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Chemical and Morphological Variability of *Taxus*

Needles, Seeds and Cell Cultures

by

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being a thesis submitted for the degree of

Doctor of Philosophy

in Pharmacognosy

at

University of Dublin

Trinity College

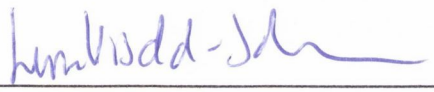
under the direction and supervision of

Ingrid Hook, B.Sc. (Pharm.), M.Sc. (N.U.I), M.R.P.S.

March 2006

Declaration

This thesis is submitted by the undersigned to the University of Dublin for examination for the degree of Doctor of Philosophy. It has not bee submitted as an exercise for a degree at any other university. I carried out all the practical work except where duly acknowledged. I wrote this manuscript with the aid of editorial advice from Ingrid Hook. I agree that the library may lend or copy this thesis upon request.



Lene Visdal-Johnsen Cand. Mag., Cand. Scient., M.Sc.

Til Mormor, Morfar og Besta

Dere vil alltid ha en plass i hjertet mitt

Takk for alt

Acknowledgements

I would like to express my sincere gratitude to my supervisor, Mrs. Ingrid Hook, for all her help, knowledge and support both during the course of the research and in the writing of this thesis. I am also truly grateful to Dr. Helen Sheridan, for her dedication, advice and constant source of motivation and encouragement throughout these studies.

I wish also to express my gratitude to Enterprise Ireland for financial support. I would like to thank Dr. John O'Brien for carrying out NMR spectroscopy and a special thanks to Dr. Paraic James (Dublin City University) for additional NMR and MS analysis. To Robert Mill (Royal Botanic Garden, Edinburgh, UK), Colin Morgan (Bedgebury Pinetum, UK), Grzegorzc Iszkuó (Polska Akademia Nauk, Kórnik, Poland), Stewart Cameron (Natural Recourses Canada, Fredericton, New Brunswick, Canada), Mathew Jebb (National Botanical Gardens, Glasnevin, Dublin) and Chris Kelly (The J. F. Kennedy Park Arboretum, New Ross, Co. Wexford) for supply of plant material. Further I would like to thank the academic staff within the School of Pharmacy for academic advice and the use of equipment. I would like to thank the executive and technical staff, in particular Joe O'Reilly for all his help and advice and his everlasting patience.

Thanks to my fellow postgrads (past and present) of the School: Gill, Adam, Martina, Aengus, Tao, Jenny and the girls, Dave, Rob, Emma, Anne, Lidia, Des, James, Richie and Emmet for all the good times. A special word of gratitude to my lab partner Cora; without your constant support and friendship, and without our coffee breaks, I don't think this would have been possible. Big thanks to Emmet for proof-reading my thesis and to Dr. Clive Mills for all his help. I also would like to thank my friends at home in Norway for all their support and encouragement. To Olaug and Hugo, Maria, Hanne Beate and Andreas, Jannicke and Jarl, Ylva and Aksel, Siv and Lars. Setter utrolig stor pris på vårt vennskap!

A dept of gratitude is owed to Mamma and Pappa, for their never-ending encouragement, love and support throughout my college years. Without their support and love this thesis would have remained an aspiration. To my sister, Hanne, for being my best friend – you are one in a million! Also thanks to my extended family Evelia, Tomás, Sergio, Cristina and Nacho for all their support.

Finally, words can not describe my gratitude to my husband, Luis.... Thank you for your infinite patience, all your love, help and support, and for your endless amount of encouragement.

Summary

The genus *Taxus* (family Taxaceae) has during the last 30-40 years been the subject of extensive scientific investigation due to the discovery of the anti-cancer drug paclitaxel (Taxol®) originally isolated from the bark of the Pacific yew tree (*T. brevifolia*). Worldwide demand for paclitaxel is high as it is currently being used to treat breast, ovarian and non-small cell lung cancers as well as AIDS-related Kaposi's sarcoma. This has led to problems with sustainable supply from renewable sources and alternative means to production of paclitaxel are still under extensive investigation. Major problems associated with paclitaxel production include (i) the correct identification of the species within the genus *Taxus* in order to comply with FDA licence agreements and (ii) production of sufficient biomass either in the form of plant material or in vivo production of taxanes.

One of the main objectives in this research was to address the problem of correct identification of *Taxus* species and cultivars and to evaluate the species delimitation within the genus. Morphological characteristics such as needle and seed dimensions combined with classical morphological characteristics (foliage colour and tree shape) were analysed from a large population of *Taxus* species and cultivars. The analysis of needle and seed dimensions was done using a computerised image analysis software-package called WinSEEDLE™. All measurements complied with literature values and no single parameter was found that could distinguish between one species and another.

Chemotaxonomic analysis of the same large *Taxus* population based on the analysis of four major taxanes (10-deacetyl baccatin III (10-DAB III), 9-dihydro-13-acetyl baccatin III (9-DHAB III), cephalomannine and paclitaxel) was carried out. The most abundant taxane was found to be 10-DAB III. The variation present in taxane content between the species as well as within each species analysed suggested that the value of taxanes as taxonomic markers was limited. The taxane 9-DHAB III, specific for *T. canadensis*, was included in the analysis; however, not all samples identified by the botanical gardens as *T. canadensis* contained this taxane. In the present study phloroglucindimethylether was isolated and structurally identified from *T. baccata* and a high concentration of this constituent is being suggested as an indicator of this *Taxus* species.

Cell cultures of *Taxus baccata* are used commercially as sources of taxanes. Suspension cultures of *Taxus baccata* 'Fastigiata' were established in 1997 and previous work was done to determine the growth kinetics and paclitaxel production of these cultures. In the present study

one of the objectives were to determine the profile of other taxane constituents of these cell cultures, including 10-DAB III, baccatin III and cephalomannine in addition to paclitaxel. Of these taxanes 10-DAB III, baccatin III and cephalomannine were found to be most abundant, whereas paclitaxel was absent or only produced in low concentrations.

Various cell culture techniques were used to enhance the taxane production of this suspension culture. Change of carbon source from sucrose to inulin resulted in a reduced osmotic pressure causing mechanical stress in the cell culture system. This led to reduction of biomass but had a positive effect on taxane accumulation. However, inulin was not found suitable for sustained growth. *In situ* product removal using a liquid-solid culture system consisting of the aqueous growth medium and Amberlite resin resulted in a significant increase in overall taxane production in the suspension cultures. Elicitation using methyl jasmonate was found to stimulate the synthesis and excretion of the taxanes 10-DAB III, baccatin III and cephalomannine in the suspension cultures. However, the biosynthesis of paclitaxel itself was not enhanced. Attempts of eliciting taxane production by addition of molybdenum and *L*-arginine were un-successful.

The induction of an oxidative burst using t*ert*-butyl hydroperoxide (tBH) in conjunction with hematin resulted in 45% enhancement of 10-DAB III and baccatin III production in the cell culture after 48 hours. An immediate burst of flavonoids observed indicated an induction of the cells defence system by the oxidative burst. The activation of defence genes was an upstream and immediate response to the treatment, whereas the accumulation of taxanes was a downstream event of signal transduction resulting from the oxidative burst.

Taxus needles are known to contain flavonoids. Another objective was to investigate the flavonoid profile of the cell culture. The major flavonoids were found to be catechin and epi-catechin, established by HPLC and verified by isolation and structure elucidation. This is the only report of the isolation of catechin and epi-catechin from cell suspension cultures of *T. b.* 'Fastigiata'. The presence of a procyanidin consisting of five units composed of catechin and epi-catechin (ratio of 4:1) was also confirmed for the first time in cell cultures of *Taxus*. Other flavonoids present in various concentrations were taxifolin, kaempferol, quercetin and amentoflavone.

Needles and seeds of *Taxus* species are known to contain unusual Δ5-olefininc acids. No previous work has examined for the presence of these in *Taxus* cell cultures initiated from seeds. They were found absent in the cell cultures. The simpler fatty acid composition of the cell culture could be due to the more protected environment created for the cells.

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Abbreviations

°	Degree
°C	Degree Celsius
±	Plus and minus
µg	Micrograms
µl	Microlitre
µM	Micromolar
2,4-D	2,4-dichlorophenoxyacetic acid
AcOOH	Acetic acid
AOS	Active oxygen species
AMP genes	Antimicrobial protein/peptide genes
ANOVA	Analyses of Variance
BAP	6-benzylaminopurine
BAW	Butanol: Acetic acid: Water
Br s	Broad singlet
Ca^{2+}	Calcium
$Ca(NO_3)_2$	Calcium nitrate
$CaCl_2$	Calcium chloride
CC	Column Chromatography
CE	Capillary electrophoresis
$CHCl_3$	Chloroform
cm	Centimetre
$CoCl_2$	Cobalt chloride
CITES	Convention on International Trade in Endangered Species
Co.	County
CI	Consistency index
Cu^{2+}	Copper
$CuSO_4$	Copper sulphate
C.V.	Coefficient of variation
cv.	Cultivars
d	Doublet
DCM	Dichloromethane
10-DAB III	10-deacetyl baccatin III
DEPT	Distortionless enhancement by polarisation transfer
9-DHAB III	9-dihydro-13-acetyl baccatin III
DAG	diacylglycerol
DMAPP	Dimethylallyl pyrophosphate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
$DPPH^{\bullet}$	1,1-diphenyl-2-picrylhydrazyl
ESI MS	Electrospray Ionisation Mass Spectrometry
ET	Ethylene
EtOAc	Ethyl acetate
EtOH	Ethanol

F	Female
FAME	Fatty acid methyl ester
FDA	The United States Food and Drug Administration
FeNaEDTA	Ethylenediaminetetra-acetic acid ferric monosodium salt
FID	Flame ionisation detector
g	Grams
GAP	Glyceraldehyde-3-phosphate
GI	Growth Index
GC	Gas Chromatography
g/l	Grams per litre
GB5	Gamborg's B5 medium
GC-MS	Gas chromatography – mass spectrometry
GDC	Glycine decarboxylase complex
GM	Murashige and Skoog medium
GPP	Geranyl pyrophosphate
GGPP	Geranyl geranyl pyrophosphate
GTP	Guanosine triphosphate
H_2O	Water
H_3BO_3	Boric acid
HCl	Hydrochloric acid
HMBC	Heteromultiple bond correlation
HMQC	Heteronucelar multiple-quantum correlation
HPLC	High performance liquid chromatography
HTS	High-throughput screening
hr	hour
HR	Hypersensitivity Response
H_2O_2	hydrogen peroxide
$HO_2^{\bullet -}$	hydroperoxyl
ICH	International conference on Harmonisation
iNOS	Nitric Oxide Synthase
IP_3	Inositol -1,4,5-triphosphate
IPP	Isopentyl pyrophosphate
IR	Infrared
ITS	Internal Transcribed Spacer Regions
JA	Jasmonic acid
JHz	Coupling constant in hertz
K_2SO_4	Potassium sulphate
KBr	Potassium bromide
KCl	Potassium iodide
kg	Kilograms
KH_2PO_4	Potassium dihydrogen orthophosphate
KI	Potassium iodide
KNO_3	Potassium nitrate
l	Litre
LCC	Liquid column chromatography
LC-MS	Liquid chromatography- mass spectrometry

Log	Logarithm
M	Molar
M	Male
m	Metre
mM	Millimolar
MALDI-TOF MS	Matrix assisted laser desorption ionization time-of-flight mass spectrometry
*mat*K	Maturase K
MAP	Microtubule-associated proteins
m.p.	Melting point
MS	Murashige and Skoog medium
MeOH	Methanol
mg	Milligrams
$MgSO_4$	Magnesium sulphate
MHz	Megahertz
Min	Minute
MJ	Methyl jasmonate
ml	Millilitre
$MnSO_4$	Manganous sulphate
Mo	Molybdenum
MS	Mass spectroscopy
N	Nitrogen
n	Sample size
NADP	Nicotinamide adenine dinucleotide phosphate
Na_2HPO_4	di-Sodium hydrogen orthophosphate
Na_2MoO_4	Sodium molybdenate
Na_2SO_4	Sodium sulphate
NAA	1-naphthaleneacetic acid
NCBI	National Centre for Biotechnology Information
n-BuOH	*n*-Butanol
N-gal	N-acetyl-D-galactosamine
N-glu	N-acetyl-D-glucosamine
$(NH_4)_2SO_4$	Ammonium sulphate
NH_4NO_3	Ammonium nitrate
nm	Nanometre
NMR	Nuclear Magnetic Resonance
NQ	Non-quantifiable
NR	Nitrate reductase
$O_2^{\bullet -}$	Superoxide anion
$OH^{\bullet}$	hydroxyl radical
PAL	Phenylalanine ammonia lyase
PDA	Photodiode array detector
Prep TLC	Preparative thin layer chromatography
PCR	polymerase chain reaction
PLC	phospholipase C
ppm	Parts per million

PR	Pathogenesis-related proteins
PVP	Polyvinylpolypyrrolidone
R	Resolution factor
RAPDs	Random amplification of polymorphic DNA
RI	Retention index
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSD	Relative standard deviation
Rt	Retention time
RO	Rugini Olive medium
s	Singlet
SA	Salicylic acid
SAR	Systemic acquired resistance
SAR	Structure-activity response/relationship
SEM	Scanning electron microscope
SFC	Supercritical fluid chromatocraphy
SOD	superoxide dismutase
t	Triplett
tBH	*Tert*-butyl hydroperoxide
TBR	Tree-Bisection-Reconnection
TLC	Thin layer chromatography
UNEP-WCMC	United Nations Environment Programme World Conservation Monitoring Centre
UK	United Kingdom
USA	United States of America
U.S.P.	United States Pharmacopoeia
UV	ultraviolet
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
WV3	Westvaco WV3 medium
WV5	Westvaco WV5 medium
$ZnSO_4$	Zinc sulphate

Origin and Scope

Research into aspects of *Taxus* and related genera has been ongoing for a number of years in the Department of Pharmacognosy, Trinity College Dublin. The following research projects have been funded:

- 'Isolation and characterisation of lectins from *Taxus* and *Cephalotaxus* species' (1994)
- 'European self-sufficiency in *Taxus* – for the isolation of the anti-cancer agent Taxol' (1994-1997) (7-EEC-AIR Programme)
- 'Yew (*Taxus baccata*) – optimisation of its field culture and establishment as a plantation crop' (1998-2000) (Forbairt-Industry Scheme)
- British Council/Enterprise Ireland Research Visit Schemes: 1998-Bedgebury Pinetum; 2001- Royal Botanical Gardens, Edinburgh
- 'Molecular systematics and chemical characterisation of *Taxus* (yews) and related genera' (1999-2002) (Enterprise Ireland Basic Research Grant, with Botany Department, Trinity College Dublin)

A number of problems became apparent during the course of these projects especially (i) in the area of identification/taxonomy of the *Taxus* species, (ii) the variability of *Taxus* species as a source of paclitaxel and (iii) the potential of cell cultures as a source of taxanes. The present research is a continuation of the projects listed above with the main objectives being the elucidation of the problems previously encountered:

- To further investigate the use of morphological characteristics in the identification and taxonomy of the different species and cultivars of the genus *Taxus*, using computerised needle- and seed-dimension analysis in addition to classical morphological characteristics on a large population of *Taxus* samples.
- To determine if taxanes or other constituents are present in the same, large population of *Taxus* samples, which would identify the species chemotaxonomically.
- To evaluate the taxane profiles of *Taxus baccata* 'Fastigiata' cell cultures.
- To enhance the production of taxanes in these cell suspension cultures by various cell culture techniques.
- To examine the effect of the induction of oxidative stress using various oxidative burst inducers and elicitors on the production of taxanes by the cell suspension cultures.
- To investigate the flavonoid profiles of the cell cultures and the influence of oxidative burst induction and elicitation on these.

1 General Introduction

1.1 Natural Products in Drug Discovery

1.1.1 Plants role in medicine - history

Traditional systems of medicine have been in existence for thousands of years and plants have been the source of medical agents needed by humans as well as animals. The poisonous or healing properties of plants were discovered by man in his search for food by trial and error. The knowledge of these plants was passed on through the generations and it became the task of the medicine man to maintain the knowledge and pass it on to his/her successor (Samuelson 1999). The first records of plants used as medicine were from Mesopotamia and date from about 2600 B.C. These were written on clay tablets and documenting *Cedrus* species (cedar), *Cupressus sempervirens* (cypress), *Glycyrrhiza galabra* (licorice), *Commiphora* species (myrrh) and *Papaver somniferum* (poppy juice), all of which are still in use today for treatment of ailments ranging from coughs and colds to infections and inflammations (Cragg and Newman 2005).

Plants are a valuable source of a wide range of both primary and secondary metabolites. The primary metabolites (carbohydrates, nucleosides, amino acids and the polymers derived from them) are compounds ubiquitous and essential for life. The secondary metabolites (phenolics, terpenoids, steroids, and alkaloids) are of restricted occurrence and were originally thought to be of no apparent utility to the plants (Mann et al. 1994). In the middle of the 20th century, improvement of analytical techniques such as chromatography allowed the recovery of more and more of these molecules, and this was the basis for the establishment of the discipline of phytochemistry. Thanks to the improvement of biochemical techniques and the rise of molecular biology, it is now generally accepted that the secondary metabolites are important for the survival of the plant in its ecosystem: their antimicrobial and anti-insect activities deter potential predators, discourage competing plant species, attract pollinators or symbionts, or further the interests of the plant in other ways (Bourgaud et al. 2001, Dixon 2001). It was also found that secondary metabolites are far more complex than primary metabolites. This is because secondary products are derived from the primary

ones by complex metabolic pathways. An example of this is shown in **Figure 1.1.1.1**. Due to the large biological activities, plant secondary metabolites have been used for centuries in traditional medicine. Nowadays, they are used as pharmaceuticals, agrochemicals, flavours, fragrances, colours, biopesticides and food additives (Ramachandra Rao and Ravishankar 2002).

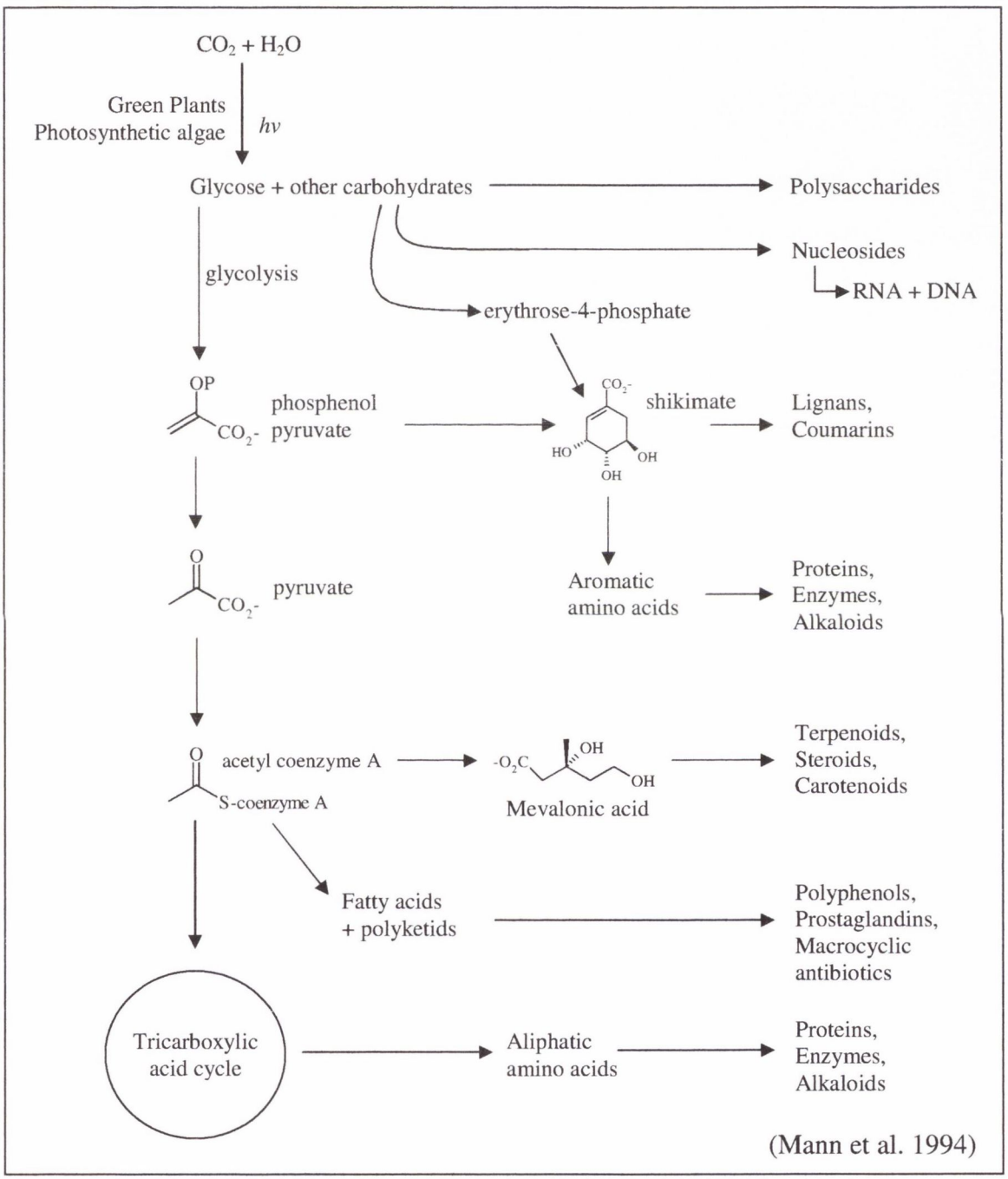
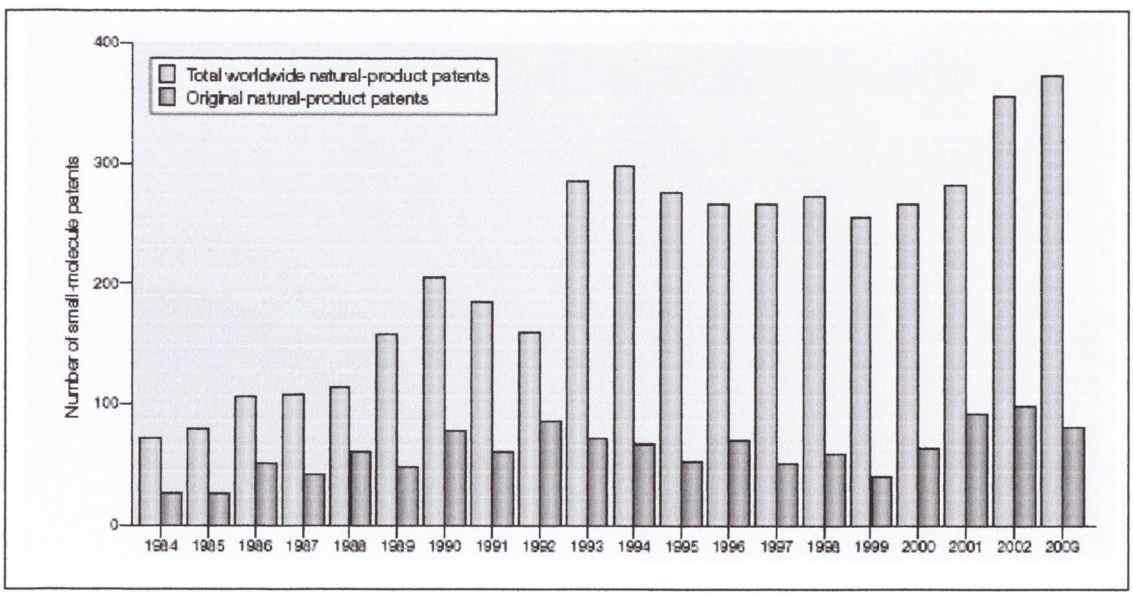


Figure 1.1.1.1 Examples of metabolic pathways leading to secondary metabolites

1.1.2 The changing role of natural products in the development of new drugs

Historically natural products have been a major source of new drugs, and many successful drugs were originally synthesised to mimic the action of molecules found in nature. Natural products are favourable as lead structures for drug discovery because of their high chemical diversity, their biochemical specificity and other molecular properties that differentiates them from synthetic and combinatorial compounds. Several studies on the difference between natural products and synthetic sources of drug leads reveal that natural products generally have more chiral centres, increased steric complexity and a more ridged structure giving them a higher stereospecificity (Feher and Schmidth 2003, Lee and Schneider 2001, Stahura et al. 2000).

The role of natural products in drug discovery experienced a decline in the last two decades. The actual downstream effect of this decline is difficult to measure precisely, because of the long product-development cycles found in the pharmaceutical industry. It can take more than 10 years from initial discovery to subsequent market launch of a new therapeutic agent. However, the worldwide pharmaceutical patent activity on small-molecule natural products (**Figure 1.1.2.1**) gives a clear illustration of the pattern from 1984-2003 (Koehn and Carter 2005). Through the 1980s there was an increasing patent activity. A flattening or even slight decline is apparent from 1990-1999, and then a rise in activity can be seen from 2000-2003.



(Koehn and Carter 2005)

Figure 1.1.2.1 Worldwide natural products patents (Koehn and Carter 2005)

A number of factors have been proposed for the decline in natural products as a basis for drug discovery seen in the 1990s:

- Introduction of high-throughput screening (HTS) against defined molecular targets. The primary role of HTS is to detect lead compounds and to supply guidelines for their optimisation. It has been developed from bench-top experiments by atomisation and design of robotic workstations, thereby permitting performance of a large number of tests in a short time. This method encouraged many pharmaceutical companies to move from natural products to synthetic libraries.
- The development of combinatorial chemistry. The principals of "rational" drug design are used in the design of combinatorial libraries to speed up the discovery of lead compounds with a desired biological activity. Simpler, more drug-like screening libraries of wide chemical diversity are produced.
- Advances in molecular biology, cellular biology and genomics. This increased the number of molecular targets and resulted in shorter drug discovery timelines.
- A declining emphasis of major pharmaceutical companies on infectious disease therapy, which was a traditional area of strength for natural products (Projan 2003).

In the early 1990s natural products drug discovery was still being done in the traditional way where crude plant extracts were tested for activity towards a drug target, and if there was a hit, the extracts were fractionated and the active compounds were isolated and identified. The process was slow and inefficient. When the drug discovery scene shifted towards high-throughput screening, automation, robotics and computing pharmaceutical companies moved away from natural products and concentrated their efforts on synthetic libraries. Combinatorial chemistry became the principal way to create new lead compounds for drug development (Rouhi and Washington 2003). However, in the early days of combinatorial chemistry the hit rate of the lead compounds made was generally low. It soon became clear that relying only on numbers and efficiency would not provided the needed leads for drug discovery (Leach and Hann 2000). The generation of combinatorial libraries are somewhat constrained by the availability of reagents and suitable reactions giving a reduced diversity and low activity hit-rate. Natural products, however, are generated in the

context of biological utility as a result of precursors and biosynthetic reactions (Feher and Schmidt 2003). Several techniques have been developed for combinatorial chemistry that can be used to reintroduce natural products as an important source for novel leads in the drug discovery process. The development of integrated methods and technologies for isolating, purifying and characterising bioactive natural products can accelerate drug discovery from natural resources.

1.1.3 Rediscovering natural products

According to Newman et al. from the National Cancer Institute (Newman et al. 2003) 61% of the 877 small-molecule new chemical entities introduced as drugs worldwide during 1981-2002 was derived from natural products. These include natural products themselves (6%), natural product derivatives (27%), synthetic compounds with natural product-derived pharmacophores (5%) and synthetic compounds designed on the basis of knowledge gained from natural products (23%).

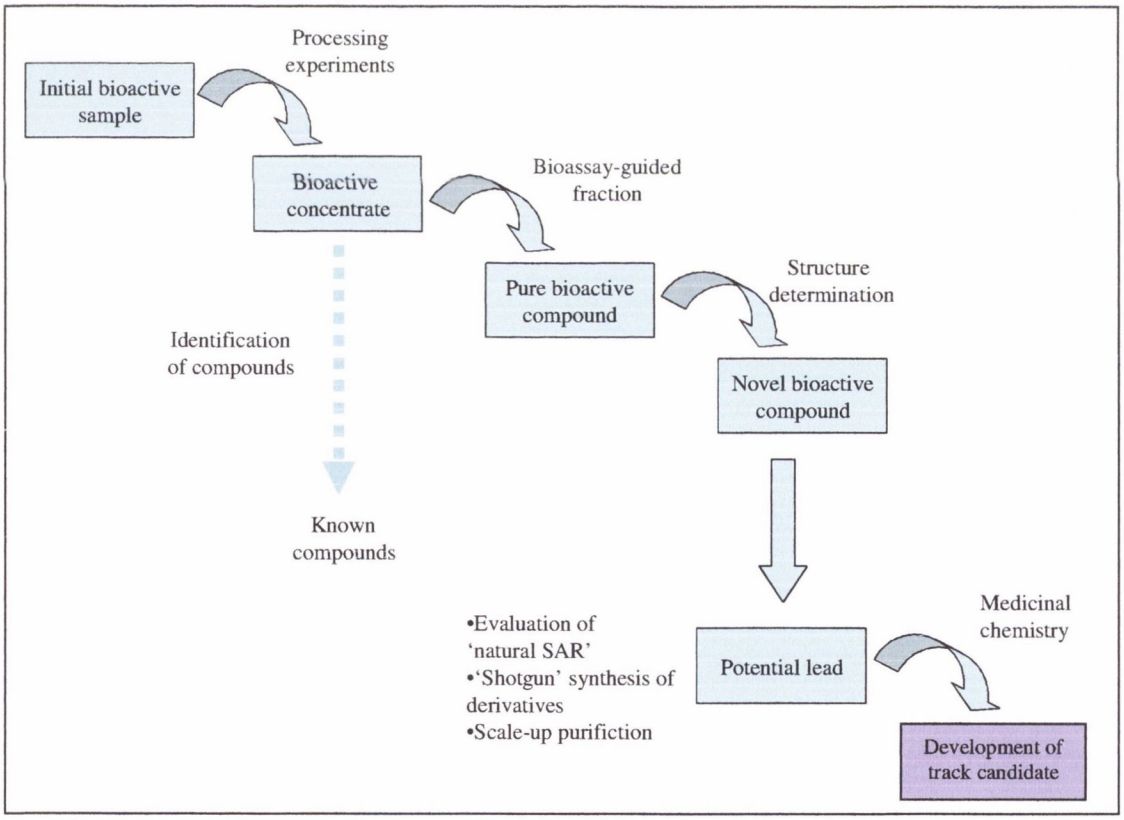


Figure 1.1.3.1 Process for natural product discovery (Koehn and Carter 2005)

The typical process of discovering natural product hits and their progression towards development can be seen in **Figure 1.1.3.1**. Historically there have been three major

difficulties in this process: (i) the identification of known compounds in order to avoid duplication. This problem has been facilitated by the development of directly coupled HPLC-mass spectrometer (LC-MS) systems and the generation of natural products databases. (ii) *De novo* structure determination of compounds. This area has been revolutionised by advances in spectroscopic techniques, particularly in high-resolution NMR and mass spectroscopy technologies. Where the biological activity profile meets the expected criteria for potency and selectivity, preliminary structure-activity response (SAR) studies are conducted and the purification process is scaled up. (iii) The major bottleneck that still affects natural product drug discovery is the isolation and purification of active compounds from complex matrixes. Although advances in separation technology, such as HPLC, supercritical fluid chromatography (SFC), and capillary electrophoresis (CE) have had a major impact on the purification problem, there are still major challenges: (1) the biological activity must be correlated with the effective compound, and (2) the separation methods must provide sufficient quantities of the compound in interest. Various innovative processes are being developed in order to solve these problems.

