and IBA.	-	< 2
5.	Randomly selected hairy root line from leaves after infection with *Agrobacterium rhizogenes* strain A4.	-	-
6.	Randomly selected hairy root line from leaves after infection with *Agrobacterium rhizogenes* strain ATCC 15834.	< 2	< 2

-: Absence of compound.

Fig. A.4 represents the chromatogram of **Withaferin-A** standard and the graphs of linearity of detector response for a range of **Withaferin-A** standards. **Fig. A.5** is the chromatogram for HPLC analysis of seedling roots showing presence of **Withaferin-A**.

Surprisingly, the R2 line of roots of the two-month old seedlings grown in liquid MS medium supplemented with IAA and IBA were found to contain withanolides while those grown in media without plant growth regulators did not produce any withanolides (**Table A.3**). Although the amount of withanolides produced is quite less than the shoot cultures (C. f. **Table 6.11**), the observation is unique and has not been reported earlier. The reason for biosynthesis of withanolides in the roots is unclear at present. Detailed studies need to be undertaken to elucidate the reason for production of withanolides in

Fig. A.4

A. HPLC chromatogram of Withaferin-A standard. It shows two distinct, baseline-resolved peaks at retention times of 11.3 min. and 15.2 min.

B. A graph showing linearity of HPLC UV detector response for various concentrations of Withaferin-A standard, for the peak eluting at retention time of 13.5 min. The graph is plotted as peak area versus mcg of Withaferin-A standard. Note that the detector response is linear for all the concentration tried of Withaferin-A standard for the peak a 11.3 min.

C. A graph showing linearity of HPLC UV detector response for various concentrations of Withaferin-A standard, for the peak eluting at retention time of 17.4 min. The graph is plotted as peak area versus mcg of Withaferin-A standard. Note that the detector response is linear for all the concentration tried of Withaferin-A standard for the peak a 15.2 min.

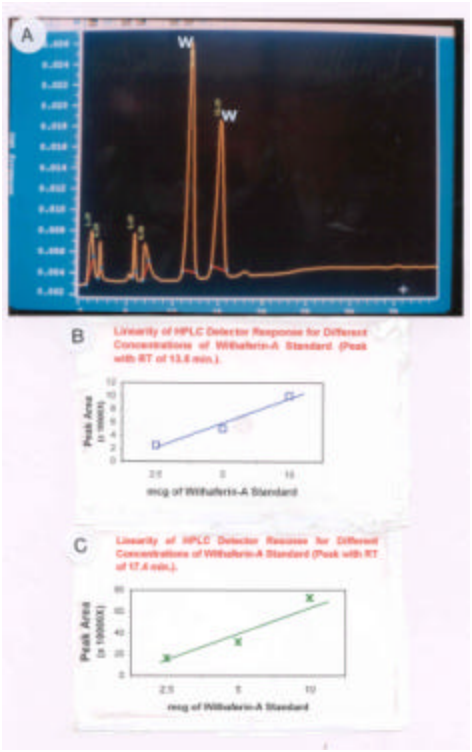
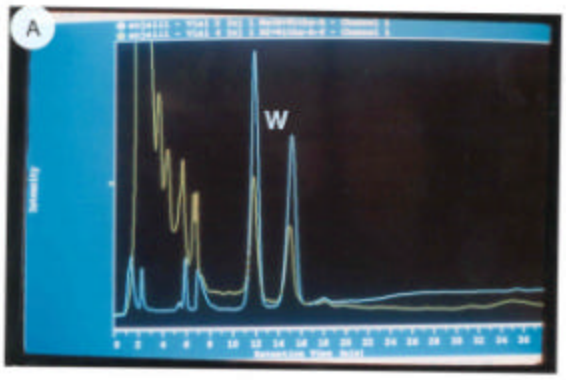


Fig. A.5 HPLC chromatogram showing presence of Withaferin-A in 2 month old seedling-derived root tissues of *Withania somnifera* (L.) Dun.

A. The blue graph shows the chromatogram for the standard of Withaferin-A while the green graph represents the spiked (along with standard) extract of seedling-derived root tissues grown in 1/2 MS medium supplemented with 0.4 mg.l^{-1} each of IAA and IBA. The peaks named as W and have identical retention times with respect to standard, represent those of Withaferin-A.



untransformed seedling root cultures, where the necessary biochemical machinery for steroidal lactone production like chloroplasts and the enzymes for steroidal biosynthesis are believed to be absent. Since the roots were grown separately from the parent plants in shake flasks, the possibility of synthesis of steroidal lactones in leaves and their subsequent transport to roots is negated.