An example of such a process is the creation of an 'online-bioassay' that utilizes continuous-flow enzymatic reactions that are capable of providing real-time read outs of inhibition of enzymatic activity (Schenk et al. 2003). The effluent from HPLC is split in two streams, one to the enzyme assay and the other in parallel to a mass spectrometer. Correlating peaks that are detected in the enzyme assay with the corresponding mass spectra provides data that are characteristic of the bioactive compound. This data can be sufficient to identify known compounds through database searching, and will provide a linkage to activity for further studies.

Another solution of the problem could be the development of natural product libraries using advanced integration of separation-, purification- and identification-technologies. An example of this are the libraries made from the stem bark of *Taxus brevifolia* by Eldridge and co-workers (Eldridge et al. 2002). The libraries were produced by integrating automated flash chromatography, solid-phase extraction, filtration, and high-throughput parallel four-channel preparative HPLC. The libraries obtained were analyzed prior to biological screening by an advanced specialised mass spectrometer to determine molecular weight, number and quantity of compounds in the fractions.

After the biological screening the active fractions were rapidly purified and the individual compounds were re-screened for confirmation of activity. Finally the active compounds could be structurally elucidated by NMR and mass spectrometry. By combining high-throughput screening with conventional natural product techniques in this way the cycle times for the discovery of natural product drug leads is shortened, and can be compared with the efficiency of combinatorial chemistry.

The continuing development of the existing separation-, purification- and identification-technologies suggests a positive trend in the forthcoming years for the discovery and development of natural product-derived pharmaceuticals. In addition there has been a positive movement in the experiments aimed at total synthesis of many natural products. The actual crucial structure of the biological activity has been defined, leading to potent and selective products derived with fewer synthetic steps, resulting in lower cost (Lin et al. 2004, Nicolaou et al. 2003).

1.1.4 Natural sources for future drug discovery

The selection of natural sources used in the search for new leads in drug discovery has traditionally followed two main paths: ethnopharmacology (medicinal herbs, substance of abuse, ordeal poisons etc) and toxicology (poisonous plants, venomous animals, arrow and fish poisons etc) (Tulp and Bohlin 2004).

The plant kingdom

Most of the active components of medicinal products from natural sources are derived from plants. Although plants have been used in screening programmes in the search for biologically active molecules for a long time, probably only 5-15% of the ~250,000 plant species in the world have been tested for some type of biological activity (Pieters and Vlietinck 2005, Verpoorte 1998).

Prominent plant-based medicines include:

- Quinine, the anti-malarial drug, from the bark of *Cinchona* species.
- Morphine, the analgesic, from the opium poppy (*Papaver somniferum*).
- Digoxin, for heart disorders, from *Digitalis lanata*.

- Reserpine, the anti-hypertensive agent, from *Rauwolfia serpentina*, traditionally used for snakebites and other ailments.
- Ephedrine, an anti-asthma agent, from *Ephredra sinica*.
- Tubocurarine, the muscle relaxant, from *Chondrodendron* and *Curarea* species, used in the Amazon as the basis for the arrow poison curare.
- Podophyllotoxin, the anti-cancer agent, from the American mayapple (*Podophyllum peltatum*).
- Vinblastine and vincristine, the anti-cancer agents, were isolated from the Madagascar periwinkle, *Catharanthus rosesus*.
- Taxol®, the anti-cancer agent, was initially isolated from the bark of the Pacific yew, *Taxus brevifolia*.

The path traditionally used for the selection of plants screened for biological activity (ethnopharmacology and toxicology) have led to the neglect of a wide structural diversity found in e.g. plants commonly seen in gardens, fruits and vegetables. Grapes (*Vitis vinifera*) and cranberries (*Vaccinium macrocarpum*) have been found to have antibiotic activity due to their proanthocyanidin content (Howell and Nicholi 1999). Extracts of orange peel has an antitumour activity, attributed to the poly-methoxy flavonoids it contains (Ghai and Rosen 2001). Common vegetables have been found to be a source of pharmacologically active molecules. Broccoli was found to contain high amounts of sulphoraphane, a compound in broccoli and its botanical kin that inhibits the development of cancer (Fahey et al. 1997). The prevention of stomach- and lung-cancer by extracts from winter rape, mustard, radish, and purslane (Cruciferae and Scrophulariaceae families) was shown by Zheng (Zheng 1999), and tumour reduction by an extract of beetroot (*Beta vulgaris*) was observed by Kapadia (Kapadia 2000).

There are various problems in using plants as sources for new drugs. More than 12.5% of the world's vascular flora is threatened by extinction due to over-harvesting, deforestation etc. Accessibility, seasonal changes, plant disease, slow growth and taxonomic difficulties are other disadvantages. The solution to many of these problems has been to use plant cell cultures instead of whole plants. There has been more than 85% success rate with cryobanking of cultured plant cells, greatly increasing

the accessibility compared to traditional methods. Furthermore, the culturing of seeds enables the investigation of rare and endangered species and variability caused by seasonality and lifecycle is reduced (Lawrence 1999). There are a multitude of manipulations possible in a plant cell culture environment, such as genetic, epigenetic, elicitation/induction, addition of hormones and enzymes making the use of cell cultures a platform for combinatorial biology approaches. This would not be possible to the same extent with whole plants because only one environment can be applied at one time.

Microorganisms

Microorganisms have been a rich source of drugs, including antibiotics, immunosuppressants and lipid-lowering statins.

Well-known medicines from microbial origin (Cragg and Newman 2005) include:

- Anti-bacterial agents from *Penicillium* species;
- Immunosuppressants, such as the cyclosporins and rapamycin, from *Streptomyces* species.
- Cholesterol lowering agents, such as mevastatin and lovastatin, from *Penicillium* species.
- Anthelmintics and antiparasitic drugs, such as the ivermectins, from *Streptomyces* species.
- A potential new antidiabetic agent from a *Pseudomassaria* fungal species found in the Congolese rainforest.

These drugs have been produced from a very small range of the world's microbial diversity. How many species of microorganisms there might exist is not known, but so far only approximately 6000 bacterial species have been named (Harvey 2000). Microorganisms are being found in extreme environments, such as polar ice (Priscu et al. 1998), geothermal vents (Deckert et al. 1998), dark caves (Harvey 2000) and deep-sea sites (Takami et al. 1999). The recent development in molecular microbial ecology has shown that the microbial diversity in nature is not reflected in the strains cultured in the laboratory, since only a very small fraction of microorganisms can be cultured under standard laboratory conditions (Courtois et al. 2003).

Many potential biomolecules from microbial fermentation are produced as a result of encountering other organisms, producing metabolites that either kill or harm other organisms, or are signalling compounds involved in inter-relationships between the organisms. So in order to get microorganisms to produce such metabolites they need to be grown in nutrient-manipulated media (Lawrence 1999).

Marine organisms

The sea covers 70% of the earth's surface and is home to an estimated 1-2 million marine species ranging from microorganisms to large animals and plants. The majority of this species diversity is found in the land-sea interface. The high concentration of species living in this relatively limited habitat makes them highly competitive and complex. As a result of this high level of competition, a large percentage of the marine organisms have developed chemical means; in the form of secondary metabolites, in order to defend against predation, defend against overgrowth by competing species, or in order to subdue motile prey species for ingestion. Unique to the secondary metabolites from the marine organisms is the high presence of covalently bound halogen atoms, mainly chlorine and bromine. This is presumably due to their ready availability in seawater (Simmons et al. 2005).

Examples of medicines from marine origin (Kijjoa and Sawangwong 2004) include:

- The pseudopterosins, with significant analgesic and anti-inflammatory properties, from the Caribbean gorgonian *Pseudopterogorgia elisabethae*
- Manoalide, an anti-inflammatory agent, from the sponge *Luffarriella variabilis*
- Ziconotide and other new pain killers derived from peptides from the cone snail venom (Cragg and Newman 2005)

A range of anticancer drugs from marine organisms are currently in clinical and pre-clinical trials. Specialist companies and institutes such as PharmaMar (Madrid, Spain), The Australian Institute for Marine Species (Townsville, Queensland, Australia) and The National Cancer Institute (USA) are currently screening large collections of marine samples for drug discovery. Some examples of anti-cancer drugs in clinical and pre-clinical trials are shown in **Table 1.1.4.1** (Newman and Cragg 2004, Simmons et al. 2005).

Table 1.1.4.1 Examples of anticancer drugs from marine sources in clinical trials

Compound	Source Organism	Chemical Class	Molecular Target	Current Status
Ecteinascidin 743 (Yondelis®)	*Ecteinascidia turbinate* (tunicate)	Tetrahydro-isoquinolone alkoloid	Tubulin	Phase III
Dolastatin 10	*Dolabella auricularia/ Symploca sp.* (mollusc/cyanobacterium)	Linear peptide	Tubulin	Phase II
Bryostatin 1	*Bugula neritina* (bryozoan)	Macrocyclic lactone	Tubulin	Phase II
Synthadotin	*Dolabella auricularia/ Symploca sp.* (synthetic analogue)	Linear peptide	PKC Tubulin	Phase II
Kahalalide	*Elysia rufescens / Bryopsis sp.* (mollusc / green alga)	Cyclic peptide	Lysosomes / erbB pathway	Phase II
Squalamine	*Squalus acanthias* (Shark)	Aminosteroid	Phosopholipid bilayer	Phase II
Dehydrodidemnin B (Aplidine®)	*Tridindenmnum solidum* (tunicate, synthetic / possible cyanobacterium)	Cyclic peptide	Ornithine decarboxylase	Phase II
Soblidotin	*Dolabella auricularia/ Symploca sp.* (synthetic analogue)	Linear peptide	Tubulin	Phase I
E7389	*Halichondria okadi* (sponge)	Macrocyclic polyether	Tubulin	Phase I
NVP-LAQ824	*Psammaplysilla sp.* (sponge)	Indolic cinnamyl hydroxamate	HDAC/DNMT	Phase I
KRN-7000	*Agelas mauritianus* (sponge)	α-Galacosylceramide	Vα24 + NKT cell activation	Phase I
Curacin A	*Lyngbya majuscula* (cyanobacterium)	Thiazole lipid	Tubulin	Pre-clinical
Salinosporamide A	*Salinospora sp.* (bacterium)	Bicyclic γ-lactam-β lactone	20S proteasome	Pre-clinical
Laulimalide	*Cacospongia mycofijiensis* (sponge)	Macrolide	Tubulin	Pre-clinical

1.1.5 Plant metabolomics – natural products in the post-genomics era

As mentioned in section 1.1.4 the biotechnological production of important secondary metabolites in plant cell and organ cultures is an attractive alternative to the extraction of whole plant material. However, cell and organ cultures have been found to produce insufficient amounts of the required metabolites, and despite treatment with elicitors, selection of high-yielding species and media manipulations only a few examples of have led to commercial success. One of the explanations for this is the lack of understanding of how the secondary metabolites are synthesised or how the synthesis

is regulated. In order to engineer the secondary metabolic pathways that knowledge is necessary (Oksman-Caldentey and Inzé 2004).

The recent completion and publication of the first complete genome sequence of a flowering plant, the brassica *Arabidopsis thaliana*, represents a giant step forward not only for plant biotechnology, but also for drug discovery (The Arabidopsis Genome Initiative 2000). The *Arabidopsis thaliana* genome is dispersed over five chromosomes and documents a complete set of genes controlling development, growth, responses to environmental variation, disease resistance and primary and secondary metabolism. This full genomic sequence provides the means for analysing gene function that is also important for other plant species, including commercial and agricultural crops (Oksman-Caldentey and Barz 2002).

The post-genomic era has opened up the world of "omics". Plant functional genomics is a rapidly developing technology that allows the identification of large sets of genes that influence a particular biological process (Fiehn et al. 2000). It includes transcriptomics; quantification of the total gene expression in a given system (Holtorf et al. 2002), proteonomics; mapping of all the proteins present (Blackstock and Weir 1999) and metabolomics; describing all metabolites and their fluxes (Fiehn et al. 2000).

A possible functional genetics approach to enhancement of secondary metabolite production was proposed by Oksman-Caldentey and Inzé (Oksman-Caldentey and Inzé 2004), shown in **Figure 1.1.5.1**. This approach was based on the assumption that the elicitor treatment not only changes the production of the desired metabolites but also activates the genes involved in their biosynthesis. One important aspect of using this method is that theoretically it is applicable to any plant or cell culture without any pre-existing gene-sequence knowledge. Many interesting secondary metabolites are produced by rare and exotic plant species and genome knowledge might not be available.

This approach was used in a study where the nicotine biosynthesis in tobacco cells was investigated (Goossens et al. 2003). Targeted secondary metabolite analysis was combined with cDNA-amplified fragment length polymorphism (cDNA-AFLP)

transcript profiling (Breyne and Zabeau 2001) following elicitation with methyl jasmonate. By using this method 591 out of 20,000 visualised genes were identified to be induced by the elicitor.

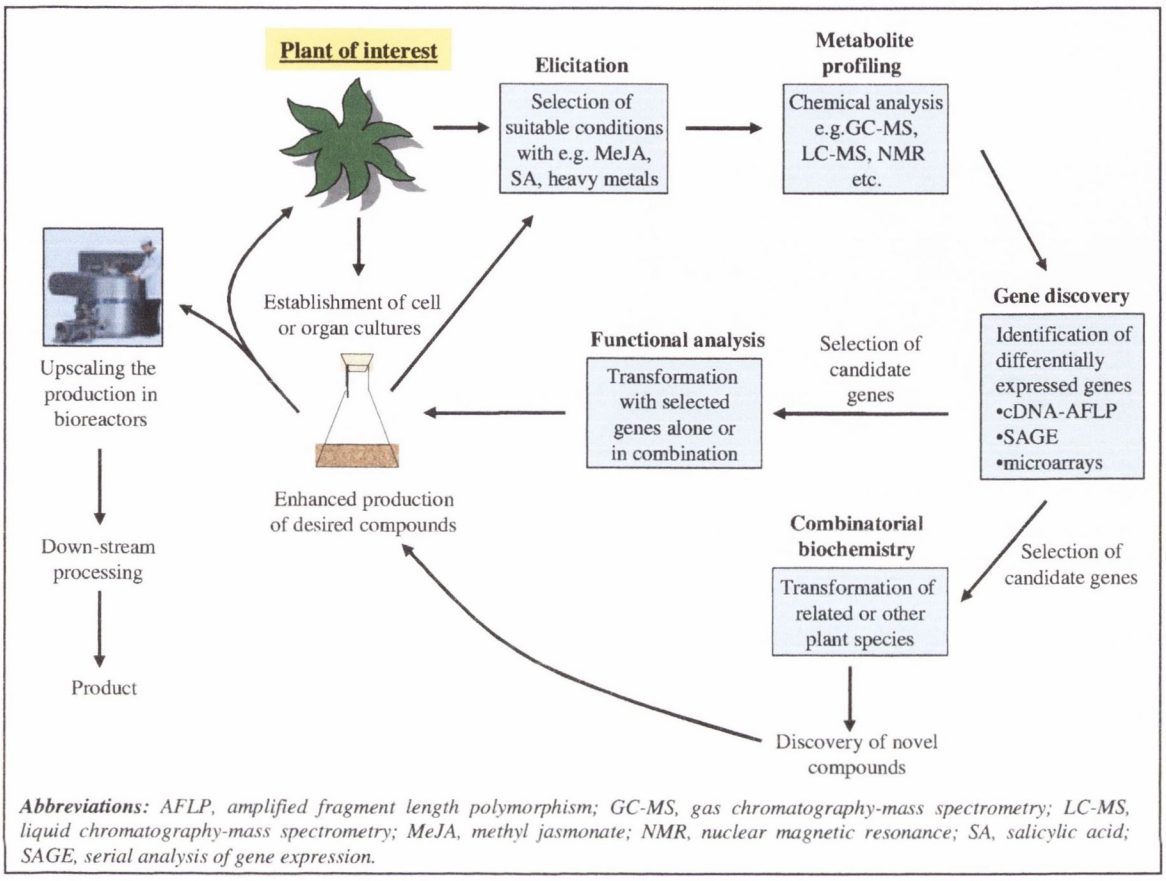


Figure 1.1.5.1 Enhancement of novel and known secondary metabolites by functional genomics (Oksman-Caldentey and Inzé 2004)

1.2 Taxol® (paclitaxel) – an anticancer drug from the yew tree (*Taxus*)

1.2.1 The history of paclitaxel – from discovery to clinical use

The search for anticancer agents from plant sources started after the Second World War with Goodman's discovery of nitrogen mustards, of value in the treatment of leukaemia (1946), Faber's discovery of aminopterin for use in childhood leukaemia (1948) and Burchenal's discovery of 6-mercaptopurine for treating leukaemia (1953) (Itokawa 2003). As a result of these findings, the United States National Cancer Institute (NCI) initiated an extensive plant collecting program in 1960, focused mainly

on the temperate regions. The plant collection program started with plants native to the US. Rather than being directed by ethnobotanical knowledge, the collection of plant material was based on the belief that diversity of morphological characteristics could possibly be mirrored by diversity in secondary metabolite production by the plants (Suffness and Wall 1995). Two samples were collected from the Pacific yew tree, *Taxus brevifolia*: PR-4959 (stems and fruit) and PR4960 (stem and bark) in 1962 by Arthur Barclay from the US Department of Agriculture (USDA). Upon screening the stem and bark sample demonstrated cytotoxic activity against KB cells, leading to fractionation studies on the *T. brevifolia* bark. The isolation of 0.5 g of pure paclitaxel from the *T. brevifolia* bark took about two years, and the first pure sample was isolated in 1966 by Wall and his team (Itokawa 2003). The elucidated structure of paclitaxel was published in 1971 (Wani et al. 1971). The further chronology from the publication of its structure to its current clinical use is as follows (Itokawa 2003, Suffness and Wall 1995):

1971	Structure of paclitaxel published for the first time (Wani et al. 1971)
1971	Paclitaxel was found in other *Taxus* species (e.g. *T. baccata* and *T. cuspidata*)
1974-75	Paclitaxel was found have B16 melanoma activity, meets development criteria
1978	Paclitaxel shows activity against human cancer cell lines – MX-1 breast xenograft
1979	Paclitaxel mode of action published, promoter of microtubule assembly
1980	Formulation studies completed and toxicology studies begins
1983	Investigational New Drug Application filed
1984	Investigational New Drug Application approved, Phase I clinical trials begins
1987	NCI contract for the collection of 60,000 lbs of *T. brevifolia* bark
1989	Phase II clinical trials show 30% improvement in refractory ovarian cancer, NCI contract for the collection of another 60,000 lbs of bark
1990	Presence of paclitaxel in the needles and other pant parts of *T. brevifolia* confirmed
1991	Phase II clinical trials show 48% shrinkage in metastatic breast cancer, Bristol-Myers Squibb is selected as a Co-operative Research and Development Agreement partner
1992	Phase III clinical trials begins
	The Pacific Yew Act becomes law
	FDA approves paclitaxel isolated from the bark of *T. brevifolia* for treatment of refractory ovarian cancer
	Taxol becomes a trade mark (Bristol-Myers Squibb); generic name: paclitaxel
1993	Supplemental New Drug Application filed by Bristol-Myers Squibb to allow needle derived paclitaxel to be used clinically
	Paclitaxel was approved as second line treatment for ovarian cancer
1994	FDA approval for the use of Taxol for treatment of metastatic breast cancer

	Total synthesis of paclitaxel achieved (Nicolaou et al. 1994) (not commercially viable)
1995	F. H. Faulding & Co., Australia's largest pharmaceutical company, launches NaPro paclitaxel in Australia under the name Anzatax.
1996	Paclitaxel approved for first line treatment of ovarian cancer
1997	Approval of Taxol for the second-line treatment of AIDS-related Kaposi's sarcoma
1997	Ivax submits a New Drug Application to FDA seeking clearance to market Paxene in the U.S. for the treatment of Kaposi's sarcoma. NaPro is their paclitaxel supplier
1997	Bristol-Myers Squibb is issued U.S. Patent and Trademark Office Patent Number 5,641,803, a use patent on the infusion of Taxol.
1998	Paclitaxel licensed for the treatment of non-small cell lung cancer
1998	Bristol-Myers Squibb and Phyton Inc. sign an agreement to commercialize Phyton's proprietary plant cell fermentation technology.
2000	IVAX Corporation received approval to market Paxene® (paclitaxel), in Canada for the treatment of AIDS-related Kaposi's sarcoma
2001	Bristol-Myers Squibb is sued over anticompetitive practicing concerning Taxol
2002	Taxol is being produced solely from the cell-culture method by Bristol-Myers Squibb, using plant cell cultures of *T. chinensis*
2004	Boston Scientific Corporation received approval from FDA to market its Taxus® Express2™ paclitaxel-eluting coronary stent system
2005	FDA approval of Abraxane® for clinical trials. In this preparation, paclitaxel is bonded to albumin as the delivery agent as an alternative to solvent delivery

1.2.2 Taxane biosynthesis

The structure of paclitaxel is quite complex, with an unusual diterpene carbon skeleton, eight oxy-functional groups, and various acyl side chains. It has a total of 11 chiral centres (Wani et al. 1971). Total synthesis of paclitaxel has been achieved (Danishefsky et al. 1996, Nicolaou et al. 1994), but the approach is not commercially viable. The supply of the drug is therefore still relying on either semisynthesis, using 10-deacetylbaccatin III (10-DAB III) or baccatin III from renewable sources (foliage) or from plant cell cultures of *Taxus* species producing taxanes. It is therefore important to understand the complex biosynthetic pathway leading to the production of these secondary metabolites. The paclitaxel biosynthetic pathway is considered to require 19 enzymatic steps from the universal diterpenoid precursor geranylgeranyl diphosphate (GGPP) which is cyclized to taxa-4(5),11(12)-diene (Long and Croteau 2005). The total biosynthetic pathway is still not fully determined, in particular the elucidation of the assembly of the C13-side chain is still under investigation.

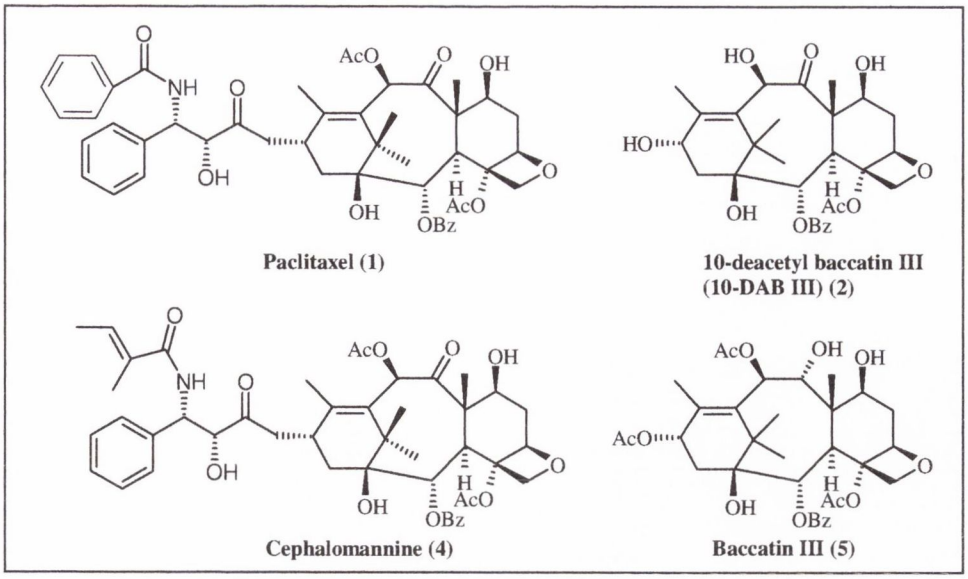


Figure 1.2.2.1 Important taxanes found in *Taxus*

1.2.2.1 The mevalonate pathway

Terpenoids, also called terpenes or isoprenoids, are a diverse, numerous and widespread group of natural products derived from head-to-tail linkage of C_5 isoprene units synthesised via the mevalonate pathway (Dewick 1997, Mann et al. 1994). Mevalonic acid is derived from three molecules of acetyl-CoA (**Figure 1.2.2.2**), and further transformed into the two different C_5 isoprene units, isopentyl pyrophosphate units (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP) in a series of phosphorylation, condensation, reduction and decarboxylation steps (**Figure 1.2.2.3**) (Dewick 1997). Successive condensations of IPP and DMAPP extend the carbon chain length providing the precursors of terpenoids and steroids found in nature.

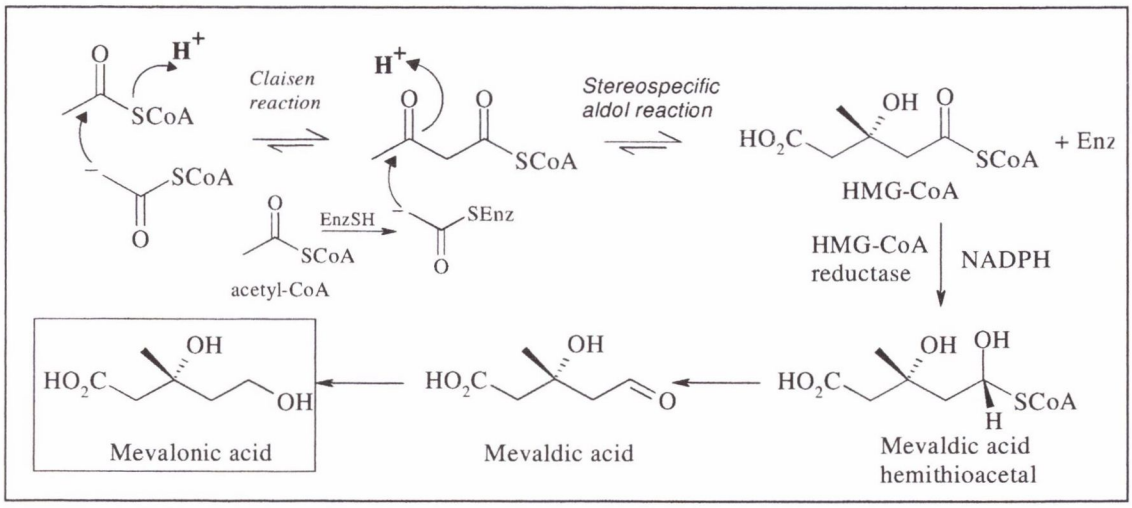


Figure 1.2.2.2 Biosynthesis of mevalonic acid

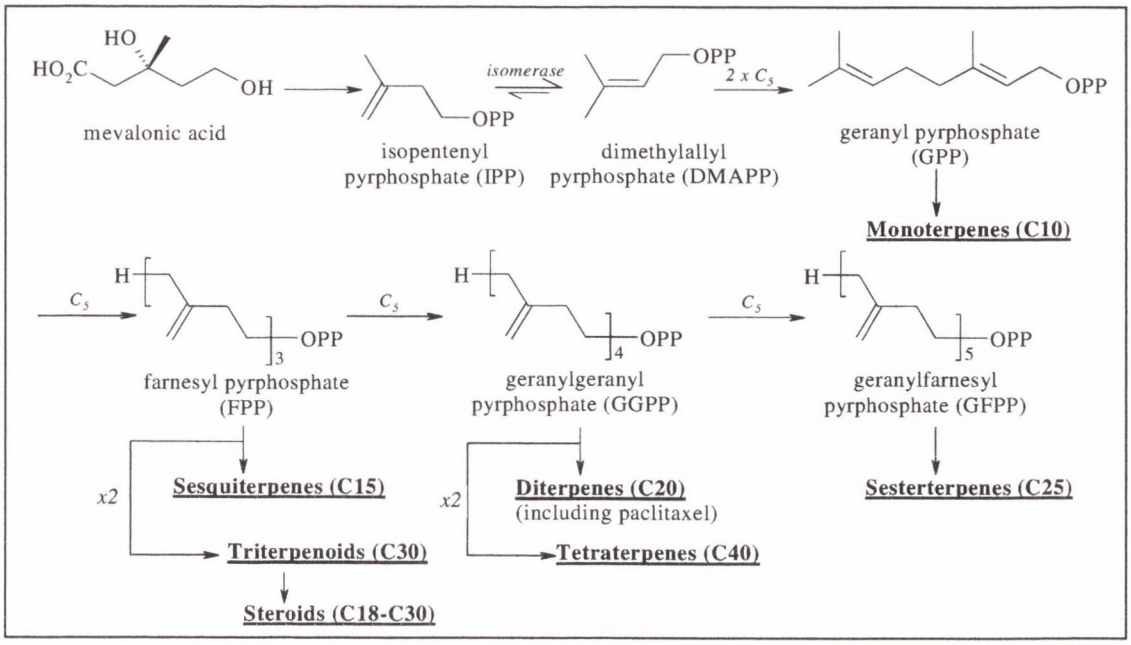


Figure 1.2.2.3 The mevalonate pathway (Dewick 1997, Mann et al. 1994)

1.2.2.2 Taxane biosynthesis

Early studies on the biosynthesis of paclitaxel involved *in vivo* feeding experiments with radiolabeled precursors. These indicated that acetate, mevalonate and phenylalanine were building blocks for paclitaxel (Hezari and Croteau 1997). However, Zenk and his associates (Eisenreich et al. 1996) showed conclusively that mevalonate is not the precursor to IPP in the taxane biosynthesis, that in this case the key intermediate IPP originates through a pyruvate / glyceraldehyde-3-phosphate (GAP) non-mevalonate pathway first described by Schwender et al (Schwender et al. 1996) (**Figure 1.2.2.4**).

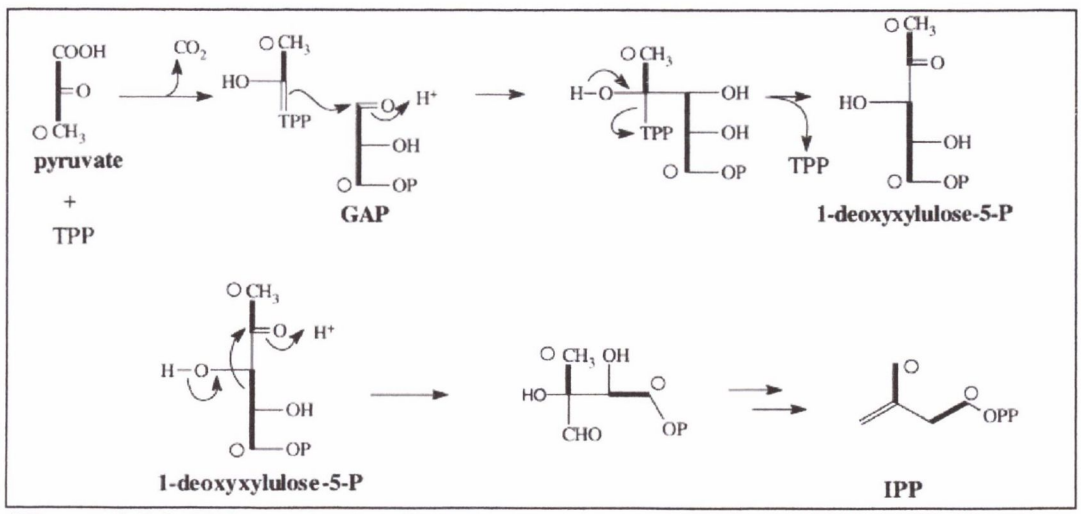


Figure 1.2.2.4 Biosynthesis of IPP from pyruvate and glyceraldehyde-3-phosphate

First step in taxane biosynthesis: taxadiene formation

The first committed step in the biosynthesis of paclitaxel and related taxanes is the cyclization of the universal diterpenoid precursor geranylgeranyl pyrophosphate (GGPP) to taxa-4(5),11(12)-diene. This cyclization is catalysed by the plastidial enzyme taxadiene synthase (Walker and Croteau 2001) (**Figure 1.2.2.5**).