In contrast, the three month old hairy root lines derived after infection of *in vitro* grown, two-month old leaves by *Agrobacterium rhizogenes* showed only traces of **Withaferin-A**. This is in contrast to earlier report (Banerjee et al., 1994) but concurs with a later report (Ray et al., 1996). Since we did not have a standard of **Withanolide-D**, its presence could not be detected.

A.4 Conclusions

Even though the work carried out on hairy root induction and **Withanolides** formation therefrom in this thesis is preliminary, the results obtained are significant and opens up the possibility for large-scale investigations and extrapolations of the know-how generated here for successful use and production of **Withaferin-A** from *in vitro* raised seedling roots of *Withania somnifera* (L.) Dun. The salient observations on the work done in this chapter could be summarized as:

1) **Hairy roots can be successfully induced on leaf and nodal explants of in vitro grown seedlings of *Withania somnifera* by *Agrobacterium rhizogenes* strains A4, ATCC 15834 and ATCC 25818.**
2) **Preliminary data obtained points out the ability of hairy root induction depending on the strain of *Agrobacterium rhizogenes* used for co-cultivation.**
3) **Withaferin-A was absent in hairy roots after 3 months in liquid culture.**
4) **Seedling roots grown for 3 months in liquid culture have significant amounts of Withaferin-A. This has important biosynthetic implications and need to be studied further.**

SUMMARY

Application of plant tissue and cell culture methods has immense potential in the large-scale propagation and conservation of the unexplored biodiversity of plants all over the world and especially in a huge country like India. This is especially true for plants with medicinal properties. A number of plants have recently been studied with an approach combining traditional medicinal knowledge with new research tools leading to elucidation of a number of phytochemicals of high potency. The present thesis details our efforts in characterization of two Indian Medicinal plants namely, ***Taxus baccata* ssp. *wallichiana* Zucc. Pilg.** and ***Withania somnifera* (L.) Dunal**. Plants of *Taxus* spp. produce a number anticancer diterpenoids of the **Taxane** class and hence are becoming endangered in their natural habitats due to over-exploitation of natural resources for the extraction of these compounds. Plants of *Withania* spp. also produce a range of antitumor steroidal lactones such as **Withaferin-A**. Natural populations of *Withania* spp. show high chemical diversity and hence need to be characterized for identification of superior chemotypes and their exploitation *in vitro* for better yields of active compounds. These plants were therefore studied with respect to aspects such as clonal propagation and secondary metabolite production *in vitro* either in undifferentiated or differentiated cultures.

During the course of the preliminary experiments, it became apparent that bacterial and fungal contamination as well as phenolic browning hindered the successful establishment of aseptic cultures. A series of experiments were carried out in order to identify the bacterial and fungal contaminants and to devise specific control measures with antimicrobial agents. These were then combined with antioxidants in a successful **'Pre-treatment Strategy'** and a non-phytotoxic **'Modified Surface Sterilization Protocol'** to achieve 90% sterile cultures *in vitro* both from mature explants of *Taxus* spp. and juvenile explants of *Withania* spp. The highlights of this work are:

1) The prevalent bacterial contaminants in both *Taxus* spp. and *Withania* spp. belong to the genera such as *Pseudomonas*, *Enterobacter-Erwinia* complex and *Serratia* spp.
2) The fungal contaminants in both *Taxus* spp. and *Withania* spp. belong to the class Ascomycota.
3) Both bacterial and fungal contamination (endophytic as well as occasional/opportunistic) associated with mature explants of *Taxus* spp. and juvenile

explants of *Withania* spp. can be controlled by use of specific anti-microbial agents in a pre-treatment solution such that no phytotoxicity problems are encountered.