Second step in taxane biosynthesis: taxadienol formation

Hydroxylation of the taxa-4(5),11(12)-diene at C-5 with migration of the double bond is the next step in the taxane biosynthesis yielding taxa-4(5),11(12)-diene-5α-ol. The responsible enzyme for this step is the cytochrome P450 associated NADPH-dependent taxa-4(5),11(12)-diene-5α-hydroxylase enzyme (Walker and Croteau 2001) (**Figure 1.2.2.5**).

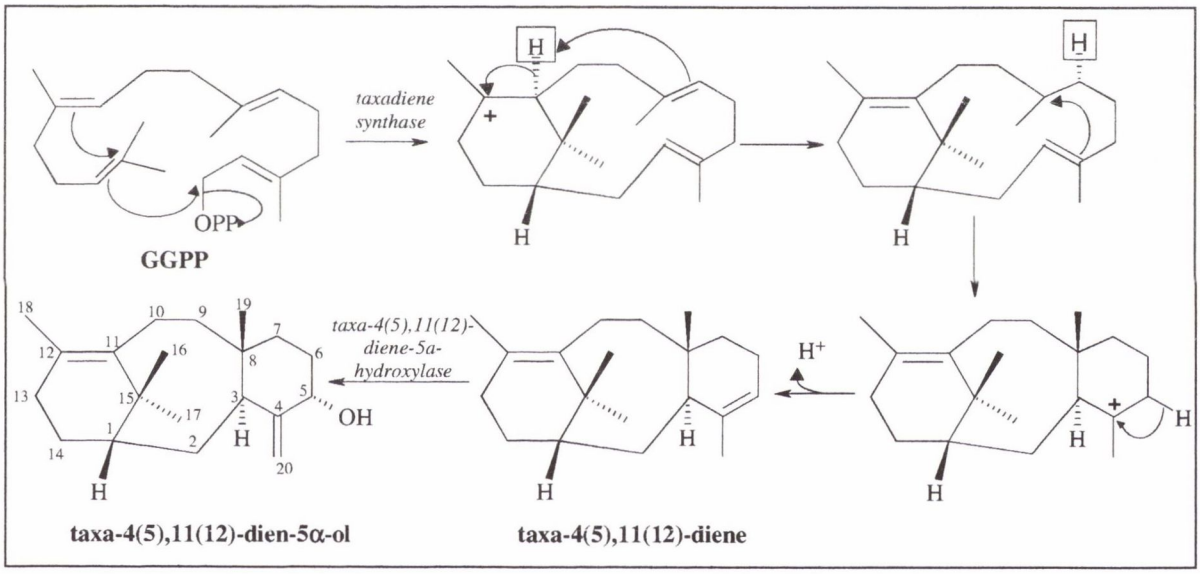


Figure 1.2.2.5 First and second step in the biosynthesis of taxanes

Third step in taxane biosynthesis: acetylation of taxadienol

Acetylation of taxa-4(5),11(12)-diene-5α-ol in the C-5 position is the next step, catalysed by taxadien-5α-ol-O-acetyltransferase, a cytochrome P450 aetyl-CoA-dependent acetyltransferase (Walker and Croteau 2001). The resulting 4(20)-5α-acetate functional group plays a further role in the construction of the oxetane ring in paclitaxel and related taxanes (**Figure 1.2.2.6**) (Hezari and Croteau 1997).

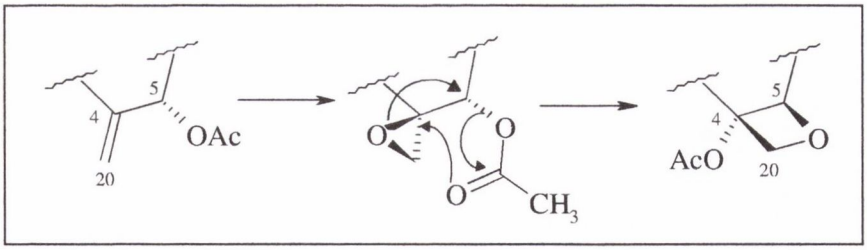


Figure 1.2.2.6 Stereospecific oxetane ring formation in taxane biosynthesis

Subsequent oxygenation steps

The subsequent oxygenation of the taxane nucleus has been suggested to follow the order of C-10, C-2, C-9 and finally C-13. Oxygenation also occurs at C-7 and C-1, but is thought to occur late in the biosynthetic pathway (Walker and Croteau 2001). The further functionalisation of the taxane ring system is still not fully determined, although the enzyme 10-DAB III-10β-O-acetyltransferase have been found to catalyse the formation of baccatin III from 10-DAB III (Menhard and Zenk 1999) (**Figure 1.2.2.7**).

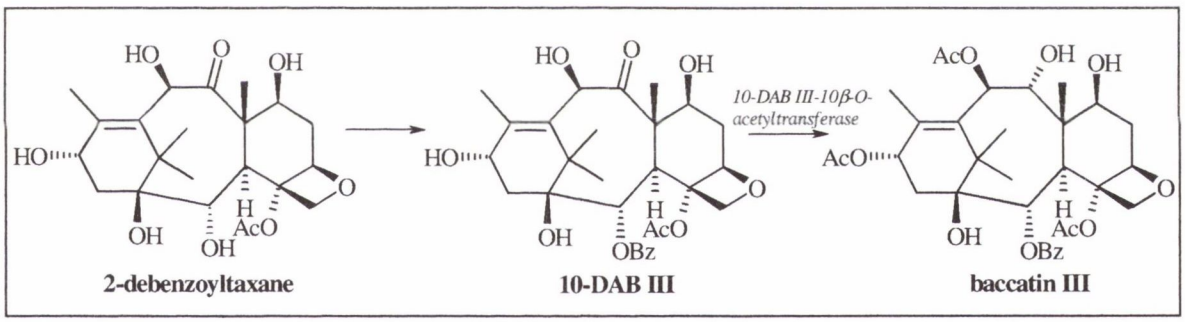


Figure 1.2.2.7 Formation of baccatin III from 10-DAB III

Formation of C-13 side chain

In vivo studies with *Taxus*, where labelled amino acids were used to elucidate the mode of assembly of the C-13 side chain, showed that the intact side chain (3-dimethylamino-3-phenyl-propanoic acid) was not incorporated directly into paclitaxel, however, β-phenylalanine (derived by mutation from α-phenylalanine), in its free amine form, is attached to baccatin III, and it is then hydroxylated and N-benzoylated to form paclitaxel (**Figure 1.2.2.8**). The enzyme catalysing the formation of β-phenylalanine from α-phenylalanine is phenylalanine aminomutase. Amino-phenylpropanoyl ligase catalyses the formation of β-phenylalanoyl CoA, which

together with baccatin III (catalysed by amino phenylpropanoyl-13-O-transferase) yields N-(3'R)-debenzoyl-2'-deoxypaclitxel. The final two steps in the paclitaxel biosynthesis are catalysed by side chain-2'hydroxylase and benzoyl-N-transferase. The immediate precursor of paclitaxel, N-debenzoyl paclitaxel, is also converted by N-tigloyl transfer to form cephalomannine (Long and Croteau 2005).

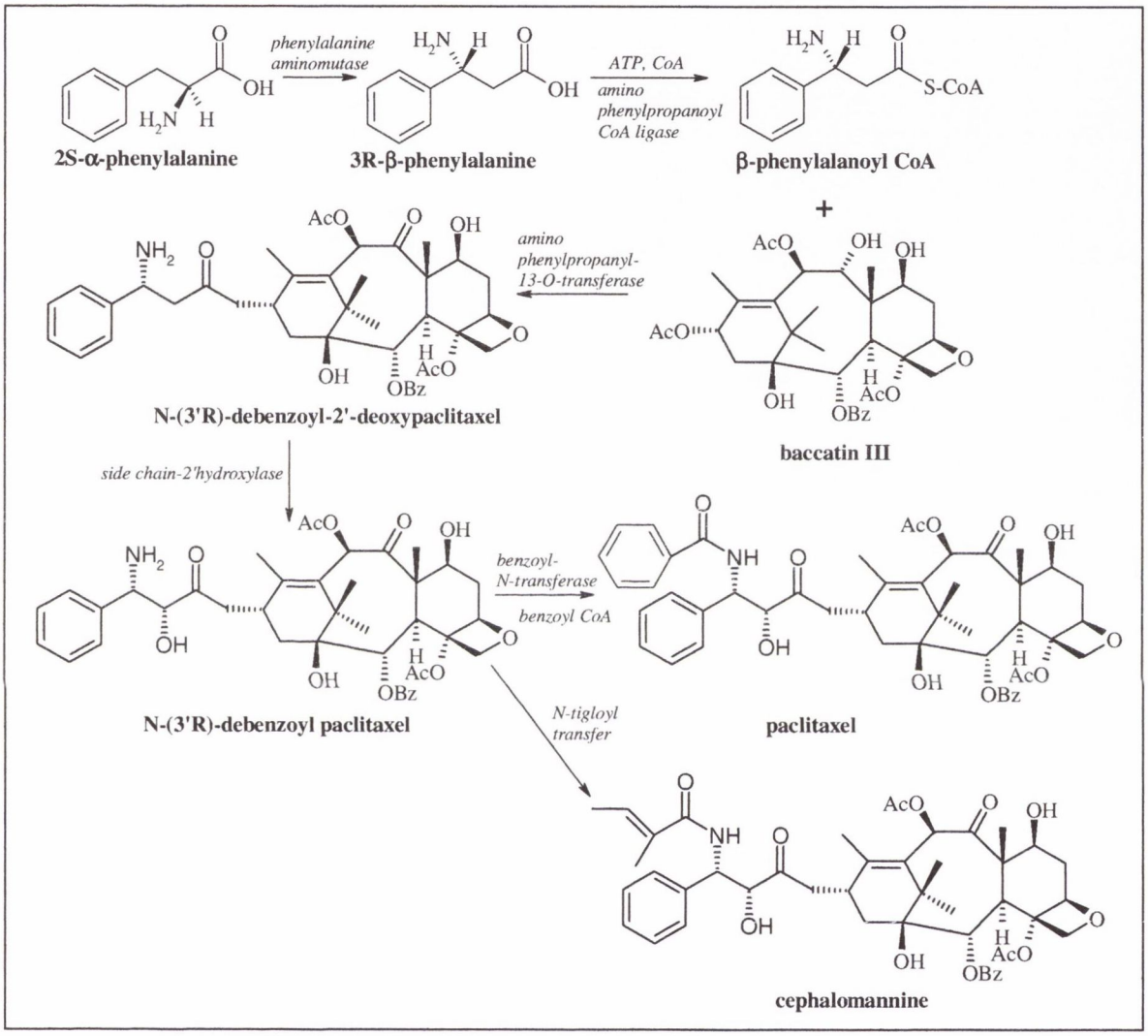


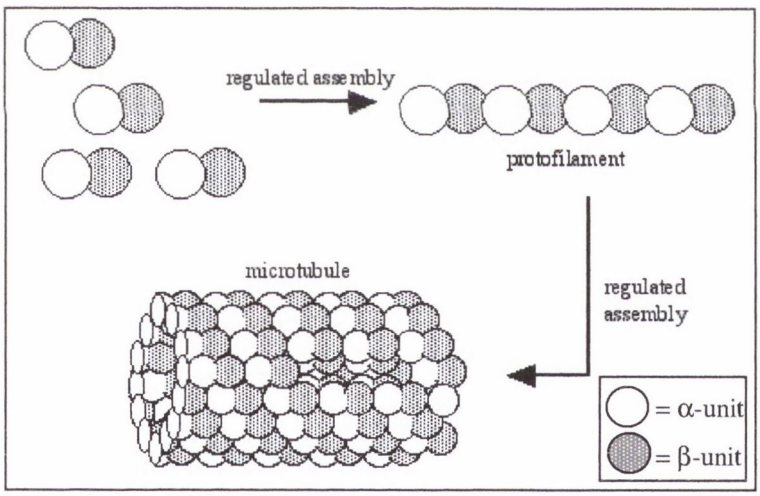
Figure 1.2.2.8 Assembly of the C-13 side chain and final formation of paclitaxel and cephalomannine

The actual regulation of the biosynthesis of either paclitaxel or cephalomannine in the cell is still under investigation, but Wang and co-workers found that salicylic acid promoted the biosynthesis of cephalomannine, whereas it had no influence on paclitaxel biosynthesis in cell cultures of *T. chinensis* (Wang et al. 2004). In the same study they reported that methyl jasmonate induced biosynthesis of paclitaxel, but had no effect on cephalomannine production.

1.2.3 Paclitaxel mode of action

The cytotoxic properties of paclitaxel were initially thought to be due to its ability to inhibit microtubule formation or to destabilise microtubules. It was thought to be just another of a series of naturally occurring 'spindle poisons' (compounds interfering with the tubulin-microtubule equilibration), such as vincristine, vinblastine, colchicines, podophyllotoxin and others (Suffness and Wall 1995). However, in 1979 Horwitz and associates found that paclitaxel induces the assembly of tubulin into microtubules, and more importantly, that the drug actually stabilises them to the extent that mitosis is disrupted (Horwitz et al. 1979).

Tubulin is a dimeric cellular protein (made up of an α-and a β-unit) that polymerizes into long chains or filaments forming microtubules in eukaryotic cells (**Figure 1.2.3.1**). Microtubules are hollow filaments which serve as cytoskeletal elements for living cells, are vital for intracellular transport and are required for the formation of the mitotic spindle in cell division in all eukaryotes (Nogales et al. 1998).



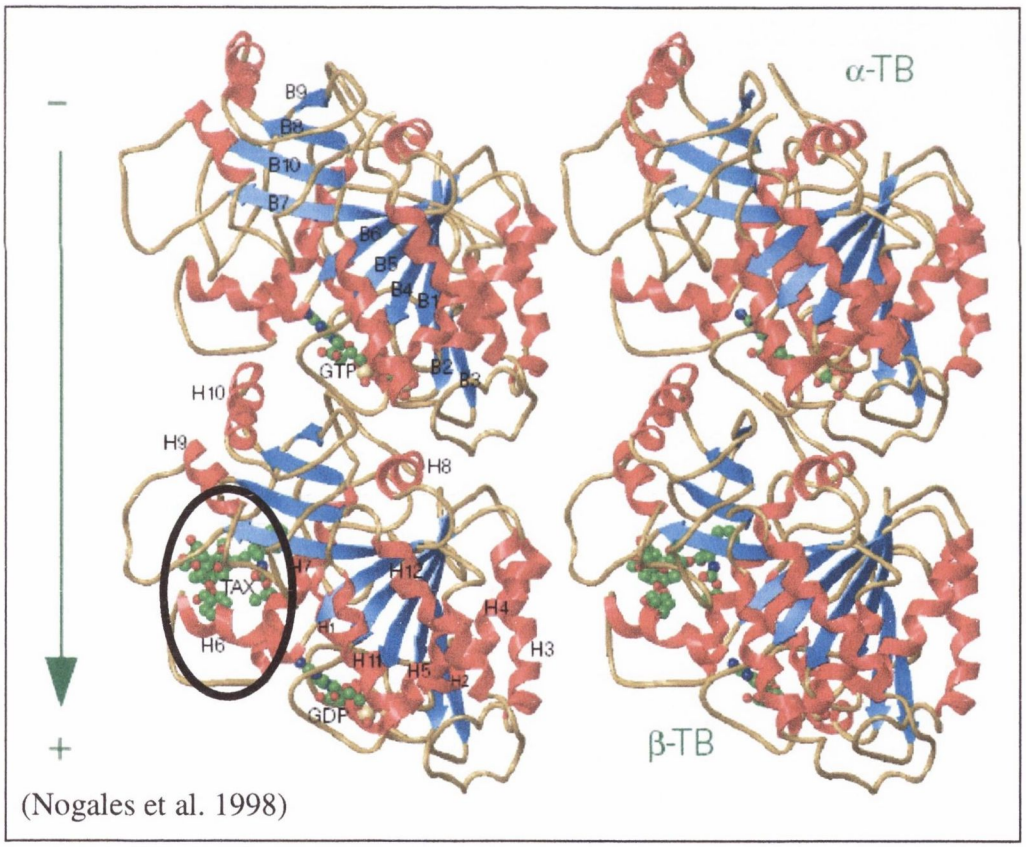
(Carpette 2005)

Figure 1.2.3.1 The organization of tubulin subunits in a microtubule

Because of the dimeric character of tubulin and the alternation of alpha and beta, one end of the protofilament is terminated by an alpha subunit and at the opposite one by a beta subunit. This provides the protofilament with a type of polarity (**Figure 1.2.3.2**). Within the microtubule, the protofilaments are associated laterally with the same polarity. Therefore, the microtubule also appears as a polar structure with a plus and a

minus end. Polarity is a very important feature for microtubule functioning. It is the basic property for direction-dependent cellular events, e.g. vesicle transport (Lodish et al. 2000).

Paclitaxel binds specifically to one site on the β-subunit of tubulin (**Figure 1.2.3.2**, circled with black in figure), and it binds preferentially to formed microtubules rather than specifically to β-tubulin alone or tubulin dimers (Nogales et al. 1998). Microtubule assembly normally requires GTP (guanosine triphosphate), microtubule-associated proteins (MAPs) and physiological temperatures (37°C). By addition of Ca^{2+} or reduction of the temperature (to ~ 8°C) depolarization occurs. Paclitaxel has the ability to induce microtubule assembly in the complete absence of GTP or MAPs. In addition paclitaxel-polymerised microtubules are stable to both Ca^{2+} and temperature reduction (Orr and Horwitz 1997).



Labels for strands (in the α-subumit) and helices (in the β-subunit) are included. The arrow indicates the direction of the protofilament and microtubule axis.

Figure 1.2.3.2 Ribbon diagram of the tubulin dimer showing α-tubulin with bound GTP (top), and β-tubulin containing GDP (guanosine diphosphate) and taxol (bottom)

The activity of the compounds that interfere with tubulin-microtubule equilibrium can be detected using a unique anti-tubulin assay (Lastaste et al. 1984, Shelanski et al. 1973). In this assay tubulin is isolated from bovine or porcine brain. It is then purified by a series of homogenisation and centrifugation steps and further pre-treated to inhibit spontaneous microtubule formation. On addition of anti-mitotic agent, such as paclitaxel or similar compounds (e.g. docetaxel), the temperature dependent *in vitro* assembly (37°C) or disassembly (4°C) can be monitored by observing the turbidimetric response at 360 nm (Chauvière et al. 1981, Lastaste et al. 1984). In the absence of polymer formation, turbidity is low and the observed degree of light scatter is reduced. Paclitaxel actively promote polymerisation causing sustained increase in optical density in the assay (**Figure 1.2.3.3**).

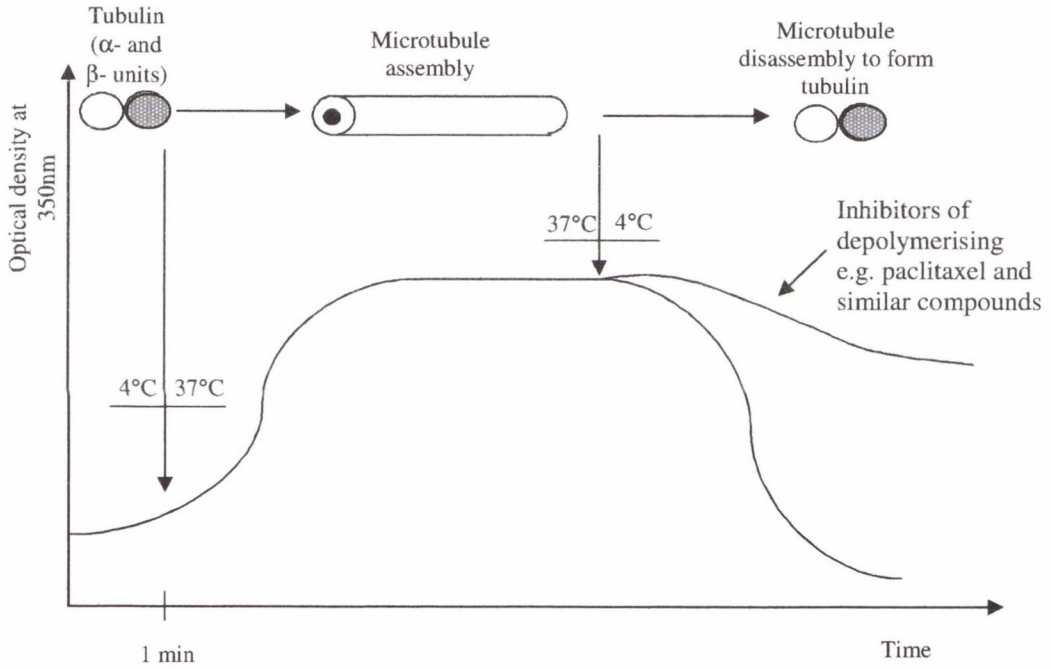


Figure 1.2.3.3 The anti-tubulin assay (Chauvière et al. 1981)

In addition to being a tool for testing the potential anti-cancer activity of novel taxanes isolated or synthesised, the anti-tubulin assay is also being used in order to elucidate the bioactive conformation of taxanes, i.e. the conformation they take when they are bound to microtubules. E.g. in the synthesis of novel C2-C3'N-linked docetaxel analogues Querolle and co-workers found that para-substituted derivatives were unable to stabilize microtubules, whereas the ortho- and meta-substituted compounds showed significant ability to inhibit depolymerisation of microtubules (Querolle et al. 2004).

PART I

2 Introduction

2.1 The Yew Tree (*Taxus*) – A Historical Overview

Several books have appeared in recent years giving the historical importance of yews (Bevan-Jones 2002, Itokawa and Lee 2003, Suffness 1995). The following is a very brief overview: The name *Taxus* comes from Greek *'toxos'*, which is similar to their word for bow (*toxon*) and *toxocon* meaning poison or toxin (Hartzell 1995). Yew has been associated with death since the Western man began to write things down. It was described as "the tree of death" because the ancients were familiar with its poisonous nature, which is already documented in the Latin and Greek literature (Brosse 1989, Riogoni-Stern 1991). Julius Caesar mentioned in his *"Gallic Wars"* that Catulvolcus, the old chief of the Celtic tribe of Eburones, committed suicide by consuming extracts from the yew tree rather than surrendering to the Romans (Cragg et al. 1994).

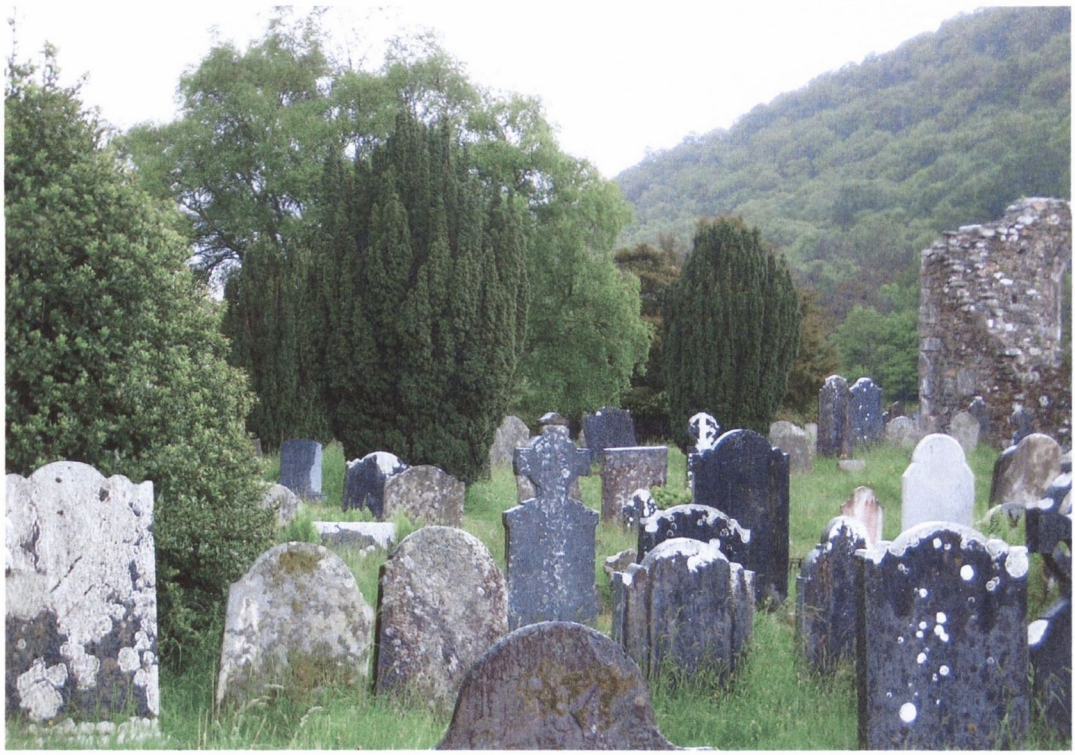


Figure 2.1.1.1 Irish yew, *Taxus baccata* 'Fastigiata', from graveyard on Monastery site of Glendalough, Co. Wicklow, Ireland

The yew tree was also a sacred tree in Celtic tradition, where religious objects, such as the druidic staff, were made of yew. The Irish yew was considered a focal point for

tribal meetings in early Ireland, because it was thought to possess memory and to have the power of witness (Bevan-Jones 2002). Yew was called Ibor in Celtic and this root has remained in the name for yew in many European languages, such as German (eibe), English (yew), French (if) and Spanish (iva) (Appendino 1992). Despite its sacred and magical associations yew wood was also used in weapon making and making of household tools. The yew long-bow was a revolution of its time, and led to the initial success of the English yeomen in the Hundred Years' War. Archaeological findings dating back over 50, 000 years throughout Europe suggest that arms from yew wood were used by the Egyptians, Greeks, Romans and Celts (Hartzell 1995). One very important finding was that of "Ötzi the Iceman", a well-preserved natural mummy of a man from about 3300 BC, found in 1991 in a glacier of the Ötztaler Alps. Amongst the items found with the Iceman were a copper axe with a yew handle and an unfinished yew longbow that was taller than he was (Suffness 1995).

Figure 2.1.1.2 *Taxus baccata* 'Fastigiata' from Glendalough

The extensive use of yew led to its near extinction in Europe, and in Austria and Poland the felling of it was forbidden even before 1600 (Appendino 1992).

2.1.1 Conservation of *Taxus*

The discovery of the anticancer agent paclitaxel (Taxol®) from bark of the Pacific yew tree (*Taxus brevifolia*) has threatened the continued existence of *Taxus* (Wani et al. 1971). As an example, a 100 year old yew tree yielding about 3 kg of bark containing only 300 mg of paclitaxel is needed to produce approximately a single dose needed for cancer chemotherapy (Jaziri and Vanhaelen 2001). Uncontrolled and unregulated harvesting would result in serious overharvesting of wild populations of most yew species worldwide, including the pacific yew (*T. brevifolia*), the native European yew (*T. baccata*) and yew species throughout Asia. The awareness of biodiversity and regulation of bio-prospecting worldwide has led to strict regulation of the harvesting of yew trees. When Bristol-Myers Squibb was given exclusive rights to provide Taxol® from the bark of *T. brevifolia* from federal lands of California, Idaho, Montana, Oregon and Washington (under a cooperative research and development agreement with the US government) in 1991 the public and concerned conservation groups objected, leading to the Pacific Yew Act of 1992. This act required the appropriate Federal agencies (US Department of Agriculture, Forest Service etc) to improve the management of Pacific yew trees on Federal lands in order to ensure continuing supplies of Taxol®, but also to assist in research on the ecology of yew, development of alternative methods of obtaining Taxol®, and the cultivation of yew in commercial settings (Goodman and Walsh 2001).

An alternative to obtaining Taxol® from the bark of harvested Pacific yew trees was found by Bristol-Myers Squibb in 1994, when they started to semi-synthetically produce Taxol® from 10-deacetyl baccatin III obtained from renewable foliage from commercially grown European Yew (*T. baccata*) (Goodman and Walsh 2001). The fact that renewable foliage could replace the usage of bark as a source for paclitaxel stopped the overharvesting of the Pacific yew, but it led to the over-exploitation of Asian yew trees, and particularly the Himalayan yew (*Taxus wallichiana*), thought to be similar to the European yew. Few data are available but it is estimated that 5,500

tonnes of leaves were exported from India in 1994 (Beherens et al. 2000). Wild trees were cut by local people and sold to traders with very little monitoring and few programmes in place to regenerate or cultivate the species. As a result of this over-exploitation *T. wallichiana* in China is considered to be endangered according to the United Nations Environment Programme World Conservation Monitoring Centre (UNEP-WCMC) threat categories, and in northern India the species has been listed as critically endangered (UNEP-WCMC 2005). All native species of *Taxus* in China are listed as "Class I" in the Convention on International Trade in Endangered Species (CITES) in 2001, prohibiting the collection without authorization of the Chinese Government. There are, however, reports of illegal harvests (CITES 2001). The ban on export of *T. wallichiana* from India in 1995, caused the overharvesting of this species in Nepal, despite the fact that Nepal had banned the export of *T. wallichiana* leaves since 1993 (Beherens et al. 2000). Another problem has arisen because it is difficult to identify *Taxus* species, and the plant material being exported from Asia is often misidentified and therefore not compliant with FDA regulation (Beherens et al. 2000). A large number of synonyms for the Himalayan yew exists in Asia (*T. wallichiana*, according to the authority Farjon (1998)): *T. baccata* 'Wallichiana', *T. nucifera*, *T. contorta*, *T. orientalis*, *T. yunnanensis*, *T. wallichiana* 'Yunnanensis' and *T. chinensis* 'Yunnanensis'. Since the ban on export does not include these synonyms, yew trees classified as e.g. *T. yunnanensis* are continuing to be exploited (CITES 2001).

The importance of correct identification is vital for the conservation of *Taxus* species for the continued protection of biodiversity in the world and in order to sustain the continued production of the anticancer agent Taxol® from natural sources. Correct identification is also needed to comply with FDA regulation. Although Bristol-Myers Squibb is currently producing Taxol® using plant cell cultures in cooperation with Phyton Catalytic, the production by other manufacturers largely relies on extraction from plant material, e.g. InB:Paxis Pharmaceuticals, Inc manufactures paclitaxel from *T. canadensis* needles, PAXETOL™ is manufactured by Xechem International, Inc from the bark and needles of *T. brevifolia* and other *Taxus* species and IVAX Corporation manufactures ONXOL™ from *T. brevifolia* or *T. yunnanensis*. The

continuing search for high-yielding *Taxus* species and the correct taxonomic species identification is therefore important from both a commercial and a botanical viewpoint.

2.1.2 Taxonomy of the Family Taxaceae (Linnaeus)

Taxonomy comes from Greek (*taxinomia*) from the words for order (*taxis*) and law (*nomos*). It refers to the classification of all living things and the principles and relationships underlying this classification (Encyclopædia Britannica 2005).

According to Aljos Farjon, the conifer specialist at Kew Royal Botanical Gardens and considered a conifer authority, *Taxus* (Linnaeus) is the type genus of the family Taxaceae, and it is the largest genus in the family (**Figure 2.1.2.4**). *Amentotaxus* (Pilger), *Austrotaxus* (Compton), *Pseudotaxus* (Cheng) and *Torreya* (Arnott) are other members of this family (Farjon 1998). All species are slow growing, small to medium sized trees adapted to an existence in the understorey of coniferous, angiosperm or mixed forest (Farjon 1998). Since the discovery of Taxol® (*syn.* paclitaxel) most members of the Taxaceae have been evaluated for taxane like compounds.

Members of the Taxaceae are distributed predominately in the Northern Hemisphere, crossing the equator to Malaysia and as far as New Caledonia. The yews are amongst the oldest living trees having originated 200 million years ago during the Jurassic period. One can suggest that their Northern Hemisphere distribution resulted from their location on the Laurasian land mass formed from the break up of the single Permian land Pangaea.