4) To control the bacterial contamination in internodal explants of *Withania* spp., a 16 hour long pre-treatment in a sterile aqueous solution containing 25 $mg.l^{-1}$ each of ofloxacin, pefloxacin and ciprofloxacin is sufficient without adversely affecting the regeneration capacity of the internodes. This proves the non-phytotoxic nature of the optimized pre-treatment.
5) It is necessary to employ a 16 hour long pre-treatment in an aqueous solution containing 1% bavistin + 0.1% 8-HQS + 500 $mg.l^{-1}$ each of augmentin, streptopenicillin + 25 $mg.l^{-1}$ each of ofloxacin, pefloxacin and ciprofloxacin to overcome the fungal and the bacterial contamination observed in mature explants of *Taxus* spp. and to get 90% sterile cultures.
6) The phenolic oxidation observed in stem and needle explants of *Taxus* spp. derived from mature trees can be reduced by incorporation of anti-oxidants in the pre-treatment solution along with the antimicrobial agents, use of half strength nutrient medium and use of activated charcoal at 0.5% in the solid medium.
7) A pre-treatment solution containing 1% bavistin + 0.1% 8-HQS + 500 $mg.l^{-1}$ each of augmentin and streptopenicillin + 25 $mg.l^{-1}$ each of ofloxacin, pefloxacin and ciprofloxacin + 2% each of soluble PVP, Citric acid and Ascorbic acid is used for 16 hours to completely overcome phenolic browning in mature explants of *Taxus* spp. and to get 90% sterile cultures free from bacterial and fungal contaminants.
8) All the chemicals used for overcoming the problems of contamination and phenolic browning did not lead to any phytotoxicity. All the treated explants showed high success rate of *in vitro* response and the regenerants were normal in morphology.

Mature explants (derived from 80-130 year old trees) of *Taxus baccata* ssp. *wallichiana* Zucc. Pilg. were used to initiate cultures *in vitro*, as they are known to yield true-to-parent plants and also for the reason that procurement of seeds due to the dioecious nature of this plant has become a limiting factor in the number of experiments that could be carried out with juvenile material. Callus cultures were established from stem bit and needle explants after optimization of nutrient media, plant growth regulators and culture conditions. These callus cultures were fast growing and could be routinely subcultured over the years. A number of callus cultures were analyzed by TLC and HPLC for the presence of anticancer diterpenoids **Taxol** and **10-DAB**. **Taxol** and **10-DAB**

hyper-producer callus lines were identified that combined growth and product yield in the same nutrient medium. Preliminary studies on suspension cultures indicated a general trend of increase in **Taxane** contents during liquid culture. The highlights of this work are as follows:

1) Callus cultures were raised from stem bits, needles (diploid calli) and endosperm (haploid calli) of mature trees of *Taxus baccata* ssp. *wallichiana* Zucc. Pilg.
2) Callus initiation was achieved on 1/2 SH, B5, DCR and WPM media each supplemented individually with 2, 4, 6, 8 and 10 $mg.l^{-1}$ 2,4-D from both stem bits and needle explants.
3) The stem bit explants formed callus with 20% efficiency as opposed to 100% for needle explants. Needle explants were therefore used for further studies.
4) B5 medium with a number of growth regulators was the best medium for survival and good growth of needle-derived calli over monthly subcultures for more than 4 years.
5) Needle explants gave rise to callus with fast growth rate and high FW on B5 medium supplemented individually with high concentrations (6, 8, 10 $mg.l^{-1}$) of auxins like 2,4-D, NAA and P. The best callus growth rate and high FW was obtained in calli grown on P containing media. Callus induction from needle explants and subsequent callus growth on B5 medium supplemented with auxins like IAA and IBA together in various concentrations was very good.
6) **Taxane** production was not detected in needle-derived calli cultured on B5 medium supplemented with various combinations of 2,4-D + KIN and NAA + KIN.
7) B5 medium supplemented with Picloram was the optimized medium for good growth as well as **Taxane** production for needle-derived callus cultures.
8) **Taxane** contents increased with increasing age of callus tissues in culture on optimized B5 medium with P. The highest **Taxol** content was 540 µg/g DW of callus in needle-derived diploid, 2 year old callus line growing on B5 medium supplemented with 6 $mg.l^{-1}$ P.
9) Needle-derived, 1 year old callus line in B5 medium supplemented with 8 $mg.l^{-1}$ P had the highest **10-DAB** content of 2585 µg/g DW of callus.
10) Thus in terms of both **Taxol** and **10-DAB** production, higher P concentrations above 4 $mg.l^{-1}$ are conducive.
11) Endosperm-derived, 6 month old callus growing on DCR medium supplemented with 8 $mg.l^{-1}$ P produced 203 µg **10-DAB**/g DW of callus.