Austrotaxus

Austrotaxus is monospecific and it is endemic to the central and northern parts of New Caledonia. It is growing in the understorey of tropical montane rainforest (Farjon 1998).

Amentotaxus

Amentotaxus consist of five species and one variety, growing in China, Taiwan, North-east India and Vietnam. They occur as understorey trees in montane deciduous or

evergreen forest (Farjon 1998). No taxane like compounds have been reported from this family (Croom 1995).

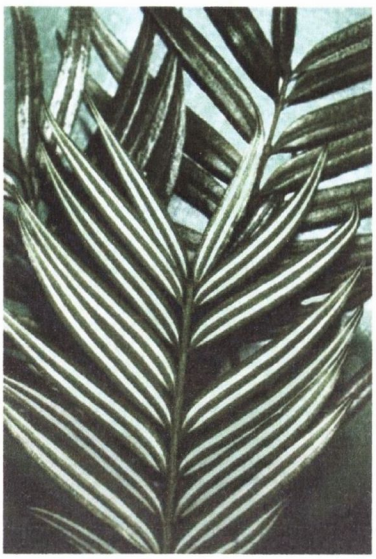


Figure 2.1.2.1 *Amentotaxus* foliage (Walker 2004)

Pseudotaxus

Pseudotaxus is also a monospecific genus endemic to the Zhejiang province in eastern China (Cope 1998). It is a rare genus and grows as an understorey shrub in cool, moist temperate montane forest dominated by evergreen or deciduous angiosperm trees (Farjon 1998).

Figure 2.1.2.2 *Pseudotaxus cheinii* foliage (Walker 2004)

Torreya

Torreya consist of five species and three varieties, distributed in North America (California (*T. californica*) and North-western Florida (*T. taxifolia*)), China (*T. jackii*, *T. grandis*) and Japan (*T. nucifera*) (Cope 1998, Farjon 1998). The genus occurs as rare and scattered understorey trees in mixed conifer and conifer-angiosperm forest from lowland to montane sites. It grows in moist, warm and cool climate (Farjon 1998).

Figure 2.1.2.3 *Torreya nucifera* foliage (Atha et al. 2005)

Taxus

"I am uncertain as to its species, but think that it is identical with the Taxus baccata of Europe" –H.B. Croom 1834 (Croom 1995). Hardy Bryan Croom's taxonomic dilemma of whether his original discovery of *Taxus* in Florida was a subspecies of the European *T. baccata* or if it deserved to be designated as a separate species illustrates the continuing debate by modern authorities on the number of species in the genus *Taxus*. According to the most recent publication on the matter by Farjon, the genus *Taxus* consists of 10 species and two hybrids, and Cope lists 190 cultivars (Cope 1998, Farjon 1998). Spjut, however, included 24 species and 55 varieties in his publication of a taxonomic key for *Taxus* (Spjut 2003). The species of *Taxus* is distributed mainly in the Northern Hemisphere, and occurs in distinct geographical regions of the world, with four species in North America (*T. brevifolia*, *T. canadensis*, *T. floridana* and *T. globosa*), one in Europe (*T. baccata*) and the remaining in Southeast Asia (*T. chinensis*, *T. fuana*, *T. cuspidata*, *T. wallichiana*, *T. sumatrana*).

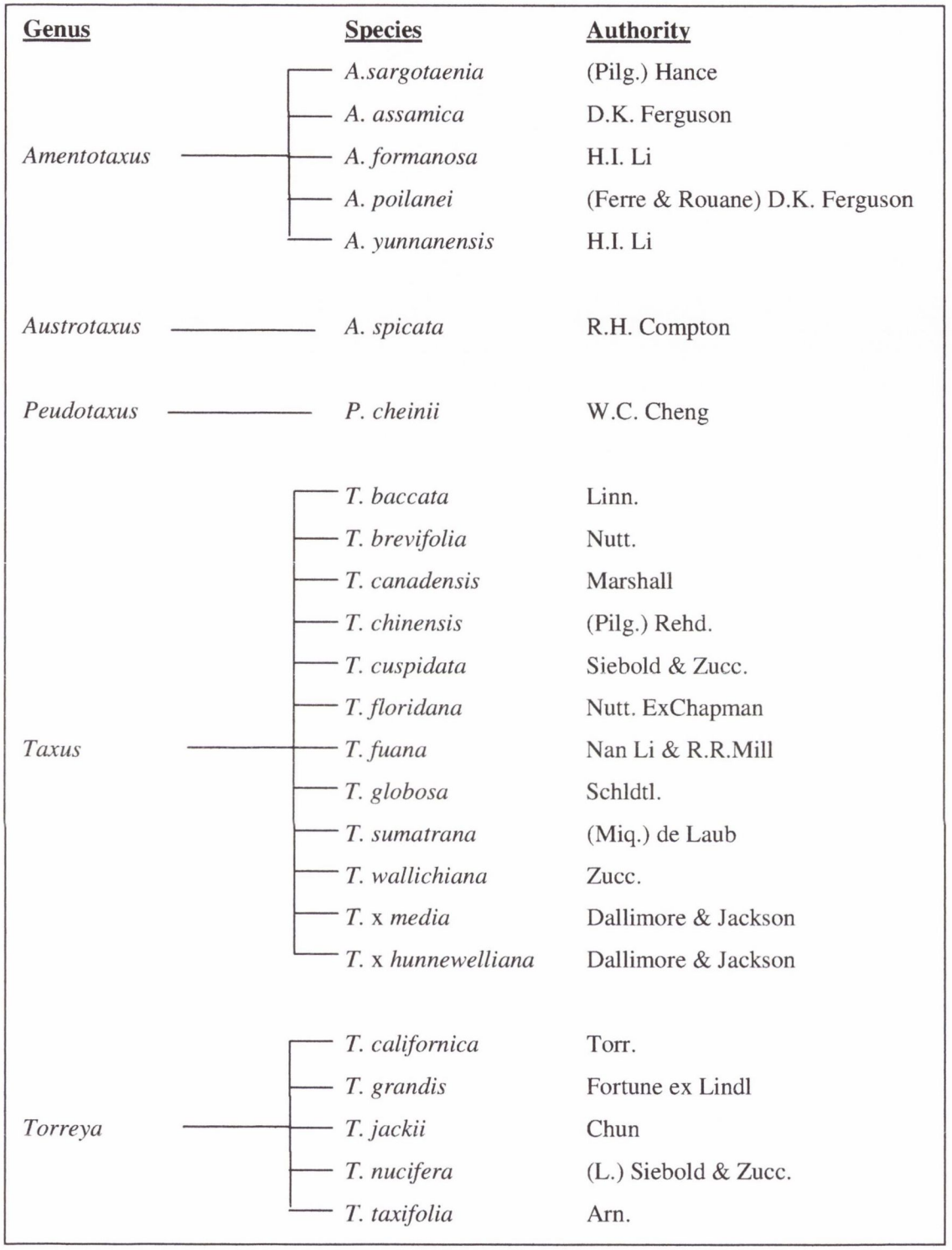


Figure 2.1.2.4 The family Taxaceae according to Farjon (Farjon 1998)

Morphologically, all species of *Taxus* are very similar and intraspecific variation is often more apparent than differences between species. The genus has been considered to consist of a number of geographical varieties of a single species (Elwes and Henry 1906), descended from *Paleotaxus rediviva*- a fossil angiosperm common in the

Triassic period (Hartzell 1995). This theory has been challenged by many (Chadwick and Keen 1976, Cope 1998, Dallimore and Jackson 1924, Farjon 1998, Strobel and Hess 1996, Vidakovic 1991). Their work was based mainly on macroscopical characteristics such as dimensions and shapes of needles and seeds and actual characteristics of the trees themselves. Another the classification relied on microscopical characteristics, such as stomatal rows and shape of epidermal cells.

2.1.3 Chemotaxonomy of the Genus *Taxus*

Chemotaxonomy is a classification system based on similarities in the structure of certain compounds among the organisms being classified (Encyclopædia Britannica 2005).

The chemical constituents of *Taxus* species have been extensively studied and important review papers have been published in recent years as a result of the occurrence of the antitumour constituents, such as paclitaxel, but also because of other biologically active constituents (Appendino 1995, Das and Anjani 1998, Olsen et al. 1998, Parmar et al. 1999). This search for novel constituents in *Taxus* species is still ongoing, with 11 new compounds having been isolated and characterised from various *Taxus* species so far in 2005 (Li et al. 2005, Shen et al. 2005a, Shen et al. 2005b, Xia et al. 2005). The cyclic diterpenoids, taxanes, are found in most yews, but the content is very variable. The most commercially important taxane is paclitaxel, but other related taxanes such as 10-DAB III, baccatin III and cephalomannine (see **Figure 1.2.2.1**, section 1.2.2) are also commercially important. An extensive chemotaxonomic review of the genus *Taxus* linking taxanes and morphological characters was published in 1999 (van Rozendaal et al. 1999). By principal component analysis the genus was divided into three groups based on chemical constituents: a North-American group (*T. canadensis*, *T. floridana* and *T. globosa*), a Eurasaian group (*T. baccata*, *T. celebica*, *T. cuspidata*, *T.* x *hunnewelliana* and *T.* x *media*) and a monospecific group containing only *T. brevifolia*. When these three groups were linked with groupings resulting from classical morphological analyses no correlation was found.

Brevifoliol was thought to be a species-specific taxane to *T. brevifolia* when it was first isolated (Balza et al. 1991). However it was later isolated from the needles of *T. wallichiana* (Georg et al. 1993), the needles and seeds of *T. baccata* (Appendino 1995, Guo et al. 1995) and the needles of *T.* x *media* (Rao et al. 1996). Although brevifoliol is present in other *Taxus* species, it is still present in a higher concentration in *T. brevifolia* making it a "marker compound" for this species.

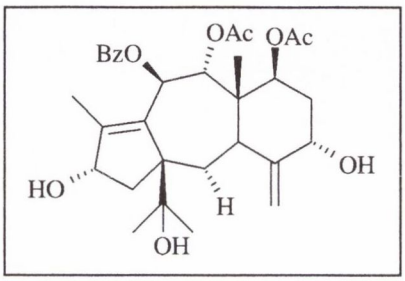


Figure 2.1.3.1 Molecular formula of brevifoliol

Another species-specific taxane is 9-dihydro-13-acetyl baccatin III, first isolated in 1992 from the needles of *T. canadensis* (Gunawardana et al. 1992, Zamir et al. 1992). This taxane is the major taxane in *T. canadensis* needles, often found at 5-7 times the concentration of paclitaxel (Shi et al. 2002).

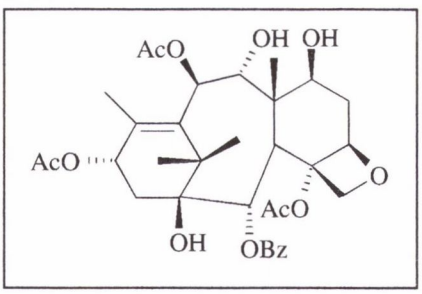


Figure 2.1.3.2 Molecular formula of 9-dihydro-13-acetyl baccatin III

Another important group of compounds isolated from *Taxus* is the taxines (**Figure 2.1.3.3**). These are the main alkaloids in *Taxus*, they are present in all parts of the tree except the arils and the toxicity of the yew is ascribed to taxines. It has been reported that taxines are relatively abundant in *T. baccata* and *T. cuspidata*, yet only minimal amounts are found in *T. brevifolia* (Suffness 1995). Taxine B has also been used as a starting material in the semi-synthesis of taxane derivatives, such as 7-deoxobaccatin III (Payré et al. 2000).

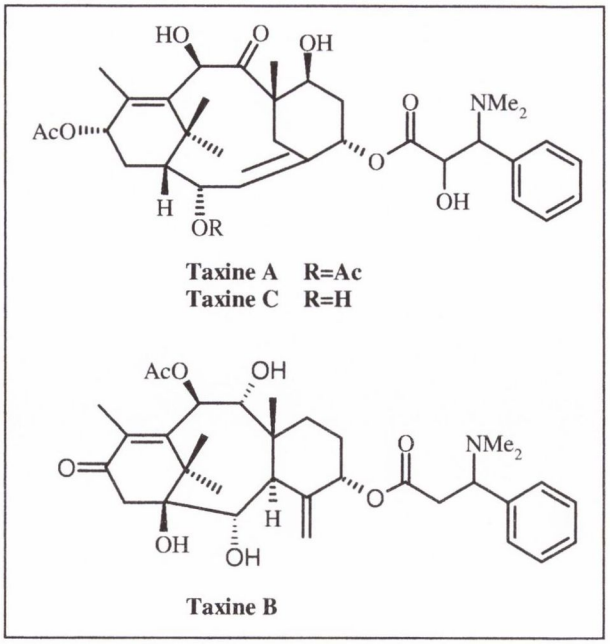


Figure 2.1.3.3 Molecular formula of taxine A, B and C

In addition to the taxanes a wide variety of chemical constituents, such as non-taxane diterpenes, isoprenoids, lignans, flavonoids, glycosides, phytosteroids and simple phenolic compounds have been isolated from *Taxus* species (Parmar et al. 1999). No attempt has been made to examine the chemotaxonomic value of these constituents.

2.1.4 Phylogenetics as a Tool in Plant Classification

Phylogenetics (Greek: *phylon* meaning race and *genetic* meaning birth) is the taxonomic classification of organisms based on how closely they are related in terms of evolutionary differences. Phylogeny (or phylogenesis) is the origin and evolution of a set of organisms, usually of a species. A major task of systematics is to determine the ancestral relationships among both living and extinct known species (Encyclopædia Britannica 2005).

Studies have been conducted in order to establish the phylogenetic relationship among the various genera in the Taxaceae (Cheng et al. 2000, Price and Quinn 2003). Cheng et al (2000) analysed maturase K (*mat*K) genes from the chloroplast and the results strongly indicated that the five taxad genera (*Amentotaxus*, *Austrotaxus*, *Taxus*, *Pseudotaxus* and *Torreya*) together with *Cephalotaxus* share a common ancestor, although strong evidence suggests that *Cephalotaxus* should remain as a separate

family. Chen et al (2000) also analysed the internal transcribed spacer (ITS = a sequence of RNA in a primary transcript that lies between precursor ribosomal subunits) and the results supported the suggestion that *Cephalotaxus* should remain separate to Taxaceae. The results also suggested that *Amentotaxus* and *Austrotaxus* should be excluded from Taxaceae. In contrast Price and Quinn (2003) analysed *mat*K genes from the genera in Taxaceae and *Cephalotaxus* and from their results suggested that *Cephalotaxus* should be included in the Taxaceae family.

The degree of genotypic distinctiveness that might exist among various *Taxus* species has been studied (Collins et al. 2003, Li et al. 2001, Zhou et al. 1998) , but this attempt at clarifying the *Taxus* classification remains largely unresolved. When 12 different populations of four *Taxus* species (*T. cuspidata*, *T. yunnanensis*, *T. chinensis* and *T. chinensis* 'Mairei') were studied by allozyme analysis (*Def*: allozymes represent different alleles of the same gene), the authors proposed that the four species should be treated as geographical variations of the same species (Zhou et al. 1998). Collins et al (2003) DNA-fingerprinted 19 samples from three species (*T. baccata*, *T. canadensis*, *T. cuspidata*) and 31 putative hybrids (*T.* x *hunnewelliana* (*T. cuspidata* x *T. canadensis*), *T.* x *media* (*T. baccata* x *T. cuspidata*)) using RAPDs (random amplification of polymorphic DNA). They were also characterised for their respective chloroplast genotype using restriction digestions of polymerase chain reaction- (PCR) amplified *trn*L-F fragments. Analysis of the results using these methods suggested that the three species were clearly separate species, and the relationship between the hybrids and the parent plants could be determined. Using sequences of the nuclear ribosomal DNA ITS region Li et al found a close relationship between *T. brevifolia*, *T. floridana* and *T. globosa* (Li et al. 2001). They also found that *T. canadensis* was more closely related to the Asian *Taxus* species, such as *T. chinensis*, *T. mairei* and *T. cuspidata*.

Although some results have been presented regarding the molecular relationship and genotypic distinctiveness between the various *Taxus* species, no comprehensive study has yet analysed the whole genus, and the dilemma of species delimitation for the genus *Taxus* still persists.

3 Materials & Methods

3.1 Plant Material

3.1.1 Needles

Twigs (ca. 20 cm long) of 57 *Taxus* species and varieties were collected in July and September 2001, and in May, June and July 2002. The same sampling protocol devised and validated in previous studies was used (Hook et al. 1999). Twigs from trees were taken from four sides and two height levels (ca. 1m and 3 m). The eight twigs were then pooled and dried intact in a Memmert™ fan assisted oven at <30°C for approximately 72 hours until completely dry. Once dried the material was separated into woody stems and needles. Samples were stored whole in sealed plastic bags at room temperature in the absence of light. To determine morphological differences in needles taken from the eight locations of the tree, the twigs were kept separate. For the morphological analysis using the WinSEEDLE™ package the needles were measured fresh (see section 3.2.1), so for this analysis the fresh plant material were stored at -20°C until time of analysis.

The twigs were collected from "authenticated" *Taxus* species and cultivars growing at four different Irish locations:

- National Botanical Gardens, Glasnevin, Co. Dublin.
- Powerscourt Estate and Gardens, Co. Wicklow.
- Mount Usher Gardens, Ashford, Co. Wicklow.
- The J. F. Kennedy Park Arboretum, New Ross, Co. Wexford.

Taxus species and cultivars were also supplied from:

- The Royal Botanical Gardens Edinburgh, Edinburgh, Scotland.
- Niedersächsische Forstliche Versuchsanstalt, Staufenberg, Germany.
- Polska Akademia Nauk, Kórnik, Poland.
- Natural Resources Canada, Fredericton, New Brunswick, Canada.
- Herstmonceaux Church, East Sussex, U.K.
- Bedgebury National Pinetum, Kent, U.K.

Table 3.1.1.1 *Taxus* species and cultivars collected for needle analysis

Genus	Species	Cultivar	Origin	Date
Taxus	*baccata*		New Ross, Ireland	10/05/02
Taxus	*baccata*		Mount Usher, Ireland	24/04/02
Taxus	*baccata*		Mount Usher, Ireland	24/04/02
Taxus	*baccata*		Powerscourt, Ireland	10/05/02
Taxus	*baccata*		Powerscourt, Ireland	10/05/02
Taxus	*baccata*		Herstmonceaux, England	21/07/02
Taxus	*baccata*	'Adpressa'	Edinburgh, Scotland	29/09/01
Taxus	*baccata*	'Adpressa'	Powerscourt, Ireland	10/05/02
Taxus	*baccata*	'Adpressa'	Krònik, Poland	15/07/01
Taxus	*baccata*	'Adpressa Aurea'	Edinburgh, Scotland	29/09/01
Taxus	*baccata*	'Aurea'	Krònik, Poland	15/07/01
Taxus	*baccata*	'Aurea'	Powerscourt, Ireland	10/05/02
Taxus	*baccata*	'Aureahoseri'	Krònik, Poland	15/07/01
Taxus	*baccata*	'Densifolia'	Glasnevin, Ireland	01/05/02
Taxus	*baccata*	'Dovastonia'	Powerscourt, Ireland	10/05/02
Taxus	*baccata*	'Dovastonia'	Mount Usher, Ireland	24/04/02
Taxus	*baccata*	'Dovastonia Aurea'	New Ross, Ireland	10/05/02
Taxus	*baccata*	'Erecta'	Krònik, Poland	15/07/01
Taxus	*baccata*	'Fastigiata'	New Ross, Ireland	17/12/04
Taxus	*baccata*	'Fastigiata'	New Ross, Ireland	17/12/04
Taxus	*baccata*	'Fastigiata'	New Ross, Ireland	17/12/04
Taxus	*baccata*	'Fastigiata'	Krònik, Poland	15/07/01
Taxus	*baccata*	'Green Mountain'	Krònik, Poland	15/07/01
Taxus	*baccata*	'Imperialis'	Krònik, Poland	15/07/01
Taxus	*baccata*	'Jacksonii'	Edinburgh, Scotland	29/09/01
Taxus	*baccata*	'Lutea'	Powerscourt, Ireland	10/05/02
Taxus	*baccata*	'Lutea'	Mount Usher, Ireland	24/04/02
Taxus	*baccata*	'Neidpathensis'	Glasnevin, Ireland	01/05/02
Taxus	*baccata*	'Pyramidalis'	Glasnevin, Ireland	01/05/02
Taxus	*baccata*	'Repandens Aurea'	Edinburgh, Scotland	29/09/01
Taxus	*baccata*	'Semperaurea'	Krònik, Poland	15/07/01
Taxus	*baccata*	'Standishii'	Edinburgh, Scotland	29/09/01
Taxus	*baccata*	'Standishii'	Krònik, Poland	15/07/01
Taxus	*baccata*	'Washingtonii'	Edinburgh, Scotland	29/09/01
Taxus	*brevifolia*		New Ross, Ireland	10/05/02
Taxus	*brevifolia*		New Ross, Ireland	17/12/04
Taxus	*brevifolia*		New Ross, Ireland	17/12/04
Taxus	*brevifolia*		Glasnevin, Ireland	01/05/02
Taxus	*brevifolia*		Krònik, Poland	15/07/01
Taxus	*canadensis*		New Ross, Ireland	10/05/02
Taxus	*canadensis*		New Ross, Ireland	17/12/04
Taxus	*canadensis*		Glasnevin, Ireland	01/05/02
Taxus	*canadensis*		Edinburgh, Scotland	29/09/01
Taxus	*canadensis*		New Brunswick, Canada	07/05/02
Taxus	*canadensis*		Kent, U.K.	July 1997

Table 3.1.1.1-continued *Taxus* species and cultivars collected for needle analysis.

Genus	Species	Cultivar	Origin	Date
Taxus	*canadensis*	'Aurea' (1)	Glasnevin, Ireland	01/05/02
Taxus	*canadensis*	'Aurea' (2)	Glasnevin, Ireland	01/05/02
Taxus	*chinensis*		Edinburgh, Scotland	29/09/01
Taxus	*cuspidata*		New Ross, Ireland	10/05/02
Taxus	*cuspidata*		New Ross, Ireland	17/12/04
Taxus	*cuspidata*		New Ross, Ireland	17/12/04
Taxus	*cuspidata*		Glasnevin, Ireland	01/05/02
Taxus	*cuspidata*		Krònik, Poland	15/07/01
Taxus	*cuspidata*	'Luteo *Baccata*'	Glasnevin, Ireland	01/05/02
Taxus	*cuspidata*	'Nana'	Krònik, Poland	15/07/01
Taxus	*cuspidata*	'Thayerae'	Glasnevin, Ireland	01/05/02
Taxus	*floridana*		New Ross, Ireland	10/05/02
Taxus	*sumatrana*		New Ross, Ireland	10/05/02
Taxus	*wallichiana*		Edinburgh, Scotland	29/09/01
Taxus	X *media*		New Ross, Ireland	10/05/02
Taxus	X *media*		Krònik, Poland	15/07/01
Taxus	X *media*	'Hicksii'	Glasnevin, Ireland	01/05/02
Taxus	X *media*	'Hicksii'	Krònik, Poland	15/07/01
Taxus	X *media*	'Rozon'	Krònik, Poland	15/07/01
Taxus	X *media*	'Stricta'	Krònik, Poland	15/07/01
Taxus	X *media*	'Wojtek'	Krònik, Poland	15/07/01

3.1.2 Seeds

Seeds were collected over a period of time. Some were purchased from international seed merchants:

- Sandeman Seeds, West Sussex, U.K.
- F.W. Schumacher Co. Inc, Massachusetts, U.S.A.
- Eichenberg & Co., Miltenberg/Main, Germany.

Seeds were also supplied/collected from:

- The J. F. Kennedy Arboretum, New Ross, Co. Wexford, Ireland.
- The National Botanical Gardens, Glasnevin, Ireland.
- Powerscourt Estate and Gardens, Co. Wicklow, Ireland.
- Polska Akademia Nauk, Kórnik, Poland.

For the seeds collected locally, the preparation, i.e. removal of the arils of the "berries", was carried out using the method suggested by Rudolf (Rudolf 1974). The

seeds were extracted by macerating the fleshy berries in warm water and floating off the pulp and seed. The seeds were then dried for about 5-7 days in a Memmert™ fan assisted oven at <30°C until dryness (± 0.05g).

Table 3.1.2.1 *Taxus* species and cultivars collected and provided for seed analysis

Genus	Species	Cultivar	Origin	Year
Taxus	*baccata*		F.W. Schumacher Co.	1996
Taxus	*baccata*		Eichenberg & Co	1996
Taxus	*baccata*		Sandeman Seeds	2001
Taxus	*baccata*		New Ross, Ireland	1995
Taxus	*baccata*		Powerscourt, Ireland	2002
Taxus	*baccata*		Krònik, Poland	2002
Taxus	*baccata*	'Adpressa'	Powerscourt, Ireland	2002
Taxus	*baccata*	'Aurea'	Krònik, Poland	2002
Taxus	*baccata*	'Aureahoseri'	Krònik, Poland	2002
Taxus	*baccata*	'Dovastonia'	New Ross, Ireland	1995
Taxus	*baccata*	'Erecta'	New Ross, Ireland	1995
Taxus	*baccata*	'Fastigiata'	Sandeman Seeds	1996
Taxus	*baccata*	'Fastigiata'	Eichenberg & Co	1995
Taxus	*baccata*	'Fastigiata'	Mount Annville, Irish	1995
Taxus	*baccata*	'Fastigiata'	New Ross, Ireland	1995
Taxus	*baccata*	'Fastigiata'	Powerscourt, Ireland	2002
Taxus	*baccata*	'Fructo Lutea'	Powerscourt, Ireland	2002
Taxus	*baccata*	'Lutea'	New Ross, Ireland	1996
Taxus	*baccata*	'Standishii'	Krònik, Poland	2002
Taxus	*baccata*	'Pyramidalis'	F.W. Schumacher Co.	1993
Taxus	*brevifolia*		Sandeman Seeds	1995
Taxus	*brevifolia*		New Ross, Ireland	1995
Taxus	*brevifolia*		Glasnevin, Ireland	2002
Taxus	*canadensis*		New Brunxwick, Canada	2002
Taxus	*canadensis*		Glasnevin, Ireland	2002
Taxus	*canadensis*	'Aurea'	Glasnevin, Ireland	2002
Taxus	*chinensis*		F.W. Schumacher Co.	1993
Taxus	*chinensis*		Sandeman Seeds	1995
Taxus	*chinensis*	'Mairei'	Sandeman Seeds	2001
Taxus	*chinensis*	'Mairei'	Sandeman Seeds	1995
Taxus	*cuspidata*		Sandeman Seeds	1995
Taxus	*cuspidata*		Krònik, Poland	2002
Taxus	*cuspidata*	'Capita'	F.W. Schumacher Co.	1993
Taxus	*cuspidata*	'Thayerae'	Glasnevin, Ireland	2002
Taxus	X *media*		Sandeman Seeds	2001
Taxus	X *media*	'Hicksii'	New Ross, Ireland	1995
Taxus	X *media*	'Hicksii'	Krònik, Poland	2002
Taxus	X *media*	'Hicksii'	Glasnevin, Ireland	2002
Taxus	X *media*	'Rozon'	Krònik, Poland	2002

3.2 Morphological Examination

3.2.1 Macroscopical Features

Needle dimensions (area, length and width) were measured for all the different *Taxus* samples (see **Table 3.1.1.1**). Seed dimensions (area, length, width and width/length ratio) were measured for some of the *Taxus* samples (see **Table 3.1.1.2**).

The needles (n=100) were collected sequentially from the left side of the twigs of the fresh plant material. Needles were taken from the bottom of the twig through to the top of the twig, in order to get a representative range of the needle dimensions. In the case of the Polish and the Canadian samples the needles were collected from pressed and dried samples.

The relatively flat needles were placed without overlap onto an optical scanner bed (ScanMaker 4700, Microtek™) and area, length, width were determined using the image analysis software WinSEEDLE™ (Regent Instruments, Inc., Quebec, Canada; http://www.regent.qc.ca/). To digitize needles or seeds, WinSEEDLE™ uses an optical scanner instead of a camera. Scanners produce high resolution images free of illumination problems. It automatically detects and analyzes needles much more precisely than conventional area meters (conveyor or camera-based type). The following parameters are obtained using WinSEEDLE™:

- Total projected area, individual and average projected area
- Individual and average straight length, individual and average curved length
- Individual and average maximum width
- Individual and average curvature
- Individual and average surface area

Additional features are:

- Measurement of straight or curved needles/seeds
- Automatic filtering of defects and debris
- Interactive image edition and the data is saved in ASCII format for easy transfer to data analysis or data visualization software
- Histogram of needle/seed length, width or area displayed on screen during measurement

The same procedure was applied to the analysis of the seeds. In addition to determining area, length and width, the width/length ratio was also measured.

The samples analysed previously (Dempsey and Hook 2000) from Bedgebury National Pinetum, U.K., were pressed and dried before analysis. The samples were then measured manually using a Vernier calipers. The needle area was assumed to be rectangular and therefore calculated by multiplying length by width (Dempsey 2000).

3.2.2 Microscopical Examination of *Taxus canadensis*

Microscopical analysis was carried out for all the seven different *T. canadensis* samples. Ten randomly chosen dried needles from each sample were analysed. Prior to analysis the needles were cleared by boiling in Chloral Hydrate solution BP. The microscopical determinations were made using an Olympus™ BH light microscope attached to a video recorder and a visual display unit (VDU).

The stomata in *Taxus* needles are covered or surrounded by a subsidiary cell arrangement called the Florin's ring, so for the purpose of this experiment the Florin's rings were counted as the stomatal pore. The stomata were counted in an area of $1mm^2$. The rows of stomata along the main vein were also counted. The stomata were counted on two fields of view on either side of the main vein of 10 needles per sample.

3.3 General Analytical Procedures

3.3.1 Chemicals and solvents

Solvents used for High Performance Liquid Chromatography (HPLC) and phytochemical extraction and isolation were Analar grade; all other solvents were laboratory grade.

Solvents used for Nuclear Magnetic Resonance (NMR) analysis were:

Dimethylsulphoxide-d_6, 99.8 atom % (Sigma-Aldrich Chemical Co., UK)

Chloroform-d, 99.9 atom %, 0.01% TMS (Sigma-Aldrich Chemical Co., UK)

Reference compounds used for comparative Thin Layer Chromatography (TLC), and High Performance Liquid Chromatography (HPLC) were:

Paclitaxel	(> 95% purity, Sigma-Aldrich Chemical Co., UK)
10-deacetylbaccatin III	(> 95% purity, Sigma-Aldrich Chemical Co., UK)
9-dihydro-13-acetylbaccatin III	(> 95% purity, Sigma-Aldrich Chemical Co., UK)
Baccatin III	(> 95% purity, Sigma-Aldrich Chemical Co., UK)
Cephalomannine	(National Cancer Institute, USA)
Catechin	(> 95% purity, Sigma-Aldrich Chemical Co., UK)
Epi-catechin	(> 95% purity, Sigma-Aldrich Chemical Co., UK)
Quercetin	(>95% purity, Sigma-Aldrich Chemical Co., UK)

Brevifoliol was kindly provided by Dr. Teris A. van Beek, Dept. of Organic Chemistry, Phytochemical section, Wageningen Agricultural University, The Netherlands (The brevifoliol was isolated in his laboratory).

Other chemicals used were:

Anhydrous sodium sulphate (Na_2SO_4)	(Scharlau Chemie S.A., Spain)
Vanillin	(AnalR, BDH Chemicals Co., UK)
Hydrochloric acid (HCl)	(Reidel-de Haën®, Germany)
Acetic acid	(Reidel-de Haën®, Germany)
Sulphuric acid (H_2SO_4)	(Reidel-de Haën®, Germany)
Formic acid	(Merck KGaA, Darmstadt, Germany)

3.3.2 Extraction procedure

The dried needles were powdered immediately prior to extraction using a Krups® Coffee grinder. The powdered material (3g) was extracted by refluxing with 50ml methanol (MeOH) for two and a half hours. The crude extract was then cooled down, filtered, and evaporated to dryness *in vacuo* using a rotary evaporator. The resulting residue was partitioned between 20ml of ethyl acetate (EtOAc) and 20ml of distilled water. The aqueous phase was separated and washed 2-3 times with ethyl acetate. The ethyl acetate fractions were combined, dried over anhydrous sodium sulphate (Na_2SO_4) and finally evaporated to dryness *in vacuo*, yielding a green/brown residue. Two replications of each needle sample was further analysed.

3.3.3 Analysis by thin layer chromatography (TLC)

Thin layer chromatography was carried out on plastic-backed or aluminium-backed pre-coated silica gel 60 F_{254} plates (Merck KGaA, Darmstadt). Samples were re-dissolved in appropriate solvent, applied as spots (10µl) and run against appropriate standards. The plates were developed over 10 cm, unless otherwise stated. Solvent systems used were (Stahl 1969, Wagner and Bladt 1996):

1. Chloroform-methanol ($CHCl_3$ – MeOH) (10:1)
2. Chloroform-methanol ($CHCl_3$ – MeOH) (30:2)
3. Chloroform-methanol-water ($CHCl_3$ – MeOH – H_2O) (75:23:2)

Taxus species were analysed by TLC (mobile phase 1)) for the identification of the following taxane compounds:

paclitaxel	$R_f = 0.61 \pm 0.05$
cephalomannine	$R_f = 0.59 \pm 0.04$
baccatin III	$R_f = 0.46 \pm 0.06$
10-deacetylbaccatin III	$R_f = 0.10 \pm 0.03$

Taxus species were analysed by TLC (mobile phase 2)) for the identification of the following non-taxane phenolic compounds:

quercetin	$R_f = 0.60 \pm 0.08$
epicatechin	$R_f = 0.40 \pm 0.09$
catechin	$R_f = 0.39 \pm 0.08$

Visualisation was achieved using UV_{254} or spraying with 1% vanillin-sulphuric acid solution followed by heating at 110°C for 10 minutes.

3.3.4 Analysis by high performance liquid chromatography (HPLC)

For the quantification of taxanes analysis by high performance liquid chromatography (HPLC) was employed. The residue was re-dissolved in 2.5 ml of HPLC grade methanol and filtered immediately prior to analysis.

3.3.4.1 Instrumentation

Analysis by HPLC was carried out using a Waters™ Multisolvent Delivery System consisting of a 600 Gradient Controller, a 2487 Dual λ Absorbance Detector, and a 746 Data Module Integrator.

3.3.4.2 Columns and chromatographic conditions

The analytical column used for the identification of taxanes from the cell and the medium was the taxane specific Phenomenex Curosil® pentafluorophenyl (PFP) (5μm, 250 x 4.6mm) column with the appropriate Phenomenex Curosil® PFP (5μm, 3.9 x 20mm) guard column. The mobile phase used was a gradient of acetonitrile-water (from 25:75 to 80:20 over a period of 45 min) as illustrated in **Table 3.3.4.1**. The injection volume was 7.5 μl for both standards and samples, and the flow rate was 1ml/min. Detection was performed at 228nm. All analyses were carried out at ambient temperature and each extract was analysed in duplicate.

Table 3.3.4.1 Gradient table for taxane analysis by HPLC

Time	Flow (ml/min)	Solvent A (%)	Solvent B (%)	Curve
Initial	1	25	75	
45	1	80	20	Linearly increasing
50	1	25	75	Linearly decreasing
55	1	25	75	

3.3.4.3 Validation of the HPLC method

The HPLC method used for quantification of taxanes was developed according to the manufacturer's guidelines (Phenomenex, UK), with slight modifications in order to obtain a suitable resolution of the principal analytes.

The validation of the HPLC method used for the analysis taxanes from *Taxus* needles was carried out in accordance with the specifications outlined in the United States Pharmacopoeia <1225> (USP26 2003), the ICH guidelines (ICH 1996), and guidelines given by the United States Food and Drug Administration (FDA 2001). According to these guidelines, the recommended validation parameters for an assay-

type analytical procedure that measures the amount of the principal analytes present in a given sample are:

1. Specificity/Selectivity
2. Precision
3. Linearity
4. Accuracy
5. Range

1. Specificity/Selectivity

Selectivity or specificity, sometimes there is some confusion over which term that should be used in characterising a method. Vessman (Vessman 1996) pointed out the differences between the two terms. Selectivity refers to a method that gives responses for a number of substances and can distinguish the analyte responses from all other responses. Specificity refers to a method that gives response for only one single analyte. In chromatography with UV-detectors it is usual that a method responds only to one analyte and therefore the term selectivity is more appropriate.

Optimum resolution is essential for the separation of both the taxanes and the flavonoids from other compounds in the extracts being analysed. The ability of the selected chromatographic methods to resolve the taxanes analysed from other structurally related compounds can be measured by determining the resolution factor, R, which, according to the USP 26, should be >1. The resolution factor, R, is given by the following equation:

$$R = \frac{2(t_2-t_1)}{W_1+W_2}$$

Where t_1 and t_2 are the retention times of the two components and W_1 and W_2 are the corresponding widths at the base of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline. In the chromatographic method developed each of the taxanes analysed were separated with resolution values >5, so the system was considered to exhibit adequate resolution. The sample was also spiked with reference standards of the taxanes in question in order to make sure the signals analysed where in fact the signals of the analytes. According to the USP 26 adequate

resolution together with comparison with a reference compound are parts of the determination of the specificity of a method. The method was therefore found to be selective for the purpose of the present analysis.

2. Precision

The precision of an analytical method is the closeness of a series of individual measurements of an analyte when the analytical procedure is applied repeatedly to multiple aliquots of a single homogenous volume of biological matrix (FDA 2001). The precision of a method that is preformed within a single laboratory is evaluated by measuring the repeatability of that method. Repeatability is obtained when one analyst using the same equipment at the same day performs the analysis in one laboratory. The precision is calculated as coefficient of variation (C.V.), i.e., relative standard deviation (RSD). According to FDA, repeatability should be tested by the analysis of a minimum of five determinations at three different concentrations (low, medium and high) in the range of expected concentrations (FDA 2001). However, according to the ICH (ICH 1996) repeatability could be measured by the analysis of three determinations at three different concentrations or through six determinations at 100% of the test concentration. The RSD should not exceed 2% for the method developed. The procedure of the ICH was followed, and a calculated average of no more than 1.3% indicated that the system was precise.

3. Linearity

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range (USP26 2003). Linearity should be established across the range of the analytical procedure, and is demonstrated by injecting a series of dilutions of the standard and plotting the recorded response versus the concentration. The degree of linearity is evaluated by analysing the regression line for fit to the following equation:

$$\mathbf{y_1 = ax_1 + b}$$

Where $\mathbf{y_1}$ is the measured response, **a** is the slope of the line, $\mathbf{x_1}$ is the concentration and **b** is the intercept. The range of concentrations should span 80-120% of the

expected test concentrations (ICH 1996). The concentrations used for the taxane quantification were: 0.055mg/ml, 0.11mg/ml, 0.22mg/ml, 0.33mg/ml, and 0.66mg/ml. The correlation coefficient of r=0.999 indicated that the method was linear (see **Figure 3.3.4.1**).

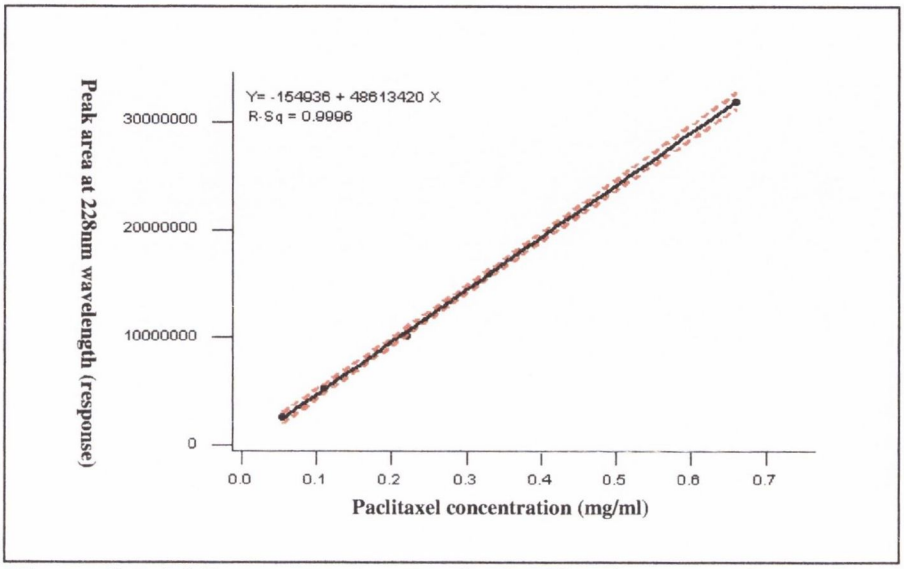


Figure 3.3.4.1 Representative standard calibration curve for paclitaxel

According to Bidlingmeyer (Bildlingmeyer 1993), a good linear correlation coefficient alone does not necessarily indicate a linear standard curve, because the standards in the lowest range can deviate from the linearity although r is high. Instead the linear coefficient should be accompanied with a graph were the response/concentration is plotted versus the logarithmic sample concentrations and the deviation in the y-axis should not exceed 5%. This was done (see **Figure 3.3.4.2**) and the highest deviation in the y-axis was 3.64%. So the results of both of these tests indicate that the method was linear.

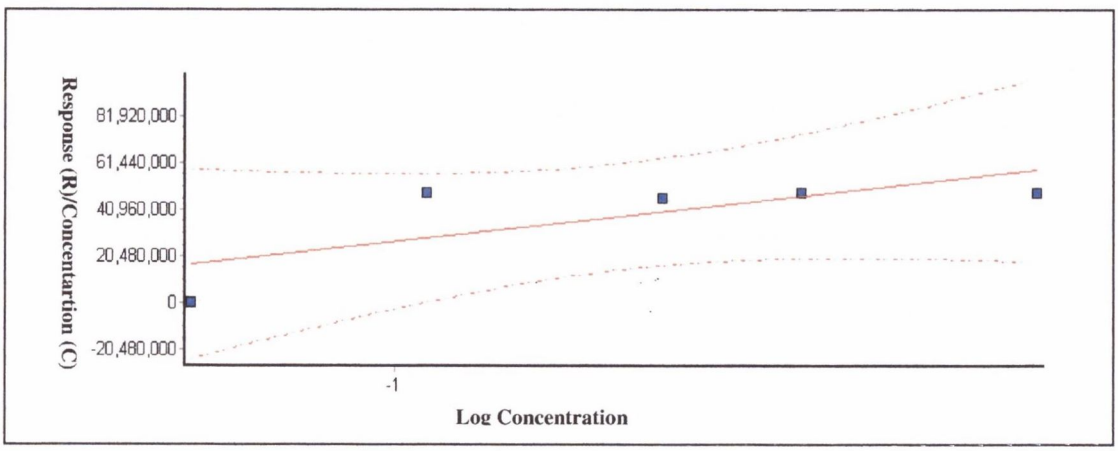


Figure 3.3.4.2 Linearity of response/concentration vs. Log of concentration (<5%)

4. Accuracy

Accuracy is defined according to the USP 26 (USP26 2003) as being 'the closeness of test results obtained by that method to the true value'. According to the ICH guidelines, 'accuracy may be inferred once precision, linearity and specificity/selectivity have been estimated' (ICH 1996). The chromatographic method has been found to be precise, selective and linear, therefore it can also be assumed to be accurate. In addition the samples analysed were all extracted in the same way, and a sample was spiked with reference standards of the taxanes analysed in order to verify that the signals analysed were the true signals of the taxanes.

5. Range

Range is defined by the ICH guidelines (ICH 1996) as 'the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity'. The precision and linearity data support the use of these methods over the concentration ranges of for taxanes: 0.055mg/ml, 0.11mg/ml, 0.22mg/ml, 0.33mg/ml, and 0.66mg/ml.

3.3.5 Quantification and statistical analysis

For the taxane analysis quantification was carried out by reference to a three-point calibration curve prepared on a daily basis with dilutions of various standards. The results were expressed in milligram per kilogram of the extracted dry weight plant material. The standards used for the quantification of taxanes were: 10-deacetylbaccatin III, baccatin III, cephalomannine, and paclitaxel (>95% purity, Sigma) (**Table 3.3.5.2**).

Table 3.3.5.1 Concentration of standards used for quantification

	Standards	**Dilution 1**	**Dilution 2**	**Dilution 3**
Taxanes	Paclitaxel, cephalomannine, baccatin III, 9-dihydro-13-acetylbaccatin III*,10-deacetylbaccatin III	0.33 mg/ml	0.22 mg/ml	0.11 mg/ml

*Only used for analysis of *Taxus canadensis*

Statistical analyses were performed using Instat®Statistical Software (Graph Pad, San Diego, USA) or Minitab Inc statistical software. All data was analysed by one-way ANOVA (Analysis Of Variance). The critical value was calculated using Tukey's "Honestly Significant Model" at a familiar significance level of 0.05. This was more appropriate than using an individual significance level of 0.05, as multiple comparisons of means were being made. The results of ANOVA were expressed as p-values. A result was significant if its p-vlaue was less than the significance level (e.i. $P<0.05$). Where many samples were compared to a control the Dunnett Multiple Comparison Test was used. ANOVA was used only if the residuals of the data were normally distributed and had a constant variance, which was not dependent on run order or any one factor.

3.4 Identification of Possible Chemotaxonomic Marker for *Taxus baccata*

3.4.1 Plant material

Branches from *T. baccata* were collected from Trinity College Dublin campus in September 2001. The same sampling protocol as before was used, and the needles were dried in the same way (see section 3.1.1).

3.4.2 Extraction procedure

The dried needles were powdered using a Brook Crompton (Series 2000) grinder immediately prior to extraction. The powdered needle material (513.19g) was then extracted exhaustively with distilled hexane, EtOAc, and MeOH in a Soxhlet extractor. The three extracts were concentrated below 50°C under reduced pressure using a rotary evaporator yielding:

- Hexane extract: Green thick residue (20.61g)
- Ethyl acetate extract: Green oily residue (24.61g)
- Methanol extract: Brown thick oily residue (121.89g)

The three extracts were all analysed by TLC and compared using solvent systems 2) and 3) (see section 3.3.3). An overlap was found between the hexane and the EtOAc extract, but only the MeOH extract contained the more polar compounds.

The different extracts were analysed using the HPLC system developed for analysis of taxanes (see section 3.3.4).

3.4.3 Liquid column chromatography (LCC) of methanol extract

A sample of the methanol extract (1.029g) was reconstituted in MeOH and pre-adsorbed onto silica (1:10 extract-silica) before introduction to a wet packed silica gel column (Silica gel 60 (0.04-0.063 mm) Merck KGaA, Darmstadt). The column dimension was 25cm x 3.5 cm.

Table 3.4.3.1 Fractions collected

Fractions	Solvent system
First 750 ml	100% $CHCl_3$
Test tube: 1-27	2% MeOH in $CHCl_3$
Test tube: 28-58	5% MeOH in $CHCl_3$
Test tube: 59-89	8% MeOH in $CHCl_3$
Test tube: 90-110	10% MeOH in $CHCl_3$
Test tube: 110-142	11% MeOH in $CHCl_3$
Test tube: 143-176	13% MeOH in $CHCl_3$
Test tube: 177-193	15% MeOH in $CHCl_3$
Last 500 ml	100% MeOH

The fractions collected were analysed by TLC using solvent system 1) and 3) (see section 3.3.3). On the basis of this the fractions were pooled and combined. Combined fractions: 1-8, 9-21, 22-39, 40-43, 44-48, 49-54, 55-63, 64-78, 79-88, 89-98, 99-108, 109-120, 121-141, 142-147, 148-190.

3.4.4 Liquid column chromatography (LCC) of ethyl acetate extract

Part of the ethyl acetate extract (10g) was re-dissolved in methanol and pre-absorbed onto silica gel (1:10 of extract-silica gel) before introduction to a wet packed column. The dimension of the column was 35cm x 3.5 cm.

The fractions were analysed by TLC using hexane-ethyl acetate (7:3) as a solvent system. Similar fractions were combined and evaporated to dryness under reduced pressure using a rotary evaporator (temperature < 40° C).

Table 3.4.4.1 Fractions collected

Fractions	Solvent system
1-196	100 % hexane
197-230	0.5 % EtOAc in hexane
231-297	1 % EtOAc in hexane
298-311	2 % EtOAc in hexane
312-338	4 % EtOAc in hexane
339-383	7 % EtOAc in hexane
384-403	9 % EtOAc in hexane
404-424 *	10 % EtOAc in hexane
425-432*	12.5 % EtOAc in hexane
433-457*	20 % EtOAc in hexane
458-461*	25 % EtOAc in hexane

*(50 ml collected)

Fractions 388-418, 419-436, and 437-440 were combined after analysis by HPLC using the HPLC method for taxanes (see section 3.3.4). These combined fractions 388-440 (0.12g) contained two compounds with retention time ~16 min and ~19 min.

3.4.5 LCC for purification of fraction 388-440

The combined fractions 388-440 (0.12g) was reconstituted in MeOH and pre-absorbed onto silica gel as before. The column dimension was 13cm x 3.5 cm, and a high-pressure pump was used. The mobile phase used for all the fractions was 10 % ethyl acetate in hexane.

Fractions 19-26 (0.04g) was analysed by TLC and HPLC (taxane system), and it was found to be a pure compound with a retention time of ~16 in the HPLC system. This fraction was further analysed by nuclear magnetic resonance spectroscopy (NMR) and gas chromatography-mass spectrometry (GC-MS).

3.4.6 Spectroscopy

3.4.6.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

Bruker DPX-400 instrument, at 400.13 MHz for proton (^{1}H) magnetic resonance and 100.61 MHz for carbon (^{13}C) magnetic resonance. Spectral analysis was performed using Bruker WIN-NMR software or Mest-Rec.

3.4.6.2 Gas Chromatography-Mass Spectrometry (GC-MS)

Low resolution GC-MS: Saturn GC/MS 2000 (CP-3800 Gas Chromatograph).

Oven temperature: initial 100°C, increasing at 15°C per minute to 300°C.

Run time: 25 minutes.

Column flow rate: 1 ml/min.

Column pressure: 10 psi.

Column: FactFour™, capillary column, VF-Xms (30m, 0.25mm, 0.25μm).

4 Results and Discussion

4.1 Morphological Examination

4.1.1 General description of *Taxus*

The Royal Horticultural Society A-Z Encyclopaedia of Garden Plants (Brickell 1996) describes yews as 'broadly rounded to upright, dioecious, evergreen, coniferous, large shrubs or small trees found in forests extending from the northern temperate areas to the Philippines and Central America'.

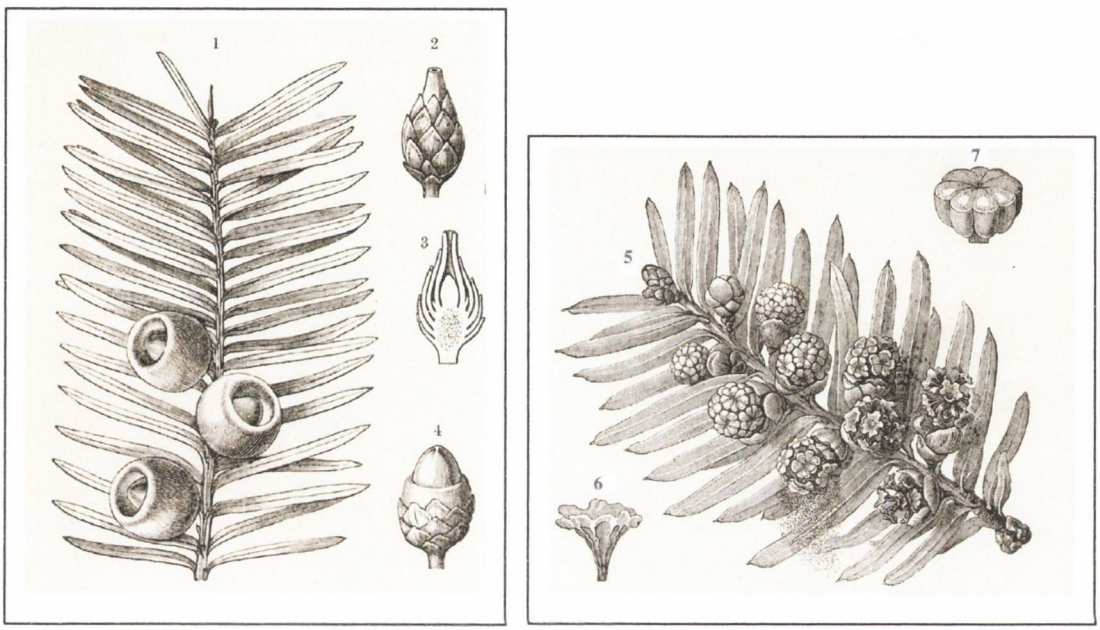
(Earle 2002)

Figure 4.1.1.1 Geographical distribution of the genus *Taxus* (marked with red on map)

Elwes and Henry in their classic text "The Trees of Great Britain and Ireland" (Elwes and Henry 1906) described *Taxus* as:

"Evergreen trees or shrubs belonging to the division Taxaceae of the order Coniferae. Bark reddish or reddish brown, thin and scaly. Branches spreading, giving off branchlets, of one kind only, irregularly alternate, surrounded at their bases by brownish scales. Buds globular or ovoid, of imbricated scales. Leaves inserted on the branchlets in a spiral order, on upright shoots spreading radially, on horizontal shoots disposed by twisting on their petioles in one plane in a pectinate arrangement, the upper and lower leaves being of same length, with their dorsal surfaces turned upwards and their ventral surfaces downwards. In fastgiate varieties all, or most, of the branchlets assume an erect position, and the leaves in consequence are arranged radially. The leaves are linear, flat, with recurved margins, dark green above, paler green below; the lower surface only bearing stomata, which never form conspicuous white bands; narrowed at the base into a short petiole, arising from a linear cushion on the twig; mucronate or acute at the apex and without a resin canal. Flowers dioecious, or in rare individuals monoaecious, on the under surface of the branchlets of the preceding year, in axils of the leaves, the female flowers being less numerous than the male flowers. Male flowers composed of stalk, girt at its base by imbricated scales, and bearing above a globose head of 6-14 stamens with short filaments. The stamen is expand above into a peltate connective, which bears on its lower surface 5 to 9 pollen sacs, united with each other and with the filament. The female flowering shoot, arising out of the axil of the leaf, is composed

of a number of imbricated scales, in the axil of the uppermost one of which is borne an ovule, placed so close to the apex of the shoot as to appear terminal; in the scale next below a bud occurs, which occasionally develops into a second ovule. The ovule, which has a small membranous disc at its base, projects out of the scales by its micropyle. Seed sessile in a fleshy, juicy cup, forming an aril (the enlarged disc), open at the top and free from the seed in its upper part. The seed variable in form, 2, 3, or 4-angled, is generally ellipsoid and has a ligneous testa, containing oily white albumen, in the upper part of which is an axil straightly cylindrical minute embryo with two cotyledons."



1 Branch of female *T. baccata* with ripe seeds each enclosed in arils, **2** tip of ovule, **3** longitudinal section of tip of ovule, **4** young seed partly enclosed in aril, **5** Branch of male *T. baccata* with flowers, **6** anther with its pollen sacs open and empty, **7** anther with its pollen sacs closed

Figure 4.1.1.2 Branches of female and male *Taxus baccata* (Kerner von Marilaun and Oliver 1904)

Elwes and Henry (1906) were of the opinion that the genus was comprised of a single species with several distinct geographical forms. Veicht in his 'Manual of the Coniferae' (Veicht 1881) described five species: adpressa, baccata, brevifolia, canadensis and cuspidata. A previous study (Dempsey and Hook 2000) failed to come to a firm conclusion as it was limited to examining only needle characteristics in a relatively small population of yews. Thus, one objective of the present study was to examine a larger number of *Taxus* species and cultivars. Additionally, needle characteristics, seed dimensions and chemical constituents were included in the analysis. Finally, for a broader morphological perspective, additional characteristics such as tree shape, foliage colour and tree gender were taken into consideration. It was

hoped that these collectively would indicate (or not) species-specific characteristics of taxonomic value.

4.1.2 Needle Dimensions

Foliar needle characteristics have been used as taxonomic tools in distinguishing between the morphologically similar species of *Taxus* (Chadwick and Keen 1976, Dallimore and Jackson 1924, Dempsey 2000, Dempsey and Hook 2000, Dempsey et al. 1999, Namba and Kuginuki 1994, van Rozendaal et al. 1999, Vidakovic 1991). Previously, size determinations have been made manually through the tedious use of a ruler (Pensa et al. 2004) or Vernier callipers (Dempsey 2000, Dempsey and Hook 2000, Dempsey et al. 1999). In this study the needle areas, lengths and widths were determined using an image analysis software; WinSEEDLE™. **Figure 4.1.2.1** and **4.1.2.2** below show examples of this analysis which was found to be more efficient, accurate and statistically relevant than the previously described methods Samples analysed by WinSEEDLE™ (n=100) were selected randomly from a pool of needles picked from top to bottom of the right hand side of ~20 cm long twigs. Eight twigs were collected from four different sides of each tree sample and at two height levels.

Figure 4.1.2.1 WinSEEDLE™ image analysis software

Figure 4.1.2.2 Magnification of a) length analysis, b) width analysis and c) area analysis using WinSEEDLE™ image analysis software

4.1.2.1 Effect of needle position on tree

The initial study was designed to determine if twig location on the trees significantly affected needle dimensions using needles (n = 100) from ten *T. baccata* trees from Niedersächsische Forstliche Verssuchsanstalt in Staufenberg Germany. The twigs were collected (as described in Materials and Methods, section 3.1.1) from four sides of each tree (denoted 1, 2, 3, and 4) at two different height levels. With their upper needle surface uppermost, needles were separated from top to bottom on the right hand side of each twig, and analysed separately. The results are shown in **Figure 4.1.2.3**

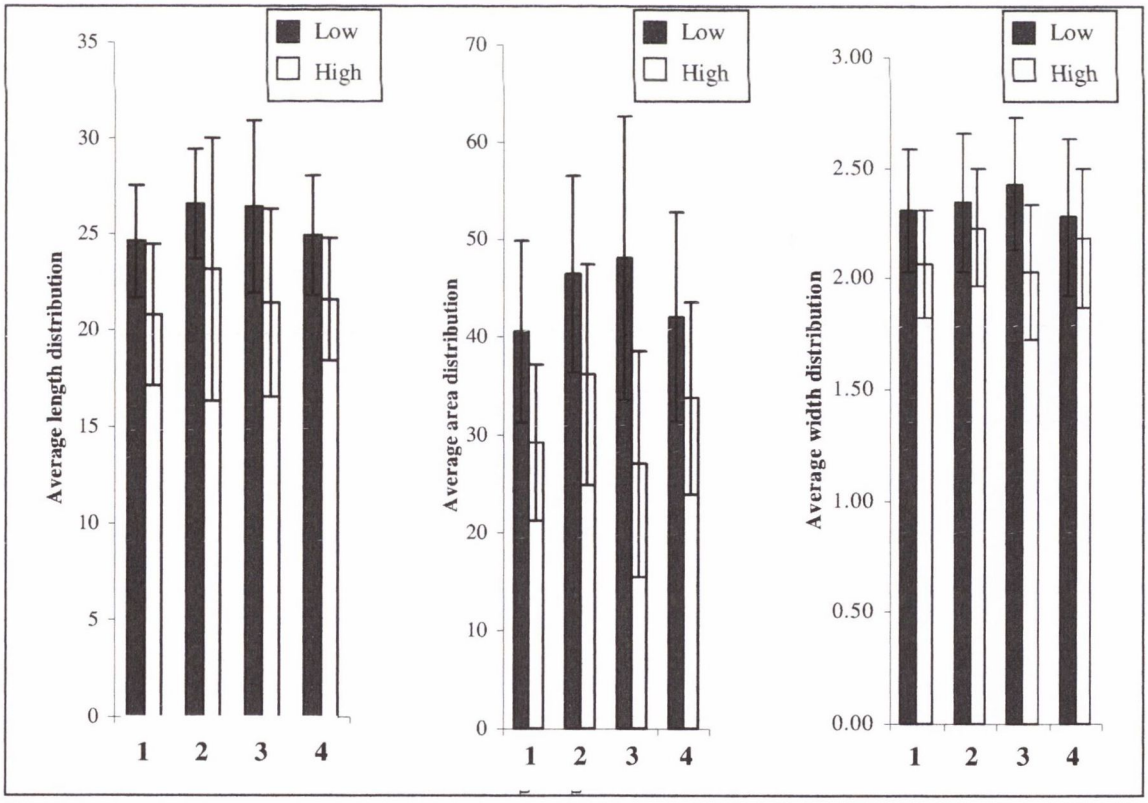


Figure 4.1.2.3 Needle dimensions (length, area and width) according to position on the tree (1, 2, 3 and 4 are the four sides of the tree)

Although results are not statistically significant, the trend suggests that height and location may affect needle size. Samples from side 2 and 3 yielded larger needles, as did lower-situated twigs. Results showed the importance of correct sampling protocol and the necessity of pooling the collected samples. Results also highlighted the importance of sampling for Herbarium collection and noting the location from which samples were taken. Lower samplings of leaves from more shaded locations are therefore longer than samples from higher (and more illuminated) locations.

4.1.2.2 Effects of drying

Both fresh and dried needles were analysed and results of dimensions are presented separately. Fresh needles were used where possible as drying resulted in shrinkage and distortion of shape. However, data from dried needles were considered significant for further comparison with Herbaria samples (if available).

The effect of drying of needles was studied using needles from locally grown *T.* x. *media*. Three collections were carried out and the needles were separated into new and old (i.e. last year's needles) growth. The samples (n = 100 of each) were initially analysed fresh and subsequently pressed, dried and re-evaluated. The results are shown in **Figure 4.1.2.4**.

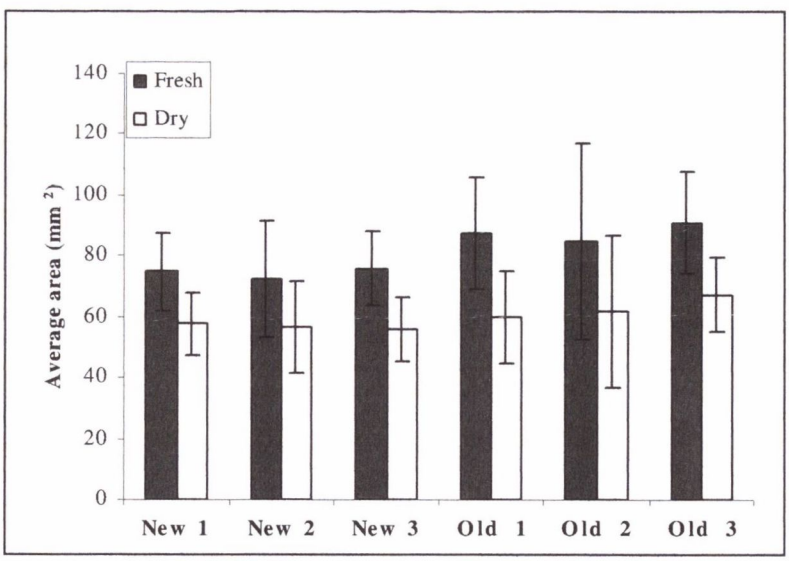


Figure 4.1.2.4 Effect of drying on needle area of new and old growth (1, 2 and 3 represents the three collections carried out)

The results showed a significant decrease in surface-area after drying both in new and old needles ($P<0.001$). The shrinkage (%) caused by drying in the new growth was on average 24 ± 2 % and in old growth was found to be 28 ± 3%. These findings clearly illustrate a significant difference in the surface-area of fresh needles of new and old growth ($P<0.001$), where fresh needles from new growth had a larger surface-area than fresh needles from old growth. Upon drying the difference in surface-area of new and old needles was not as significant, although the water content was larger in needles from old growth. Also these results prove that pooling of samples prior to analysis is necessary, and they show that data from fresh and dry needles must be analysed and presented separately.