12) **Taxanes** were not detected in the spent solid or liquid nutrient media used for growing either haploid or diploid callus cultures.
13) **Taxane** contents increased many-fold in three Cell Suspension Line Nos. 1, 3 and 5 except for the Cell Suspension Line No. 2.
14) **Taxol** and **10-DAB** presence was detected even in **Taxane** non-producer 2 year old callus grown as cell suspension in B5 medium supplemented with 10 $mg.l^{-1}$ P. The contents of both these compounds increased with the increase in liquid culture period from 0 days to 18 days.

Efforts were made to standardize a micropropagation protocol with enhanced axillary budding using mature explants of the Himalayan *Taxus* spp. Such a protocol will provide the planting material to replenish the dwindling natural stocks of this plant. The present protocol is applicable to plant material of different age and collected from different locations. In addition, precocious embryo germination and seedling development was achieved at a high frequency from seeds collected at different locations in the Himalayas. Fast growing and **10-DAB** producing callus lines were established from haploid endosperm explants of *Taxus* spp. for the first time. The highlights of this work are as follows:

1) A protocol has been standardized for enhanced axillary budding of mature stem bit explants of *Taxus baccata* ssp. *wallichiana* Zucc. Pilg. Primary and secondary shoots have been obtained at a high frequency within six months. This protocol is applicable to plant material collected from trees of different age groups, growing in different locations in the Himalayas.
2) Basal media with low total ionic salt strengths and lower ratios of ammonium nitrogen to nitrate nitrogen such as 1/2 WPM and 1/2 DCR are conducive for enhanced axillary budding of mature explants of *Taxus* spp.
3) Organic nitrogen in the form of an amino acid like glutamine does not enhance axillary sprouting in *Taxus* spp.
4) 1/2 WPM supplemented with 0.05 $mg.l^{-1}$ BAP gave 83.33% sprouting while 1/2 WPM supplemented with 0.05 $mg.l^{-1}$ KIN gave 75.77% sprouting in plant material collected from Shimla. The former percentage of sprouting was significantly higher than the latter.

5) 1/2 DCR medium supplemented with lower concentrations of BAP (0.05, 0.1 $mg.l^{-1}$) gave 100% sprouting percentage as compared to 58.89% and 54.21% sprouting obtained on 1/2 DCR medium supplemented with lower concentrations of KIN (0.05, 0.1 $mg.l^{-1}$) in plant material collected from Shimla. The results are statistically significant.
6) 1/2 DCR medium without plant growth regulators (1/2 DCR-H) gave 50% sprouting while 1/2 DCR supplemented with lower concentrations of BAP (0.05, 0.1 $mg.l^{-1}$) gave identical sprouting percentage (100%) that is similar to sprouting percentage obtained with 1/2 DCR supplemented with 10% CM. Thus apparently the absence of low concentrations of BAP in 1/2 DCR medium can be compensated by addition of CM.
7) BAP is a better cytokinin with 1/2 WPM to achieve high sprouting but shoot quality and elongation capacity is not as good as shoot quality and elongation ability obtained with KIN. So KIN is the cytokinin of choice with 1/2 WPM.
8) Higher cytokinin concentrations combined with any of the basal media are not conducive for sprouting.
9) TDZ is not conducive for sprouting due to the inability of the primary sprouts to elongate.
10) Combination of an auxin with a cytokinin is not conducive for enhanced axillary budding of mature stem explants of *Taxus* spp.
11) 1/2 WPM-H gave 50% sprouting with plant material collected from Khajjiyar; statistically similar to 52.94% sprouting achieved with plant material collected from Shimla on the identical medium. Although 75.77% sprouting was achieved on 1/2 WPM with 0.05 $mg.l^{1}$ KIN with plant material collected from Shimla, sprouting percentage did not differ statistically with KIN concentrations for plant material collected from Khajjiyar.
12) 1/2 DCR-H gave significantly higher sprouting at 90% with plant material collected from Khajjiyar while plant material collected from Shimla had only 50% sprouting on the same medium. 1/2 DCR supplemented with BAP gave higher sprouting response with Shimla material as compared to Khajjiyar material.
13) 1/2 WPM-H gave 48% sprouting with plant material collected from Almora; statistically different than 52.94% sprouting achieved with plant material collected from Shimla on the identical medium. A big standard error of mean was obtained with Almora material hinting at underlying variabilities of the plant material. Although

75.77% sprouting was achieved on 1/2 WPM with 0.05 mg.l^{-1} KIN for plant material collected from Shimla, sprouting percentage did not differ statis tically with KIN concentrations with plant material collected from Almora.