4.1.2.3 Analysis of fresh needles

The average values for all fresh needles are presented according to species in **Figure 4.1.2.5**. The needle dimensions for *T. baccata* samples are given in **Table 4.1.2.1a**). **Table 4.1.2.1b)** shows the needle dimensions of other *Taxus* species and cultivars.

The following legends indicate the origin of samples used in analyses:

1: The J. F. Kennedy Park Arboretum, New Ross, Co. Wexford, Ireland.

2: Mount Usher Gardens, Ashford, Co. Wicklow, Ireland.

3: Powerscourt Estate and Gardens, Co. Wicklow, Ireland.

4: National Botanical Gardens, Glasnevin, Dublin, Ireland.

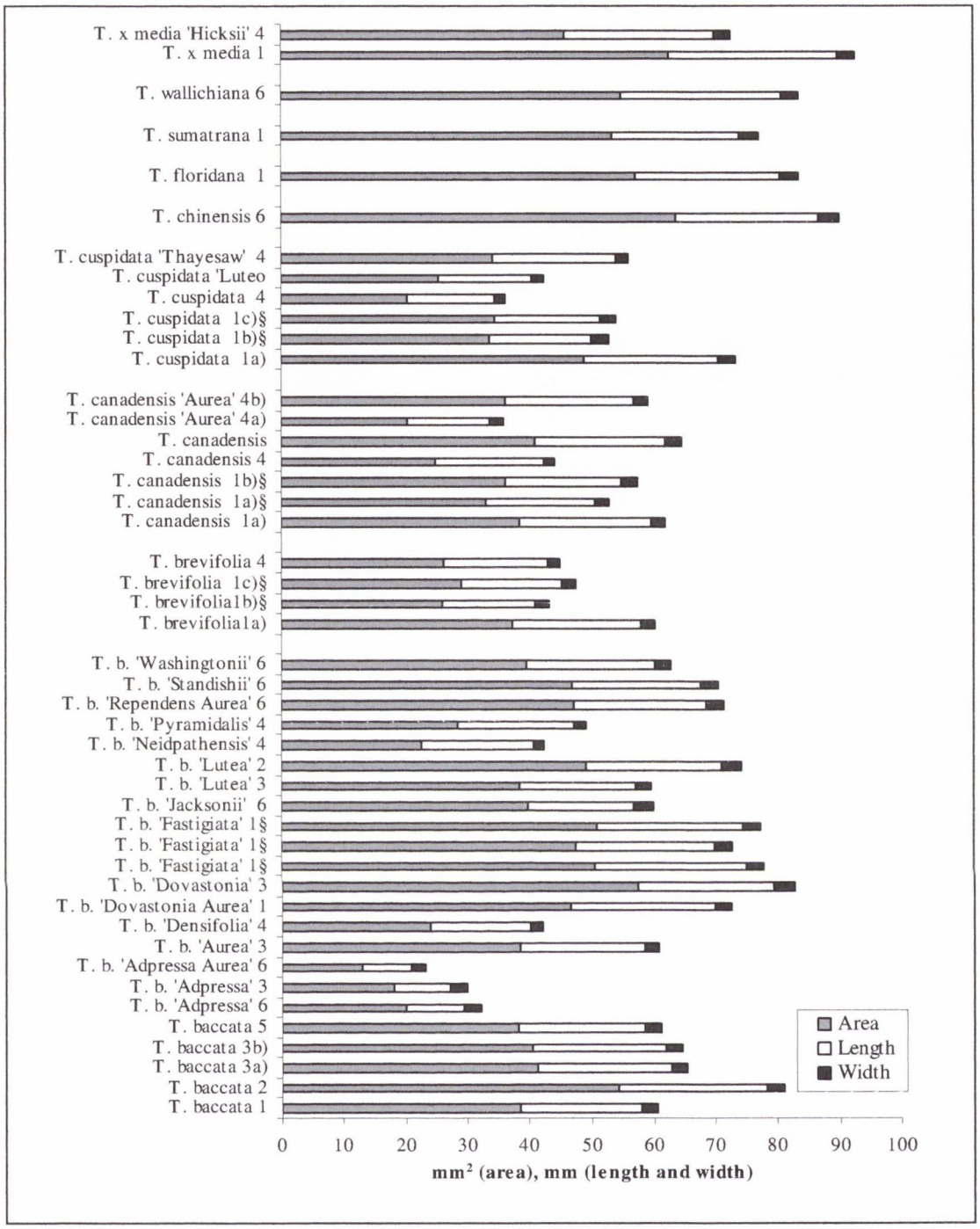
5: Herstmonceaux Church, East Sussex, U.K.

6: The Royal Botanical Gardens Edinburgh, Edinburgh, Scotland.

7: Begebury National Pinetum, Kent, UK.

8: Natural Resourses Canada, Fredericton, New Brunswick, Canada.

9: Polska Akademia Nauk, Krónik, Poland.



(The legends for origin are given in section 4.1.2.3)

Figure 4.1.2.5 Needle dimensions of fresh *Taxus* species and cultivars

Taxus baccata **Figure 4.1.2.6 (a)** and **(b)** below show typical samples of *T. baccata* and *T. baccata* 'Adpressa' illustrating the large variation in needle dimensions observed within this species.

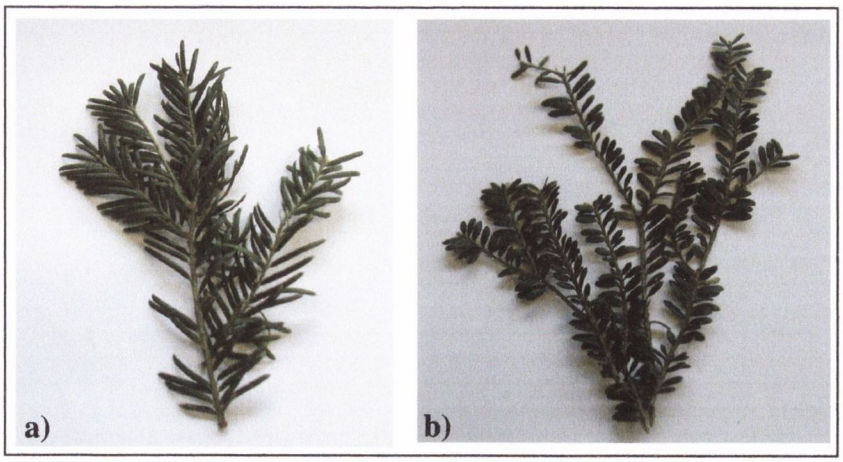


Figure 4.1.2.6 a) *T. baccata* and **b)** *T. baccata* 'Adpressa'

Fresh samples from *T. baccata* (x5) and *T. baccata* cultivars (x18) were analysed. The largest needles were found in *T. baccata* from Mount Usher (**2**) with an average area of (54.40 ± 10.88 mm^2; P<0.001). Although the needles from this sample were significantly longer (23.76 ± 2.69 mm) and wider (2.97 ± 0.36 mm) than any of the other *T. baccata* needles, they were within the range quoted by Dallimore and Jackson i.e. 12.50 – 31.75 mm (Dallimore and Jackson 1924). The average length (21.42 ± 1.54 mm) of all the *T. baccata* samples in this study were within these limits. Vidakovic (Vidakovic 1991) quoted widths for *T. baccata* needles from 2 to 2.5 mm. The average width of *T. baccata* analysed here was found to be slightly above this range (2.58 ± 0.23 mm), and in particular, the sample from Mount Usher **(2)** which was significantly wider (2.97 ± 0.36, P<0.001). These results question therefore the identity of this particular sample.

The oldest yew sample was from Herstmonceux Church, East Sussex, U.K. (**5**) and is considered by locals to be approximately 2000 years old. Needles analysed from this yew **(5)** were found to be the smallest, with a length of 20.51 ± 2.15 mm and a width of 2.39 ± 0.21 mm. Although the needles were the shortest and narrowest observed both values lay within the range give by Dallimore and Jackson, as well as Vidakovic (Dallimore and Jackson 1924, Vidakovic 1991). This sample can therefore be assumed an authentic *Taxus baccata* sample.

Table 4.1.2.1 a) Needle dimensions of fresh *Taxus baccata* needles

Sample	Area (mm^2)	Length (mm)	Width (mm)	Source code*	Origin
Taxus baccata	38.42 ± 6.57	19.64 ± 2.63	2.60 ± 0.18	9L7	1
Taxus baccata	54.40 ± 10.88	23.76 ± 2.69	2.97 ± 0.36		2
Taxus baccata	41.25 ± 12.50	21.59 ± 2.88	2.50 ± 0.56		3a)
Taxus baccata	40.55 ± 9.07	21.60 ± 2.26	2.46 ± 0.42		3b)
Taxus baccata	38.24 ± 7.76	20.51 ± 2.15	2.39 ± 0.32		5
T. b. 'Adpressa'	20.01 ± 3.38	9.37 ± 1.11	2.83 ± 0.21	699296	6
T. b. 'Adpressa'	18.19 ± 3.72	8.98 ± 1.23	2.79 ± 0.23	t	3
T. b. 'Adpressa Aurea'	13.15 ± 2.85	7.84 ± 1.05	2.31 ± 0.25	071024	6
T. b. 'Aurea'	38.61 ± 14.38	19.93 ± 4.15	2.49 ± 0.38		3
T. b. 'Densifolia'	24.21 ± 5.31	16.07 ± 2.82	1.93 ± 0.19	6563	4
T. b. 'Dovastonia'	57.63 ± 11.67	21.60 ± 2.95	3.38 ± 0.29	25	3
T. b. 'Dovastonia Aurea'	46.74 ± 11.55	23.10 ± 3.57	2.75 ± 0.33	9L30	1
T. b. 'Fastigiata' §	50.81 ± 15.30	24.31 ± 4.39	2.78 ± 0.49	9L4	1a)§
T. b. 'Fastigiata' §	47.57 ± 13.37	22.36 ± 3.91	2.79 ± 0.35	9L5	1b)§
T. b. 'Fastigiata' §	51.11 ± 13.10	23.45 ± 3.90	2.87 ± 0.32	9L6	1c)§
T. b. 'Jacksonii'	39.84 ± 10.16	17.22 ± 2.82	2.86 ± 0.27	19688018	6
T. b. 'Lutea'	38.47 ± 7.71	18.80 ± 3.26	2.62 ± 0.32		3
T. b. 'Lutea'	49.24 ± 12.06	21.92 ± 2.44	2.92 ± 0.53		2
T. b. 'Neidpathensis'	22.65 ± 7.04	18.17 ± 2.62	1.76 ± 0.35	6569	4
T. b. 'Pyramidalis'	28.54 ± 7.08	18.76 ± 2.53	2.06 ± 0.37	6594	4
T. b. 'Repandens Aurea'	47.27 ± 15.53	21.37 ± 4.68	2.75 ± 0.31	19688019	6
T. b. 'Standishii'	46.91 ± 8.36	20.73 ± 2.41	2.92 ± 0.19	19588809	6
T. b. 'Washingtonii'	39.72 ± 7.35	20.75 ± 2.66	2.47 ± 0.20	19699305	6

§ collected in December a), b) and c) shows different trees of same species from same origin *Source code is code given by Botanical gardens for identification of trees

Analysis of the fresh needles from *T. baccata* cultivars exhibited high degrees of variation in area, length and width, as illustrated in **Figure 4.1.2.7**. The small-leaved variety of the Common yew, *T. b.* 'Adpressa', and the much longer leaved variety *T. b.* 'Dovastonia' may account for this high degree of variation. The *T. b.* 'Adpressa Aurea' sample from Edinburgh **(6)** had the smallest needle area of all the samples analysed in this study (13.15 ± 2.85 mm^2). The main reason for this was the length of the needles (average of all 8.73 mm), where *T. b.* 'Adpressa Aurea' **(6)** only had a needle-length of 7.84 ± 1.05 mm. Veicht considered *Taxus adpressa* to be a separate species to *Taxus baccata* (Veicht 1881). The largest area was found in *T. b.* 'Dovastonia' from Powerscourt **(3)** (57.63 ± 11.67 mm^2). Not only were the needles longer (21.60 ± 2.95 mm, $P<0.001$), but also significantly wider (3.38 ± 0.29 mm, $P<0.001$) than in any of the other *T. baccata* cultivars. It is obvious from these results

that there is a significant variation in needle sizes of *T. baccata* even when extreme values (i.e. smallest and largest) are not included in the calculations.

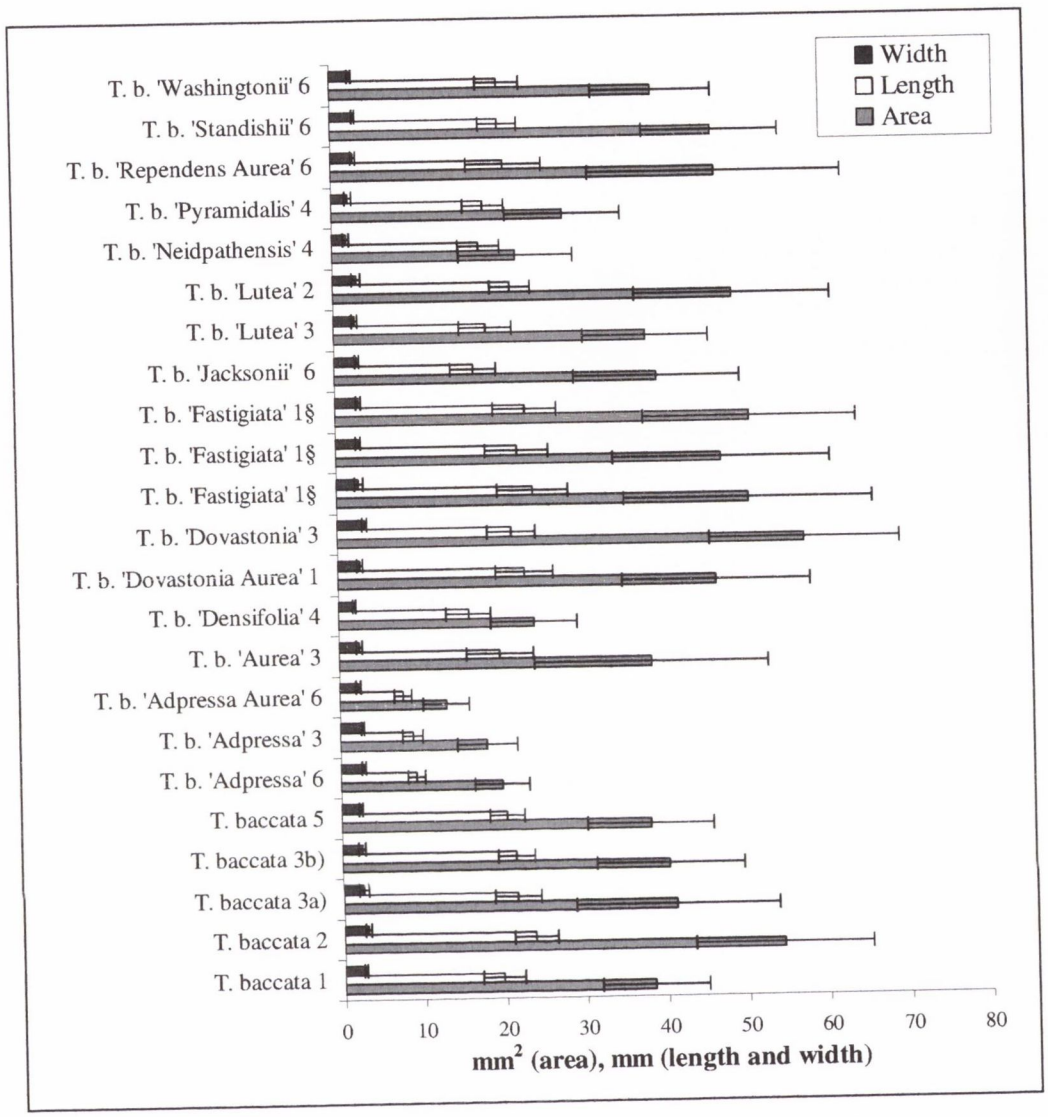


Figure 4.1.2.7 Needle dimensions of *Taxus baccata* cultivars

Dallimore and Jackson found that needles from *T. b.* 'Adpressa' had a length range 6.35-12.70 mm (1924), while Vidakovic reported a needle area in the range of 5-9mm x 2-4mm. The results of the samples analysed (8.7 ± 0.8 x 2.6 ± 0.3 mm) in the present study all fell within these expected ranges. Vidakovic also examined the needles of *T. b.* 'Dovastonia' and *T. b.* 'Fastigiata' and found their lengths to be in the ranges 20-35 and 20-30 mm, respectively (1991). Similar results obtained in the current study (average 22.65 ± 1.06 and 23.37 ± 0.98 mm long, respectively) confirmed these results.

Other *Taxus* species and cultivars

A number of other *Taxus* species and cultivars were analysed in our study, including several samples of *T. brevifolia*, *T. canadensis* and *T. cuspidate*, and are discussed separately below. Only one sample each of the Himalayan yew (*T. wallichiana*), Chinese (*T. chinensis*), Sumatran (*T. sumatrana*) and Florida (*T. floridana*) yews were obtained. The hybrid *T.* X *media* (*T. cuspidata* x *T. baccata*) and its variant *T.* X *media* 'Hicksii' were also analysed, but here also a limited number of authentic samples were available.

Table 4.1.2.1 b) Needle dimensions of fresh needles from *Taxus* species and cultivars

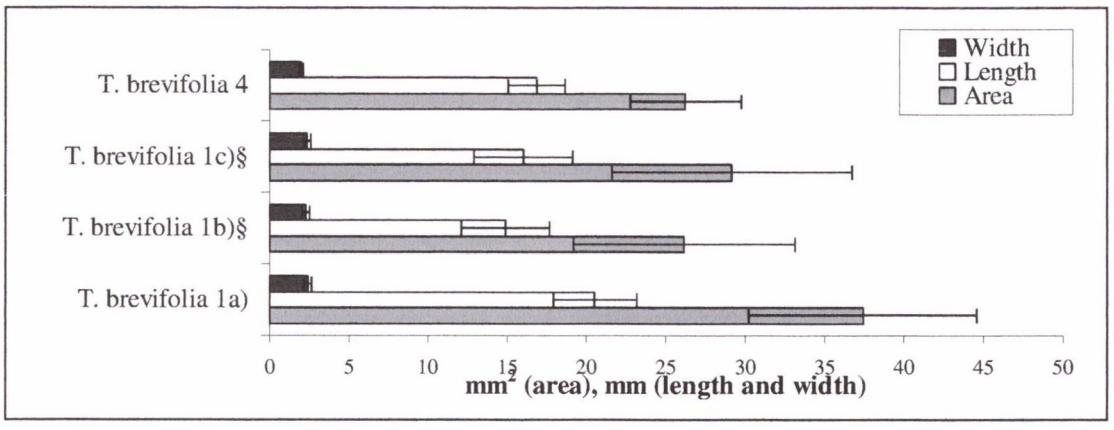
Sample	Area (mm^2)	Length (mm)	Width (mm)	Source Code	Origin
T. brevifolia	37.44 ± 7.18	20.57 ± 2.66	2.42 ± 0.26	8M34	1a)
T. brevifolia §	26.18 ± 6.99	14.93 ± 2.78	2.31 ± 0.24	8L33	1b)§
T. brevifolia §	29.17 ± 7.56	16.04 ± 3.14	2.38 ± 0.22	8L35	1c)§
T. brevifolia	26.26 ± 3.47	16.88 ± 1.79	2.00 ± 0.11	6759	4
T. canadensis	38.56 ± 7.83	21.17 ± 2.56	2.44 ± 0.32	8M5	1a)
T. canadensis §	33.10 ± 10.82	17.58 ± 3.18	2.43 ± 0.31	8M5	1a)§
T. canadensis §	36.12 ± 7.47	18.85 ± 2.70	2.51 ± 0.25	8M4	1b)§
T. canadensis	24 86 ± 3.90	17.60 ± 2.00	1.81 ± 0.15	6556	4
T. canadensis **	41.02 ± 5.61	21.04 ± 1.72	2.52 ± 0.20	271013	6
T. canadensis 'Aurea'	20.41 ± 5.13	13.38 ± 2.24	2.05 ± 0.21	6549	4a)
T. canadensis 'Aurea'	36.27 ± 6.16	20.53 ± 2.30	2.27 ± 0.28	6570	4b)
T. chinensis	63.64 ± 13.76	22.92 ± 3.23	3.49 ± 0.31	311011	6
T. cuspidata	49.10 ± 10.80	21.43 ± 3.57	2.97 ± 0.38	8M9	1a)
T. cuspidata §	33.70 ± 6.91	16.50 ± 2.38	2.67 ± 0.24	8M10	1b)§
T. cuspidata §	34.47 ± 7.72	17.11 ± 2.42	2.63 ± 0.28	8M11	1c)§
T. cuspidata	20.27 ± 3.21	14.15 ± 1.65	1.87 ± 0.13	6601	4
T. cuspidata 'Luteo Baccata'	25.41 ± 9.85	15.09 ± 3.72	2.06 ± 0.31	6571	4
T. cuspidata 'Thayerae'	34.15 ± 6.25	19.90 ± 2.50	2.16 ± 0.22	6593	4
T. floridana	57.29 ± 17.43	23.13 ± 4.70	3.15 ± 0.38	7M49	1
T. sumatrana	53.56 ± 11.79	20.30 ± 3.23	3.32 ± 0.22	8M8	1
T. wallichiana	55.06 ± 13.21	25.65 ± 4.95	2.86 ± 0.24	19902824	6
T. X *media*	62.58 ± 12.95	27.24 ± 4.01	2.95 ± 0.28	8M54	1
T. X *media* 'Hicksii'	45.83 ± 14.73	24.14 ± 3.91	2.50 ± 0.48	6584	4

§ collected in December a), b) and c) shows different trees of same species from same origin *Source code is code given by Botanical gardens for identification of trees ** Labellec *T.b.* 'Procumbens' by Botanical Garden (but according to Farjon (Farjon 1998) this is the same as *T. canadensis*

Taxus brevifolia

Figure 4.1.2.8 A sample of *T. brevifolia*

It is clear that a degree of variation exists between the samples of this species as seen in **Figure 4.1.2.9**. In four fresh *T. brevifolia* samples analysed, sample (**1a**) from New Ross had a significantly larger area (37.44 ± 7.18 mm^2, $P<0.001$) than any of the other samples, principally due to their significantly greater length (20.57 ± 2.66 mm). Dallimore and Jackson (1924) reported needle lengths of *T. brevifolia* to be within the range 12.7-19.05 mm and Vidakovic quoted (1991) that this species can exhibit lengths of up to 20 mm. Sample **(1a)** from New Ross had a length above the expected range, while the remaining samples fell well within expected ranges. It again questions the correct identification of the specimen of *T. brevifolia*.



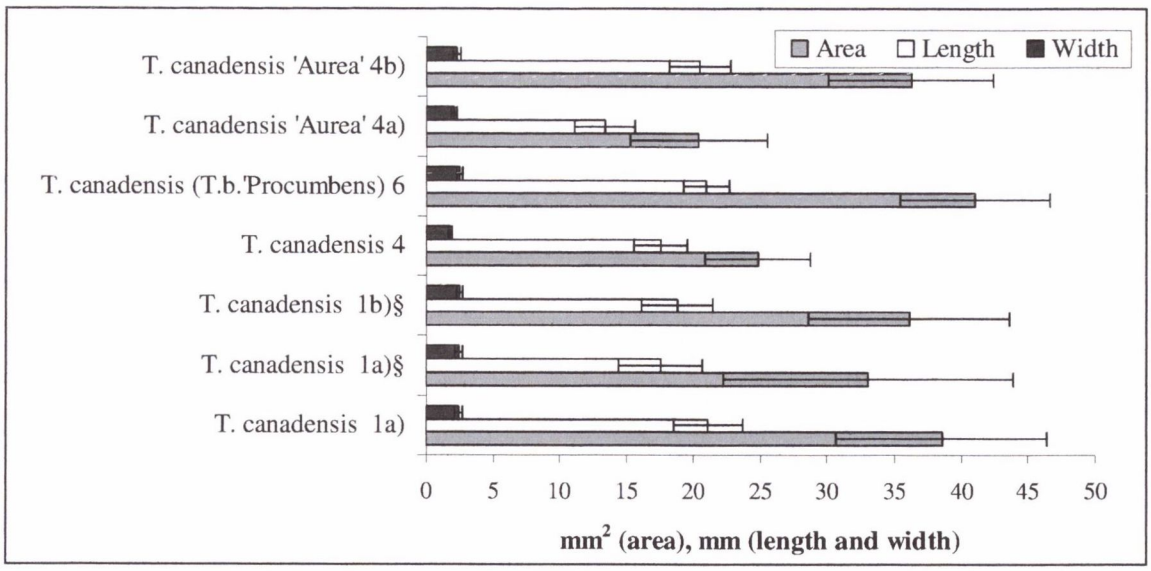
§ represents samples collected in December

Figure 4.1.2.9 Needle dimensions of fresh *Taxus brevifolia*

Taxus canadensis

Figure 4.1.2.10 A sample of *T. candensis*

A considerable variation in the needle areas were found between the five fresh *T. canadensis* samples and the two cultivars analysed. The largest areas were found in the *T. canadensis* sample(s) obtained from Edinburgh **(6)** (41.02 ± 5.61 mm^2) and (**1a**) New Ross (38.56 ± 7.83 mm^2). The sample from Edinburgh **(6)** was named *T. baccata* 'Procumbens', but according to Farjon (Farjon 1998) *T. canadensis* is the accepted name for *T. b.* 'Procumbens' and no significant difference between these two samples was observed. The *T. canadensis* 'Aurea' (**4a**) sample was found to be significantly smaller (20.41 ± 5.13 mm^2) than any of the other samples analysed (P<0.001).



§ represents samples collected in December

Figure 4.1.2.11 Needle dimensions of fresh *Taxus canadensis* cultivars

T. canadensis needles were expected to fall within the ranges reported by Dallimore and Jackson (1924) (12.70-19.05 mm in length and 1.59-2.12 mm in width) and Vidakovic (1991) (13-20 x 0.5-2mm). The average value of the samples analysed in the present study was in the upper end of this range (18.59 ± 2.76 x 2.29 ± 0.27 mm). Most of the samples were significantly wider (average width of 2.29 ± 0.27 mm) than the width given by both Dallimore and Jackson and Vidakovic. The large variation between the samples of this species analysed from this species begs the question if in fact they are all authentic samples of *T. canadensis*.

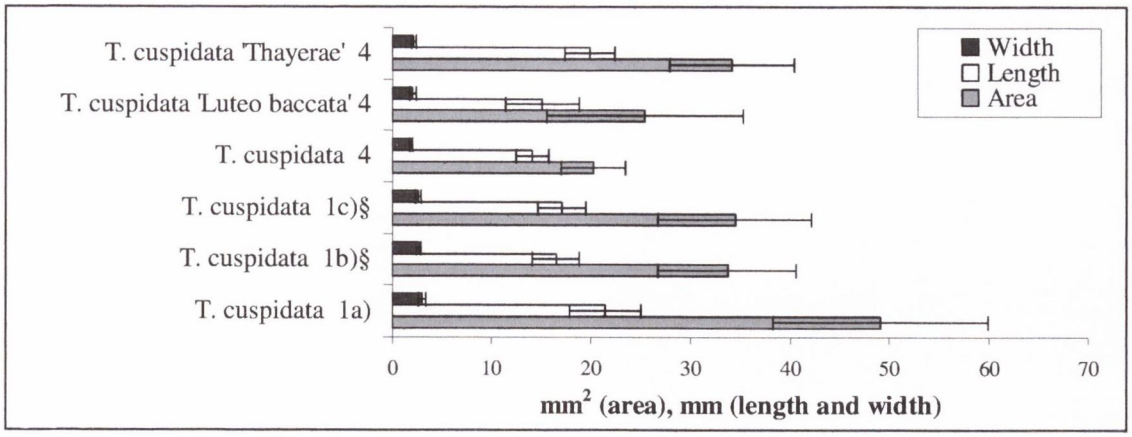
Taxus cuspidata

Figure 4.1.2.12 A sample of *T. cuspidata*

Four different samples of *T. cuspidata* (**Figure 4.1.2.13**) from two different locations and additionally, two varieties, 'Luteo baccata' and 'Thayerae', were analysed. From **Figure 4.1.2.13** it is apparent that the sample (**1a**) from New Ross was significantly bigger (49.10 ± 10.80 mm^2), longer (21.43 ± 3.57 mm) and wider (2.97 ± 0.38 mm) than needles of any of the other samples ($P<0.001$).

The average needle dimensions for the *T. cuspidata* samples in this study (17.30 ± 3.03 mm long and 2.54 ± 0.47 mm wide) were within the range reported by Dallimore and Jackson (12.7-25.4 mm long and 2.12-3.18 mm wide). Vidakovic reported a range of 15-25 x 3mm, Chadwick and Keen (Chadwick and Keen 1976) observed slightly longer needles (25mm) and Namba and Kuginuki (Namba and Kuginuki 1994) quoted

20 x 2 mm. The samples from the two varieties of analysed also fell within the expected range.



§ represents samples collected in December

Figure 4.1.2.13 Needle dimensions of fresh *Taxus cuspidata* cultivars

Taxus chinensis, Taxus sumatrana and Taxus wallichiana

Figure 4.1.2.14 Samples of (a) *T. chinensis*, (b) *T. sumatrana* and (c) *T. wallichiana*

The three remaining Asian samples (**Figure 4.1.2.15**) analysed all had large needles, with the *T. chinensis* sample being significantly larger than the other two (P<0.001), due mainly to their width (3.49 ± 0.31 mm). While there was no statistically significant difference between the areas of *T. sumatrana* and *T. wallichiana*, *T. wallichiana* had longer needles (25.65 ± 4.95 mm, P<0.001)) than both *T. sumatrana* (20.30 ± 3.23 mm) and *T. chinensis* (22.92 ± 3.23 mm). Dallimore and Jackson (1924) reported the Chinese yew (*T. chinensis*) to exhibit needle lengths of 12.7-25.4 mm and widths of 2.12-3.18mm. The *T. chinensis* sample analysed in this study was slightly above this range (22.92 ± 3.23 x 3.49 ± 0.31 mm).

Vidakovic (1991) examined needles from *T. celebica*, finding a needle range of 12-40 x 2-2.5 mm. There is significant ambiguity in the nomenclature of this species. According to Farjon (Farjon 1998) *T. sumatrana* is the accepted name for *T. celebica*, but *T. celebica* has been mistakenly used to describe the Chinese yew, *T. chinensis*. Thus, in the current study the annotation *T. sumatrana* will be used. The length of *T. sumatrana* needles (20.30 ± 3.23 mm) in our study fell within the range given by Vidakovic, but the width was significantly greater (3.32 ± 0.22 mm, P<0.001). However, the large range in length reported by Vidakovic illustrates how variable the needle sizes are within this species.