14) 1/2 DCR-H gave identical sprouting at 50% with plant material collected from Almora as well as Shimla. 1/2 DCR medium supplemented with BAP gave significantly higher sprouting response with Shimla material as compared to Almora material.

15) Thus although the general trends such as suitability of low salt media and low cytokinin concentrations for sprouting are identical in plant material collected from Shimla, Khajjiyar and Almora, the best sprouting response is observed with plant material collected from Shimla. Only plant material collected from Khajjiyar gave 90% sprouting on 1/2 DCR-H that is significantly higher than the results of sprouting obtained with plant material collected from Shimla.

16) Elongation of primary sprouts into shoots of length 3 cm was possible on respective basal media without plant growth regulators, in three months time, for the cultures established from plants of all the locations.

17) Establishment of primary shoots was possible on respective basal media supplemented with 10% CM and 1 g.l^{1} CH in the fourth month.

18) Secondary sprouting of buds developed *in vivo* on primary shoots was achieved on 1/2 WPM supplemented with 0.1 mg.l^{-1} KIN, 10% CM and 1 g.l^{-1} CH at a frequency of 25% during the fifth month.

19) Precocious embryo germination and seedling development has been achieved at a frequency of 90% from immature seeds collected from Khajjiyar on DCR basal medium at pH 5.8, in light and without plant growth regulators.

20) Precocious embryo germination and seedling development was found to vary with developmental age of seeds (as judged by aril development and embryo size) and the location of collection.

21) Precocious embryo germination and seedling development has been achieved at a frequency of 91% from mature seeds collected from Almora on B5 basal medium without plant growth regulators. The optimum medium was found to vary depending on the location of collection and developmental stage (as judged by aril development and embryo size) of the seeds.

22) Fast growing, haploid callus lines have been established from solid endosperm of seeds collected from Almora on the optimal DCR medium supplemented with various

concentrations of Picloram (P) and higher concentrations of P were conducive for faster growth of calli.

Morphogenetic studies in *Withania* spp. were carried out in order to standardize a high efficiency propagation protocol to be applicable to large scale propagation of selected chemotypes without any seasonal constraints, to check for the presence of **Withaferin-A** in *in vitro* raised cultures and to identify the origin of production of **Withaferin-A** in organized or unorganized cultures. The highlights of this work are:

1) Direct regeneration of plants has been achieved from a number of explants like leaves, nodes, internodes, hypocotyls and embryos. 70% of leaf explants regenerated shoots on an optimal medium while all the remaining explants showed 100% shoot formation response on media standardized during the course of the present work.
2) By the protocol standardized in this study, 1600 and 432 plantlets per year per explant could be produced from leaves and hypocotyl explants respectively.
3) *De novo* regeneration of 24 shoots from a single node explant could be obtained with TDZ. TDZ was found to be the best plant growth regulator among the tested plant growth regulators.
4) All the regenerants showed a high success rate on acclimatization and hardening.
5) Both the seedlings and *in vitro* regenerated shoots from nodes contained high amounts of **Withaferin-A**. *In vitro* grown seedlings had 10 times more **Withaferin-A** than that reported in literature for *in vivo* tissues of *Withania somnifera* (L.) Dun. *In vitro* regenerated shoots growing on MS medium supplemented with 0.5 $mg.l^{-1}$ BAP had 100 times more **Withaferin-A** than the *in vivo* tissues and to our knowledge such high amounts of **Withaferin-A** reported here are the first in literature. Presence of **Withaferin-A** was not detected in callus tissues suggesting that production of **Withaferin-A** is related to tissue organization.

Preliminary studies on hairy root induction with *Agrobacterium rhizogenes* in *Withania* spp. were carried out basically to check for the presence of **Withaferin-A** in the hairy root cultures. The highlights of this work are presented below:

1) Hairy roots can be successfully induced on leaf and nodal explants of *in vitro* grown seedlings of *Withania somnifera* with *Agrobacterium rhizogenes* strains A4, ATCC 15834 and ATCC 25818.

2) Preliminary data obtained points out the ability of hairy root induction depending on the strain of *Agrobacterium rhizogenes* used for co-cultivation.
3) **Withaferin-A** was absent in hairy roots after 3 months in liquid culture.
4) Seedling roots grown for 3 months in liquid culture have significant amounts of **Withaferin-A**. This has important biosynthetic implications and need to be studied further.

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