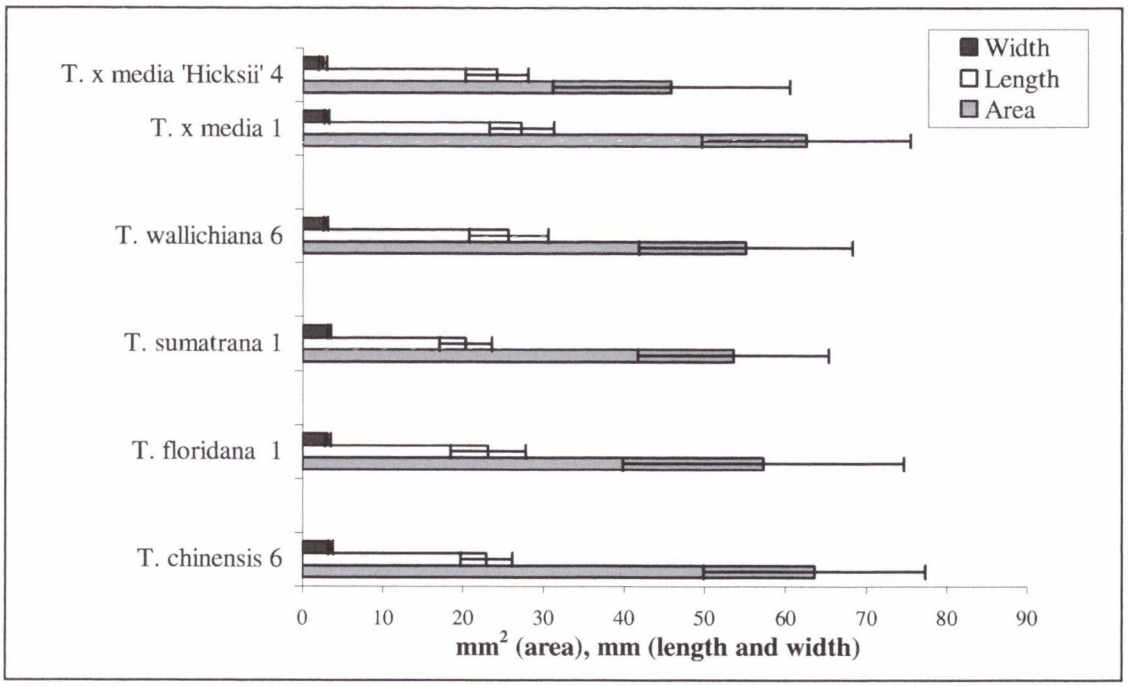


Figure 4.1.2.15 Needle dimensions of various fresh *Taxus* species and cultivars

Taxus floridana

Figure 4.1.2.16 A sample of *T. floridana*

Dallimore and Jackson (1924) reported that the needles from this species were 19.05-25.4 mm long and 1.27-1.58 mm wide. The single sample of *T. floridana* (**Figure 4.1.2.15**) analysed in the present study was very large (57.29 ± 17.43 mm^2) and well above the expected range (especially the width) given by Dallimore and Jackson due to the greater length (23.13 ± 4.70 mm) and width (3.15 ± 0.38 mm) of the needles. Thus, given the large disparities in the experimental results presented here and those of the literature, sample authenticity must be questioned. However, given that these results are from a single sample, they must remain inconclusive.

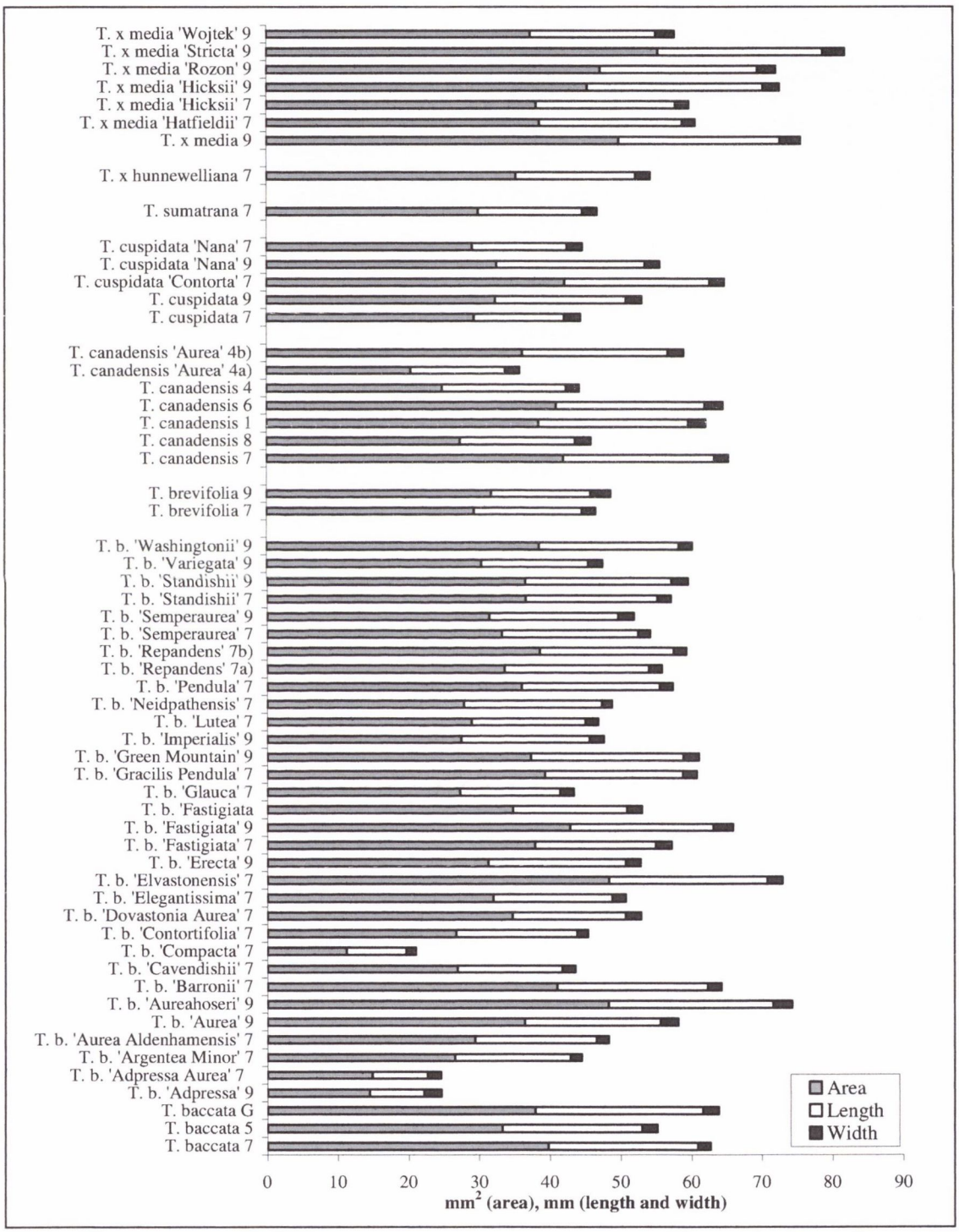
Taxus* X *media

Figure 4.1.2.17 A sample of *T.* X *media*

The *T.* X *media* (**Figure 4.1.2.15**) analysed had a significantly larger area than the variant *T.* X *media* 'Hicksii' ($P<0.001$), with needles exhibiting both significantly longer and wider needles. Although *T.* X *media* is a hybrid of *T. cuspidata* and *T.*

baccata it was found to be significantly larger (P<0.001) than the two "parent plants". Finally, Chadwick and Keen (Chadwick and Keen 1976) quoted needle lengths in excess of 25.4 mm from *T.* X *media* 'Hicksii' however, the sample used in this study was shorter than the expected (24.14 ± 3.91 mm long).

4.1.2.4 Analysis of dried needles



The code for origin is given in section 4.1.2.3.

Figure 4.1.2.18 Needle dimensions of dry *Taxus* species and cultivars

Figure 4.1.2.18 presents the average values of all the dry needles analysed in the current study. The needle dimensions for *T. baccata* samples are given in **Table 4.1.2.2(a)**. **Table 4.1.2.2(b)** includes the needle dimensions of the remaining *Taxus* species and cultivars.

Taxus baccata Three authenticated samples of *T. baccata* and 32 cultivars were examined in this study, including samples from Bedgebury (Kent, UK) (**7**) previously measured manually by Dempsey (Dempsey 2000) and the German sample (**G**) from Niedersächsische Forstliche Verssuchsanstalt in Staufenberg used in section 4.1.2.1. **Figure 4.1.2.19** shows the degree of variation between the samples analysed.

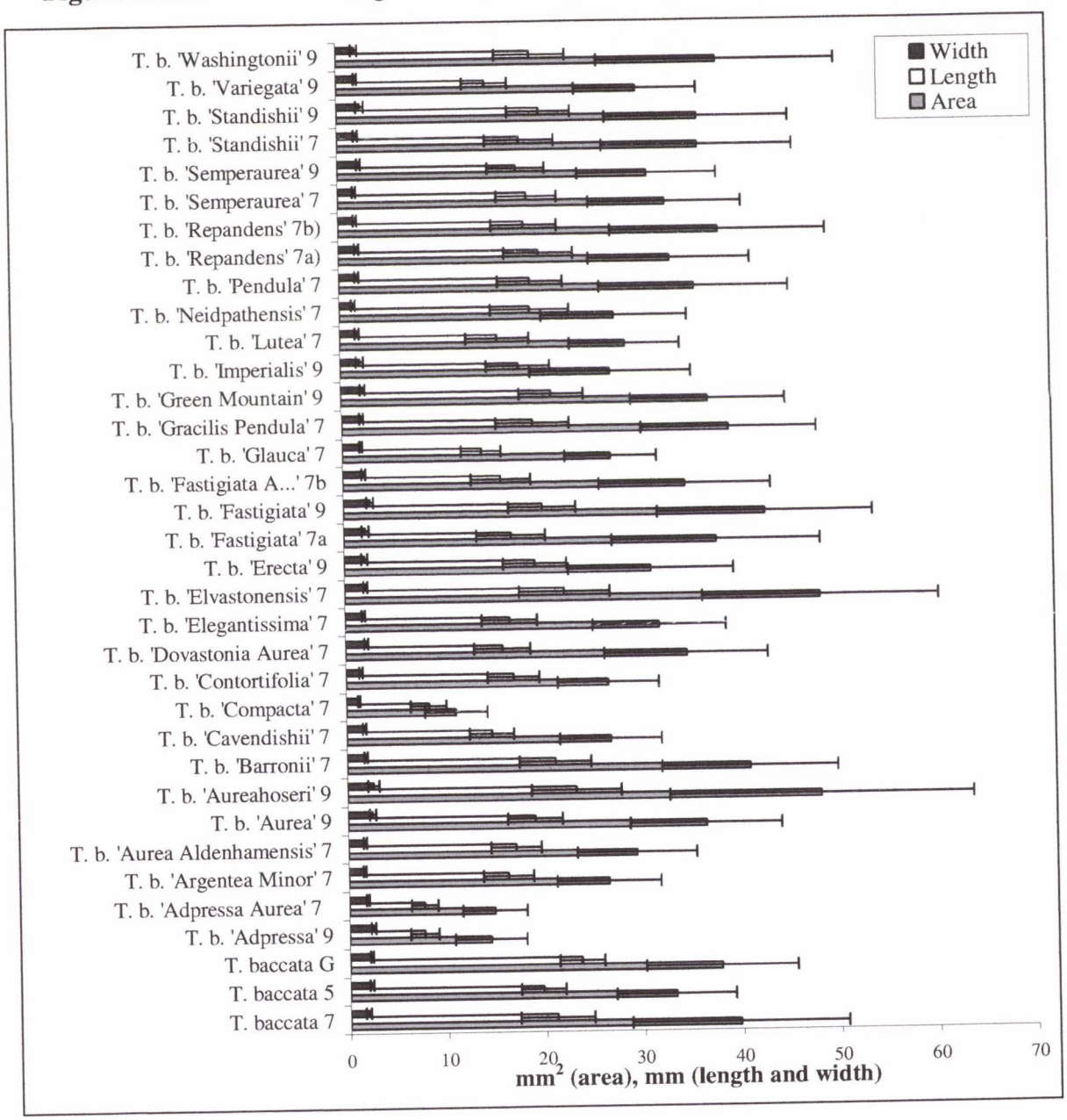


Figure 4.1.2.19 Needle dimensions of dried *Taxus baccata* needles

Table 4.1.2.2 a) Dimensions of dried *Taxus baccata* needles

Sample	Area (mm^2)	Length (mm)	Width (mm)	Origin
T. baccata	39.75 ± 11.01	21.11 ± 3.76	1.86 ± 0.28	7
T. baccata	33.23 ± 6.04	19.74 ± 2.25	2.18 ± 0.19	5
T. baccata	37.92 ± 7.71	23.71 ± 2.28	2.23 ± 0.14	G
T. b. 'Adpressa'	14.44 ± 3.65	7.70 ± 1.42	2.45 ± 0.25	9
T. b. 'Adpressa Aurea'	14.89 ± 3.29	7.73 ± 1.32	1.91 ± 0.15	7
T. b. 'Argentea Minor'	26.53 ± 5.29	16.35 ± 2.57	1.62 ± 0.16	7
T. b.' Aurea Aldenhamensis'	29.42 ± 6.05	17.15 ± 2.57	1.70 ± 0.16	7
T. b. 'Aurea'	36.47 ± 7.72	19.10 ± 2.78	2.52 ± 0.32	9
T. b. 'Aureahoseri'	48.24 ± 15.41	23.36 ± 4.56	2.67 ± 0.57	9
T. b. 'Barronii'	41.04 ± 8.94	21.25 ± 3.65	1.92 ± 0.17	7
T. b. 'Cavendishii'	26.93 ± 5.16	14.83 ± 2.27	1.82 ± 0.17	7
T. b. 'Compacta'	11.21 ± 3.15	8.43 ± 1.79	1.31 ± 0.13	7
T. b. 'Contortifolia'	26.73 ± 5.12	17.12 ± 2.65	1.55 ± 0.17	7
T. b. 'Dovastonia Aurea'	34.69 ± 8.31	16.02 ± 2.88	2.14 ± 0.18	7
T. b. 'Elegantissima'	31.98 ± 6.76	16.78 ± 2.83	1.90 ± 0.17	7
T. b. 'Elvastonensis'	48.35 ± 11.99	22.40 ± 4.59	2.15 ± 0.20	7
T. b. 'Erecta'	31.23 ± 8.41	19.46 ± 3.22	2.08 ± 0.32	9
T. b. 'Fastigiata'	37.90 ± 10.60	17.06 ± 3.51	2.22 ± 0.33	7a
T. b. 'Fastigiata'	42.88 ± 10.90	20.29 ± 3.43	2.73 ± 0.35	9
T. b. 'Fastigiata Aureomarginata'	34.78 ± 8.71	16.12 ± 3.06	2.13 ± 0.20	7b
T. b. 'Glauca'	27.32 ± 4.67	14.14 ± 2.01	1.93 ± 0.15	7
T. b. 'Gracilis Pendula'	39.34 ± 8.93	19.45 ± 3.75	2.02 ± 0.21	7
T. b. 'Green Mountain'	37.34 ± 7.80	21.49 ± 3.26	2.27 ± 0.27	9
T. b. 'Imperialis'	27.47 ± 8.14	18.13 ± 3.24	2.01 ± 0.36	9
T. b. 'Lutea'	28.98 ± 5.55	16.07 ± 2.34	1.79 ± 0.19	9
T. b. 'Neidpathensis'	27.90 ± 7.37	19.42 ± 3.98	1.43 ± 0.20	7
T. b. 'Pendula'	36.08 ± 9.61	19.48 ± 3.32	1.85 ± 0.21	7
T. b. 'Repandens'	33.64 ± 8.15	20.37 ± 3.47	1.87 ± 0.24	7a)
T. b. 'Repandens'	38.6 ± 10.91	18.92 ± 3.34	1.77 ± 0.19	7b)
T. b. 'Semperaurea'	33.25 ± 7.72	19.23 ± 3.08	1.71 ± 0.19	7
T. b. 'Semperaurea'	31.47 ± 7.06	18.21 ± 2.89	2.21 ± 0.21	9
T. b. 'Standishii'	36.60 ± 9.66	18.58 ± 3.52	1.95 ± 0.22	7
T. b. 'Standishii'	36.58 ± 9.31	20.61 ± 3.21	2.37 ± 0.40	9
T. b. 'Variegata'	30.43 ± 6.15	15.14 ± 2.31	2.00 ± 0.15	7
T. b. 'Washingtonii'	38.59± 12.03	19.75 ± 3.61	1.92 ± 0.31	7

G=Germany (the average of 10 German *T. baccata* samples) a) and b) show different samples of same species from same origin

As the *T. baccata* sample from Herstmonceux church (**5**) was assumed to be an authentic *T. baccata* sample, it was dried, pressed and used as a reference standard for the dried *T. baccata* samples used in this study. As observed in section 4.1.2.2 (*Effect of drying*), shrinkage in area between the fresh and the dried sample was observed (~13%). The sample from Bedgebury (**7**) and the German sample (**G**) were both found to be significantly larger ($P<0.001$) than the authentic sample (**5**), although all lengths

measured were within the expected ranges reported by Dallimore and Jackson (1924) (12.7-31.75 mm) and widths within the range (2-2.5 mm) quoted by Vidakovic (1991).

Generally, needle sizes (especially length) showed significant variation (11.21 ± 3.15 mm^2 (*T. b.* 'Compacta') to 48.35 ± 11.99 mm^2 (*T. b.* 'Elvastonensis)) within species. Even with omission of outliers such as the very small-leafed varieties such as *T. b.* 'Adpressa' and *T. b.* 'Compacta', and very long-leaved varieties such as *T. b.* 'Aureahoseri' and *T. b.* 'Elvastonensis' this intraspecific variation was still considered significant ($P<0.001$). The two samples of *T. b.* 'Adpressa' analysed both fell within the expected range given by Dallimore and Jackson (1924) (6.35-12.7 mm long) and Vidakovic (1991) (5-9 x 2-4 mm) having an average needle length of 7.72 ± 0.02 mm and a width of 2.18 ± 0.38 mm. The other small-leafed variety, *T. b.* 'Compacta', also had comparable needle dimensions (8.43 ± 1.79 x 1.31 ± 0.13 mm) to those quoted by Vidakovic (5-10 x 1-1.5 mm).

The *T. b.* 'Fastigiata' sample from Poland **(9)** (20.29 ± 3.43 mm long and 2.73 ± 0.35 mm wide) was found to be significantly larger than the other two samples analysed (**7a** and **b**), but was the only sample to fall within the expected range (Dallimore and Jackson 1924) (20-30 mm long). The other two samples (**7a** and **b**) had a lower average length than expected (17.06 ± 3.51 mm and 16.12 ± 3.06 mm long, respectively).

Finally, Vidakovic (1991) also examined *T. b.* 'Dovastonia' and *T. b* 'Semperaurea' needles (20-35 mm and 10-20 mm long, respectively). The sample of *T. b.* 'Dovastonia Aurea' analysed in our study had a shorter needle length than expected (16.02 ± 2.88 mm), while the *T. b.* 'Semperaurea' samples analysed had an average length that was within the expected range (18.72 ± 0.72 mm).

Other *Taxus* species and cultivars

Dried needles from a number of other *Taxus* species and cultivars were analysed in the study, including several samples of *T. brevifolia* and *T. cuspidata.* Only one sample of the Sumatrana yew (*T. sumatrana*) was obtained, so therefore no comparison can be made with literature values. The fresh samples of *T. canadensis* analysed previously (section 4.1.2.3) were dried and pressed so that they could be directly compared to the

authenticated dried and pressed sample sent from Canada. The hybrid *T.* X *media* (*T. cuspidata* x *T. baccata*) and its varieties were also analysed. Finally needle dimensions of the hybrid *T.* X *hunnewelliana* (*T. cuspidata* x *T. canadensis*) were determined.

Table 4.1.2.2 b) Dimensions of dried needles from *Taxus* species and cultivars

Sample	Area (mm^2)	Length (mm)	Width (mm)	Origin
T. brevifolia	29.45 ± 6.90	15.19 ± 2.82	1.92 ± 0.18	7
T. brevifolia	31.85 ± 6.46	14.03 ± 1.98	2.85 ± 0.41	9
T. canadensis	42.08 ± 9.42	21.33 ± 3.58	1.96 ± 0.21	7
T. canadensis	27.46 ± 5.74	16.27 ± 2.02	2.24 ± 0.32	8
T. canadensis	38.56 ± 7.83	21.17 ± 2.56	2.44 ± 0.32	1
T. canadensis	41.02 ± 5.61	21.04 ± 1.72	2.52 ± 0.20	6
T. canadensis	24.86 ± 3.90	17.60 ± 2.00	1.81 ± 0.15	4
T. canadensis 'Aurea'	20.41 ± 5.13	13.38 ± 2.24	2.05 ± 0.21	4a)
T. canadensis 'Aurea'	36.27 ± 6.16	20.53 ± 2.30	2.27 ± 0.28	4b)
T. cuspidata	29.41 ± 6.11	12.76 ± 2.00	2.30 ± 0.22	7
T. cuspidata	32.42 ± 7.05	18.46 ± 2.37	2.19 ± 0.25	9
T. cuspidata 'Contorta'	42.25 ±10.75	20.50 ± 3.75	2.05 ± 0.25	7
T. cuspidata 'Nana'	32.63 ±10.06	20.97 ± 3.78	2.10 ± 0.36	9
T. cuspidata 'Nana'	29.15 ± 5.32	13.41 ± 2.09	2.18 ± 0.24	7
T. sumatrana	30.01 ± 7.15	14.77 ± 2.64	2.01 ± 0.15	7
T. x *hunnewelliana*	35.39 ± 9.06	16.86 ± 3.23	2.10 ± 0.27	7
T. x *media*	49.84 ± 13.68	22.90 ± 4.17	2.83 ± 0.42	9
T. x *media* 'Hatfieldi'	38.66 ± 8.05	20.17 ± 3.14	1.90 ± 0.15	7
T. x *media* 'Hicksii'	38.21 ± 7.89	19.71 ± 3.25	1.92 ± 0.15	7
T. x *media* 'Hicksii'	45.41 ± 11.23	24.89 ± 3.67	2.37 ± 0.33	9
T. x *media* 'Rozon'	47.24 ± 14.13	22.30 ± 4.25	2.65 ± 0.34	9
T. x *media* 'Stricta'	55.42 ± 14.63	23.36 ± 4.26	3.04 ± 0.52	9
T. x *media* 'Wojtek'	37.42 ± 8.97	17.71 ± 3.17	2.69 ± 0.33	9

a) and b) shows different trees of same species from same origin

Taxus brevifolia, Taxus cuspidata and Taxus sumatrana

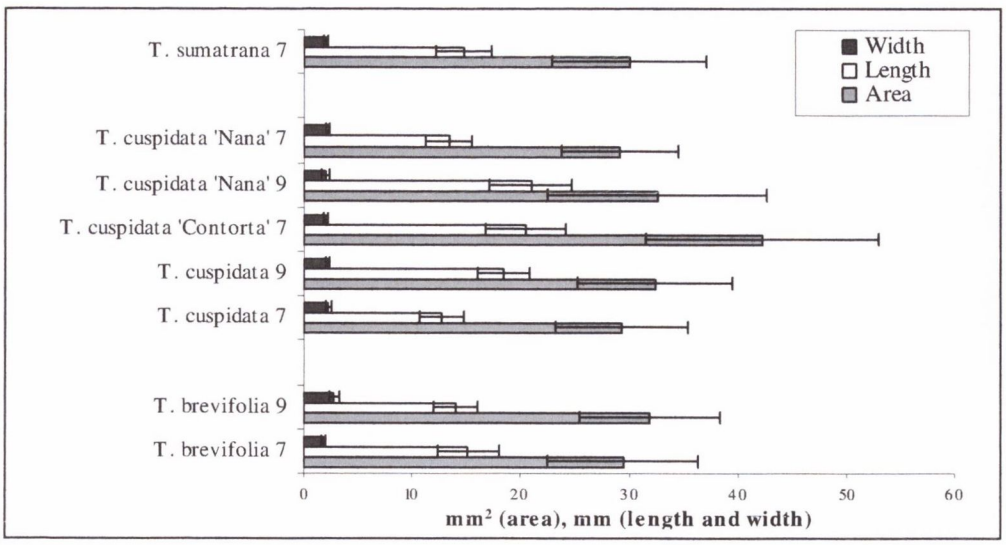


Figure 4.1.2.20 Needle dimensions of *T. brevifolia*, *T. cuspidata* and *T. sumatrana*

The sample of *T. brevifolia* obtained from Bedgebury Pinetum (**7**) was labelled *T. b.* 'Brevifolia'. However, Dempsey (Dempsey 2000) found that the needle dimensions (15.19 ± 2.82 x 1.92 ± 0.18 mm) were significantly ($P<0.001$) above that suggested by Dallimore and Jackson for this variety (rarely above 12.5 mm long). It did, however, agree with the length quoted by Vidakovic (up to 20 mm) for *T. brevifolia*. Since, according to Farjon (Farjon 1998) *T. baccata* 'Brevifolia' is the same as *T. brevifolia*, the latter name will be used for further discussion. The needles from the Polish sample (**9**) also fell within the expected length range (14.03 ± 1.98 mm).

The sample of *T. cuspidata* analysed here had an average needle length of 17.22 ± 3.90 mm and an average width of 2.16 ± 0.10 mm. These results fell within the lower end of the ranges reported by Dallimore and Jackson (12.7-25.4 x 2.12-3.18 mm) and Vidakovic (15-25 x 3 mm). The Polish *T. cuspidata* (**9**) was significantly longer (18.46 ± 2.37 x 2.19 ±0.25 mm, $P<0.001$) than the sample from Bedgebury (**7**) (12.76 ± 2.00 x 2.30 ± 0.22) and much closer to the expected range given above. This was also the case for the Polish *T. cuspidata* 'Nana' samples (**9**) compared to the *T. cuspidata* 'Nana' sample from Bedgebury (**7**) analysed (20.97 ± 3.78 x 2.10 ± 0.36 mm and 13.41 ± 2.07 x 2.18 ± 0.24 mm, respectively).

The needle dimensions of the *T. sumatrana* (**7**) sample analysed fell into the lower end of the range quoted by Vidakovic (12-40 x 2-2.5 mm) with a length of 14.77 ±2.64 mm and width of 2.01 ± 0.15 mm. No conclusion can be made from this result since only one sample of *T. sumatrana* (**7**) was obtained; the results can only be taken as an implication of what dimensions the needles have.

Taxus* X *hunnewelliana* and *Taxus* X *media

T. X *hunnewelliana* (16.86 ± 3.23 x 2.10 ± 0.27 mm) has both shorter and narrower needles than *T.* X *media* (22.9 ± 4.17 x 2.83 ± 0.42 mm). This could be a reflection of that *T.* X *hunnewelliana* is a hybrid of the smaller *T. canadensis* and *T. cuspidata* rather than the larger *T. baccata* and *T. cuspidata*, which is the case of *T.* X *media*.

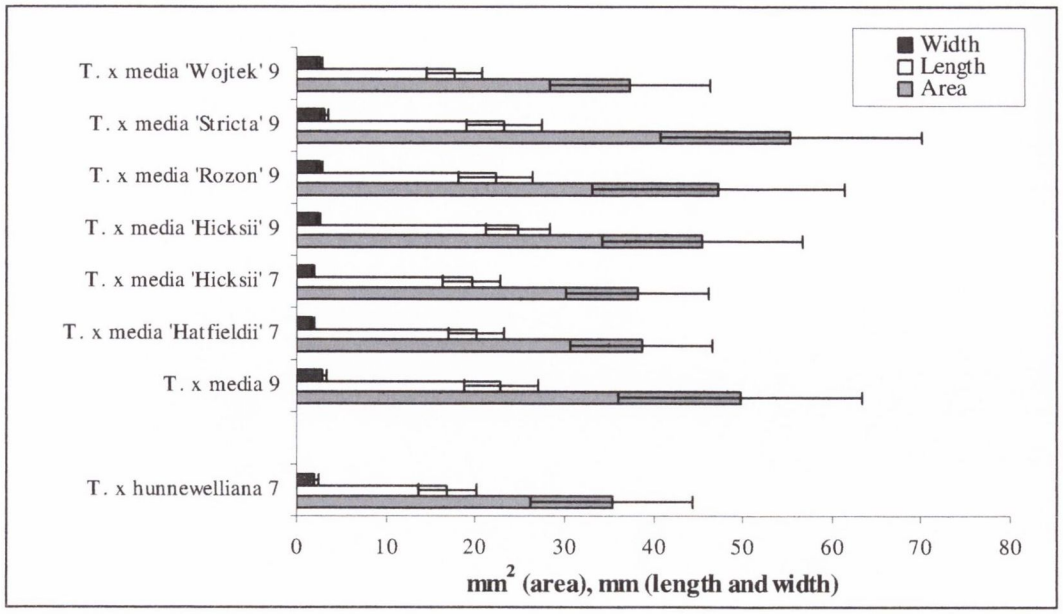


Figure 4.1.2.21 Needle dimensions of dried *Taxus* hybrids

Taxus canadensis In the fresh *T. canadensis* samples analysed a very significant variation was observed both in the length and the width measurements. The samples were therefore dried and pressed in order to be directly compared to an authentic sample obtained from Canada (**8**). **Figure 4.1.2.22** shows the needle dimensions obtained.

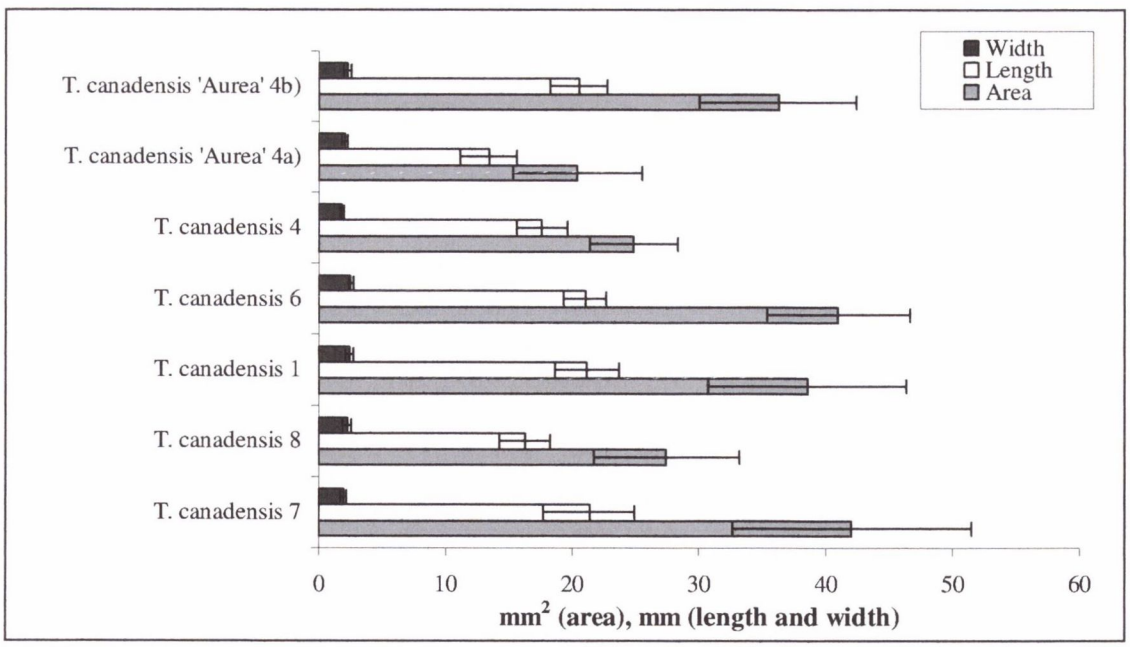


Figure 4.1.2.22 Needle dimensions of dried *T. canadensis*

The authentic *T. canadensis* sample (**8**) had a needle length of 16.27 ± 2.02 mm and fell within the needle length expected according to Dallimore and Jackson (1924) quoting a range of 12.7-19.05 x 1.59-2.12 mm. However, the needles were slightly wider than expected (2.24 ± 0.32 mm). The only other samples that fell within the expected range were *T. canadensis* from Glasnevin (**4**) (higher end: 17.60 ± 2.00 x 1.81 ± 0.15 mm) and *T. canadensis* 'Aurea' **4a**) from Glasnevin (lower end: 13.38 ± 2.24 x 2.05 ± 0.21 mm).

This again questions whether some of the samples collected as *T. canadensis* have been misidentified. In order to attempt to clarify this, a further microscopical and molecular examination of this species was performed. The results of this examination are discussed in section 4.2.

4.1.2.5 Discussion of needle examination

The morphological examination of the needle dimensions showed a high degree of variation both within and between the analysed species. Since needle dimensions are the most common form of morphological examination, the high degree of variation within the various species analysed makes one question the accuracy of these identities. It also highlights the problem of variation caused by geographical location and/or nutritional status, time of year, light intensity, etc.

In a morphological and chemotaxonomical study van Rozendaal et al (van Rozendaal et al. 1999) tried to find groupings within the various species analysed. On the basis of their morphological characteristics five groups were formed:

1) Aurea group: variegated *T. baccata* cultivars
2) Media group: cultivars ascribed to *T.* X. *media.*
3) Wild group: wild trees and cultivars similar to wild trees of *T. baccata* and *T. cuspidata.*
4) Nana group: mainly dwarf plants and other deviating cultivars.
5) Fastigiata group: cultivars belonging to *T. baccata*, easily recognised by their peculiar leaf arrangement, positioned radially around the branchlets.

On the basis of the chemical analysis they divided the species into two groups:

1) a North-American group: *T. canadensis*, *T. floridana*, *T. globosa* and *T. brevifolia* (excluding the species specific taxane brevifoliol).
2) an Eurasian group: *T. baccata*, *T. sumatrana*, *T. cuspidata*, *T.* X. *hunnewelliana* and *T.* X. *media*.

These two groupings are natural groupings because the Eurasian group has no real boarders between the species. They are clearly separated from the North-American group by the Atlantic and the Pacific oceans. They did not find a correlation between the two sets of groups. As they included 18 different morphological characteristics in their analysis, a direct comparison of our results to the five groups based on morphological characters would be of limited value. Amalgamation of the needle data obtained from individual determinations is presented in **Table 4.1.2.3** below.

Table 4.1.2.3 Amalgamated needle dimensions of *Taxus* species and cultivars

Sample	Sample condition	Area (mm^2)	Length (mm)	Width (mm)	No. of samples
T. baccata	Fresh	42.57	21.42	2.58	5
T. baccata cv	Fresh	37.82	18.60	2.63	18
T. baccata	Dry	36.97	21.52	2.09	3
T. baccata cv	Dry	32.54	17.50	2.00	27
T. brevifolia	Fresh	29.76	17.11	2.28	4
T. brevifolia	Dry	30.65	14.61	2.39	2
T. candensis	Fresh	34.73	19.25	2.34	5
T. canadensis cv	Fresh	28.34	16.96	2.16	2
T. candensis	Dry	34.80	19.48	2.19	5
T. canadensis cv	Dry	28.34	16.96	2.16	2
T. chinensis	Fresh	63.64	22.92	3.49	1
T. cuspidata	Fresh	34.39	17.30	2.54	4
T. cuspidata cv	Fresh	29.78	17.50	2.11	2
T. cuspidata	Dry	30.92	15.61	2.25	2
T. cuspidata cv	Dry	34.68	18.29	2.11	3
T. floridana	Fresh	57.29	23.13	3.15	1
T. sumatrana	Fresh	53.56	20.30	3.32	1
T. sumatrana	Dry	30.01	14.77	2.01	1
T. wallichiana	Fresh	50.06	25.65	2.86	1
T. x *hunnewelliana*	Dry	35.39	16.86	2.10	1
T. x *media*	Fresh	62.58	27.24	2.95	1
T. x *media cv*	Fresh	45.83	24.11	2.50	1
T. x *media*	Dry	49.84	22.90	2.83	1
T. x *media cv*	Dry	43.73	21.36	2.43	6

When the results of the needle dimension examination from the present study were divided into Eurasian species (*T. baccata*, *T. sumatrana*, *T. cuspidata*, *T. chinensis*, *T.*

wallichiana, *T.* X. *hunnewelliana* and *T.* X. *media*) and North-American species (*T. canadensis*, *T. floridana*, *T. globosa* and *T. brevifolia*), a clear division within the groupings themselves was observed. In the Eurasian group *T. baccata* and *T. cuspidata* had a smaller needle area than the other species (fresh amalgamated values: 42.57 mm^2 and 34.39 mm^2, respectively), and *T.* X. *media* (a hybrid of *T. baccata* and *T. cuspidata*) had a considerably larger needle area than the rest (fresh amalgamated values: 62.58 mm^2). *T. chinensis* and *T. sumatrana* had considerably broader needles than the rest (fresh amalgamated values: 3.49 and 3.32 mm, respectively) and *T. wallichiana* had longer needles than the rest (fresh amalgamated values: 25.65 mm). Similarly a division was seen in the North-American group, where *T. brevifolia* and *T. canadensis* had similar needle dimensions (fresh amalgamated values: 29.76 and 34.73 mm^2, respectively), and *T. floridana* had considerably larger needles, both longer and wider than in the two other species (fresh amalgamated values: 23.13 x 3.15 mm). The only conclusion this division could bring was that the two North-American species *T. brevifolia* and *T. canadensis* have smaller needles than the rest of the species analysed.

Another approach is to divide the species into:

1) 'Pacific rim' group (species growing close to the Pacific Ocean): *T. brevifolia*, *T. chinensis*, *T. cuspidata*, *T. sumatrana* and *T. wallichiana*.
2) 'Atlantic rim' group (species growing close to the Atlantic Ocean): *T. baccata*, *T. canadensis*, *T. floridana, T. globosa, T.* X. *hunnewelliana* and *T.* X. *media*.

In the 'Pacific rim' group *T. chinensis* and *T. sumatrana* had considerably broader needles than the rest (fresh amalgamated values: 3.49 and 3.32 mm, respectively), *T. wallichiana* had longer needles than the rest (fresh amalgamated values: 25.65 mm), where as *T. brevifolia* and *T. cuspidata* had similar needle dimensions (fresh amalgamated values: 29.76 and 34.39 mm^2, respectively). In the 'Atlantic rim' group *T. floridana* and *T.* X. *media* had larger needles than the rest (fresh amalgamated values: 57.29 and 62.58 mm^2, respectively) and *T. canadensis* had the smallest needles (fresh amalgamated values: 34.73 mm^2). From these results it can be concluded that when the results are grouped in this way, too many differences are present within the group in order to be able to call the group homogenous.

Overall, it was concluded that needle sizes were not of major taxonomic value because of the significant variation in sizes observed. Also because of the difficulties encountered in finding type-specimens for each species, only old reference texts could be referred to.

4.1.3 Seed dimensions

Seed characteristics, as with needle dimensions, have also been used as taxonomic tools in morphological descriptions of species within a genus. However, seed dimensions have not been recorded with the same degree of detail for the genus *Taxus* as they have for needle dimensions (Chadwick and Keen 1976, Dallimore and Jackson 1924, Vidakovic 1991).

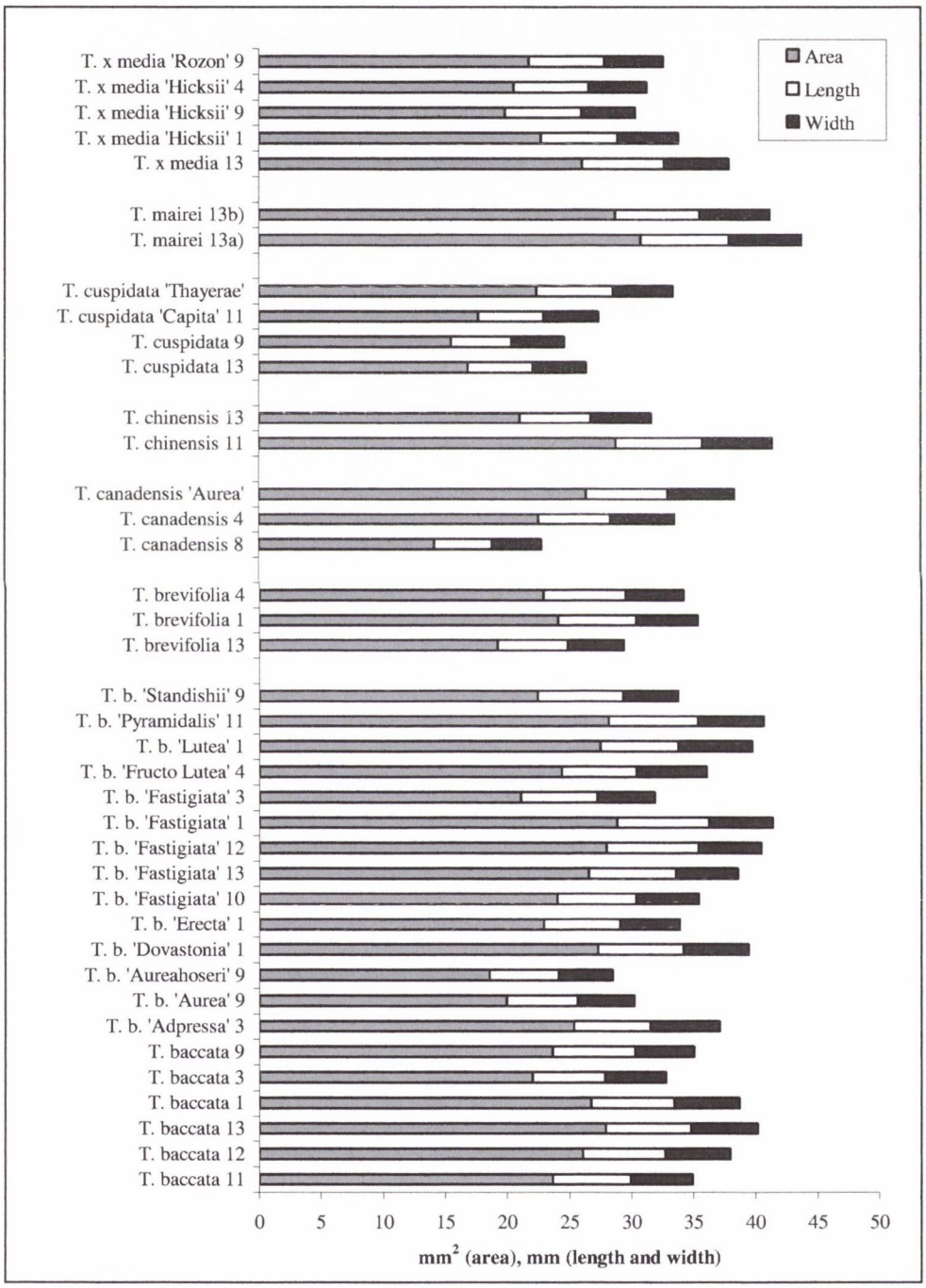
4.1.3.1 Analysis of dried seeds

The seed dimensions including area, length, width and length/width ratio of various *Taxus* samples were determined using WinSEEDLE™ in the same manner as described previously for needles. Some seeds were purchased from seed merchants while others were collected locally (see Materials and Methods, section 3.1.2). Seeds that were collected locally were picked from four sides and at two height levels of the tree and were prepared as suggested by Rudolf (Rudolf 1974) (see Materials and Methods, section 3.1.2). The seeds analysed (n = 100, if possible) were taken randomly from the pooled sample.

The following legends indicate the origin of samples used in analyses:
1: The J. F. Kennedy Park Arboretum, New Ross, Co. Wexford, Ireland.
3: Powerscourt Estate and Gardens, Co. Wicklow, Ireland.
4: National Botanical Gardens, Glasnevin, Dublin, Ireland.
6: The Royal Botanical Gardens Edinburgh, Edinburgh, Scotland.
8: Natural Resources Canada, Fredericton, New Brunswick, Canada.
9: Polska Akademia Nauk, Krónik, Poland.
10: Mount Anville School, Dublin, Ireland.
11: F.W. Schumacher Co. Inc., Massachusetts, U.S.A.
12: Eichenberg & Co., Miltenberg/Main, Germany.

13: Sandeman Seeds, West Sussex, U.K.

The average values for area, length and width of the samples analysed are presented in **Figure 4.1.3.1**, while **Figure 4.1.3.2** shows the width/length ratios for all the seeds. Additionally, seed dimensions for *T. baccata* samples are given in **Table 4.1.3.1a),** and **Table 4.1.3.1b)** including seed dimensions of the remainder of the *Taxus* species and cultivars.



The code of origin is as given in section 4.1.3.1

Figure 4.1.3.1 Dimensions of seeds from *Taxus* species and cultivars

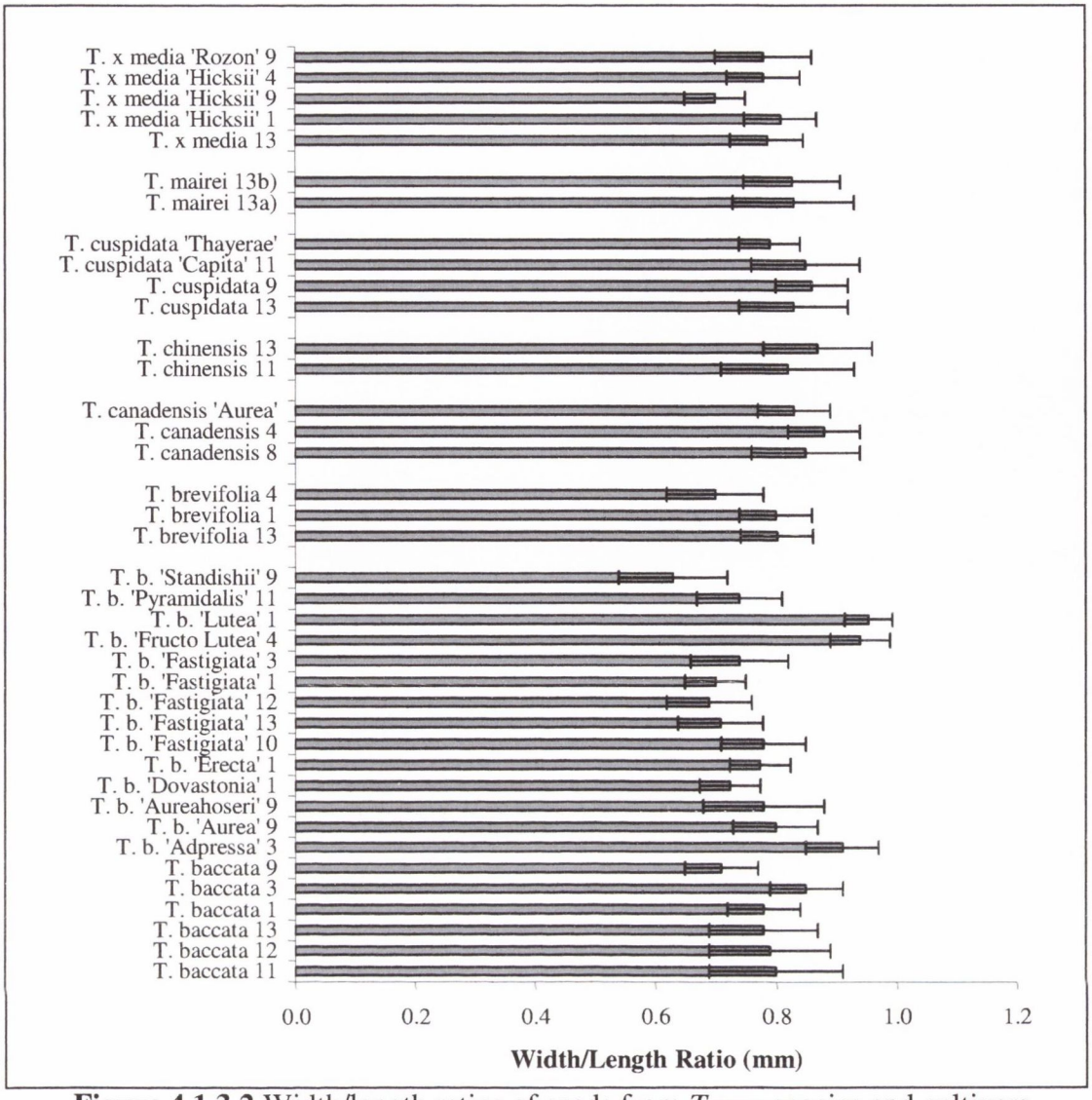


Figure 4.1.3.2 Width/length ratios of seeds from *Taxus* species and cultivars

Taxus baccata

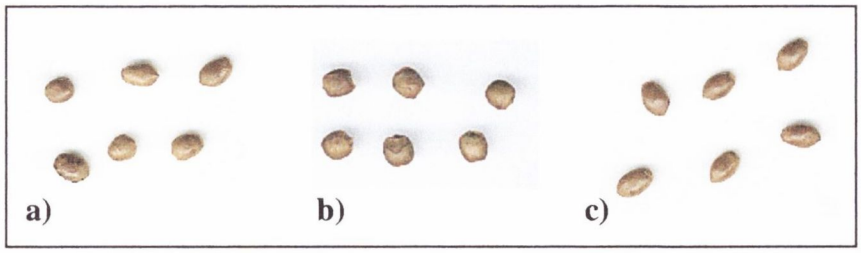


Figure 4.1.3.3 Seeds from a) *T. baccata*, b) *T. b.* 'Adpressa' and c) *T. b.* 'Fastigiata'

Table 4.1.3.1 a) Seed dimensions of *Taxus baccata* seeds

Sample	Area (mm^2)	Length (mm)	Width (mm)	Width/ Length Ratio	Origin
T. baccata	23.68 ± 2.54	6.30 ± 0.55	4.98 ± 0.41	0.80 ± 0.11	11
T. baccata	26.13 ± 4.72	6.63 ± 0.74	5.20 ± 0.52	0.79 ± 0.10	12
T. baccata	27.93 ± 3.53	6.92 ± 0.75	5.35 ± 0.42	0.78 ± 0.09	13
T. baccata	26.76 ± 2.13	6.75 ± 0.32	5.23 ± 0.32	0.78 ± 0.06	1
T. baccata	22.00 ± 1.63	5.91 ± 0.32	4.91 ± 0.31	0.85 ± 0.06	3
T. baccata	23.64 ± 1.57	6.68 ± 0.36	4.71 ± 0.35	0.71 ± 0.06	9
T. b. 'Adpressa'	25.40 ± 1.75	6.11 ± 0.27	5.57 ± 0.36	0.91 ± 0.06	3
T. b. 'Aurea'	19.94 ± 2.27	5.71 ± 0.36	4.58 ± 0.45	0.80 ± 0.07	9
T.b. 'Aureahoseri'	18.58 ± 2.86	5.55 ± 0.49	4.37 ± 0.60	0.78 ± 0.10	9
T. b.'Dovastonia'	27.34 ± 2.83	6.83 ± 0.54	5.29 ± 0.37	0.72 ± 0.05	1
T. b. 'Erecta'	22.94 ± 1.50	6.18 ± 0.22	4.78 ± 0.26	0.77 ± 0.05	1
T. b.'Fastigiata'	24.09 ± 2.14	6.36 ± 0.42	4.96 ± 0.32	0.78 ± 0.07	10
T. b.'Fastigiata'	26.56 ± 3.67	7.04 ± 0.76	4.99 ± 0.37	0.71 ± 0.07	13
T. b.'Fastigiata'	28.01 ± 8.43	7.40 ± 1.60	5.04 ± 0.50	0.69 ± 0.07	12
T. b.'Fastigiata'	28.86 ± 2.00	7.39 ± 0.32	5.17 ± 0.31	0.70 ± 0.05	1
T. b.'Fastigiata'	21.08 ± 2.69	6.18 ± 0.47	4.62 ± 0.54	0.74 ± 0.08	3
T. b. 'Fructo Lutea'	24.40 ± 1.58	6.02 ± 0.30	5.67 ± 0.24	0.94 ± 0.05	4
T. b. 'Lutea'	27.54 ± 2.34	6.26 ± 0.28	5.97 ± 0.35	0.95 ± 0.04	1
T. b.'Pyramidalis'	28.20 ± 3.02	7.15 ± 0.51	5.29 ± 0.40	0.74 ± 0.07	11
T. b. 'Standishii'	22.46 ± 4.37	6.90 ± 0.93	4.41 ± 0.73	0.63 ± 0.09	9

The code of origin is as given in section 4.1.3.1

Six seed samples of *T. baccata* and 14 cultivars were included in this study. Vidakovic (1991) examined the seeds of some *Taxus* species and cultivars. Amongst them were the seeds of *T. baccata*, with a length of 6-7 mm and a width of 5 mm. The average of the *T. baccata* seeds analysed in the present study fell within this range (6.53 ± 0.36 x 5.06 ± 0.24 mm), although the seeds were slightly wider than expected. The *T. baccata* seeds were described as drupe-like, and this description corresponds well with the width/length ratio observed (0.79 ± 0.05). The seed areas for *T. baccata* cultivars analysed did not vary to any great extent (average area 24.78 ± 2.97 mm^2) with only the *T. b.* 'Aurea' and *T. b.* 'Aureahoseri' seeds being slightly smaller than the rest (19.94 ± 2.27 and 18.58 ± 2.86 mm^2, respectively).

The major difference in the seed dimensions of *Taxus baccata* cultivars was the shape of the seeds, reflected in the width/length ratios observed. Seeds from *T. b.* 'Lutea', *T. b.* 'Fructo lutea' and *T. b.* 'Adpressa' all had a rounder shape, with a width/length ratio close to 1, than the rest (0.95 ± 0.04, 0.94 ± 0.05 and 0.91 ± 0.06, respectively), whereas seeds from *T. b* 'Standishii' (0.63 ± 0.09) and most of *T. b.* 'Fastigiata'

(average 0.71 ± 0.02) seeds analysed were much more oval. The average width/length ratio of all seeds from the *T. baccata* cultivars was 0.78 ± 0.08.

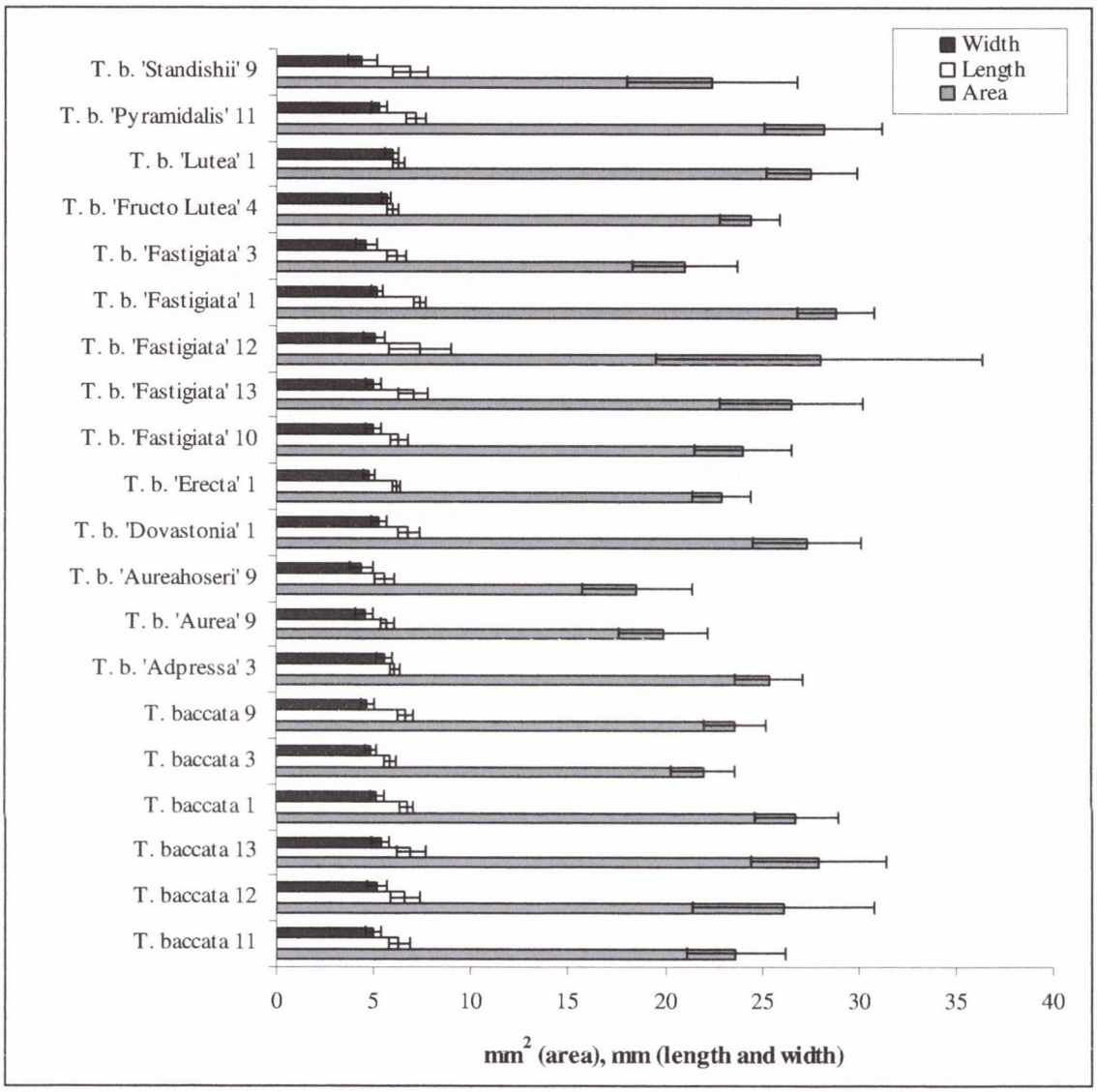


Figure 4.1.3.4 Seed dimensions of *Taxus baccata* seeds

Other *Taxus* species and cultivars

The seed dimensions for other *Taxus* species, cultivars and hybrids are presented in **Table 4.1.3.1b)** below, including *T. brevifolia*, *T. canadensis*, *T. chinensis*, *T. mairei*, *T. cuspidata*, *T.* X. *media*.

Table 4.1.3.1 b) Seed dimensions of seeds from *Taxus* species and cultivars

Sample	Area (mm^2)	Length (mm)	Width (mm)	Width/ Length Ratio	Origin
T. brevifolia	19.21 ± 1.73	5.65 ± 0.32	4.53 ± 0.29	0.80 ± 0.06	13
T. brevifolia	24.07 ± 3.58	6.30 ± 0.78	5.00 ± 0.30	0.80 ± 0.06	1
T. brevifolia	22.90 ± 2.59	6.63 ± 0.44	4.68 ± 0.51	0.70 ± 0.08	4
T. canadensis	14.14 ± 1.93	4.67 ± 0.36	3.99 ± 0.43	0.85 ± 0.09	8
T. canadensis	22.56 ± 1.95	5.84 ± 0.31	5.15 ± 0.30	0.88 ± 0.06	4
T. canadensis 'Aurea'	26.42 ± 1.50	6.53 ± 0.35	5.42 ± 0.24	0.83 ± 0.06	4'
T. chinensis	28.80 ± 6.02	6.96 ± 1.35	5.60 ± 0.47	0.82 ± 0.11	11
T. chinensis	21.07 ± 4.89	5.70 ± 0.94	4.90 ± 0.46	0.87 ± 0.09	13
*T. mairei**	30.83 ± 4.20	7.10 ± 0.97	5.82 ± 0.40	0.83 ± 0.10	13
*T. mairei**	28.74 ± 3.37	6.81 ± 0.57	5.63 ± 0.40	0.83 ± 0.08	13
T. cuspidata	16.87 ± 3.22	5.23 ± 0.75	4.29 ± 0.39	0.83 ± 0.09	13
T. cuspidata	15.52 ± 1.22	4.89 ± 0.26	4.21 ± 0.26	0.86 ± 0.06	9
T. cuspidata 'Capita'	17.67 ± 4.12	5.28 ± 0.87	4.42 ± 0.45	0.85 ± 0.09	11
T. cuspidata 'Thayerae'	22.39 ± 1.85	6.18 ± 0.39	4.86 ± 0.24	0.79 ± 0.05	4
T. X *media*	26.05 ± 1.70	6.64 ± 0.29	5.21 ± 0.30	0.79 ± 0.06	13
T. X *media* 'Hicksii'	22.74 ± 1.81	6.15 ± 0.42	4.97 ± 0.25	0.81 ± 0.06	1
T. X *media* 'Hicksii'	19.85 ± 1.62	6.19 ± 0.22	4.35 ± 0.30	0.70 ± 0.05	9
T. X *media* 'Hicksii'	20.56 ± 1.56	6.03 ± 0.31	4.67 ± 0.28	0.78 ± 0.06	4
T. X *media* 'Rozon'	21.76 ± 2.76	6.10 ± 0.47	4.77 ± 0.56	0.78 ± 0.08	9

* According to Farjon (Farjon 1998) *T. mairei* is another name for *T. chinensis* (Pilg.) Redher var. mairei
' = Same sample as *T. canadensis* 'Aurea' 4b) in needle discussion
The code of origin is as given in section 4.1.3.1

Taxus brevifolia* and *Taxus canadensis

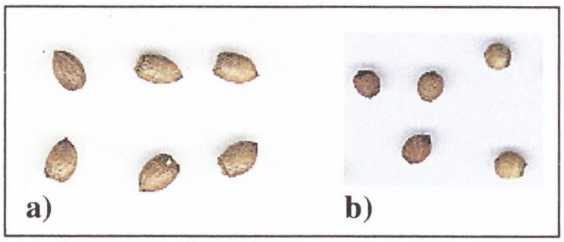


Figure 4.1.3.5 Seeds from a) *T. brevifolia* and b) *T. canadensis*

The mean seed length (6.19 ± 0.50 mm) for the *T. brevifolia* samples analysed was below the length of 8 mm recorded by Vidakovic (1991). The value reported by Dallimore and Jackson (1924) was even longer (12.7 mm long). The shape of the seeds was similar to *T. baccata* seeds, having a mean width/length ration of 0.77 ± 0.06. Dallimore and Jackson described the seeds as ovoid, which corresponds to the

observations made in this study. Vidakovic (1991) described the *T. canadensis* seeds as being short and wide, and dimensions (5x4 mm) given by Chadwick and Keen (1976) corresponds with that statement. The dimensions of the seeds from the authentic *T. canadensis* were found to be in the higher end of the expected range (4.67 ± 0.36 x 3.99 ± 0.43 mm), whereas the two other samples analysed, *T. canadensis* (4) and *T. canadensis* 'Aurea' (4b)) were significantly larger than expected (22.56 ± 1.95 and 26.42 ± 1.50 mm^2, respectively ($P<0.001$)). All the *T. canadensis* seeds analysed were considerably rounder (average width/length ratio of 0.85 ± 0.03) than the *T. baccata* and the *T. brevifolia* seeds.

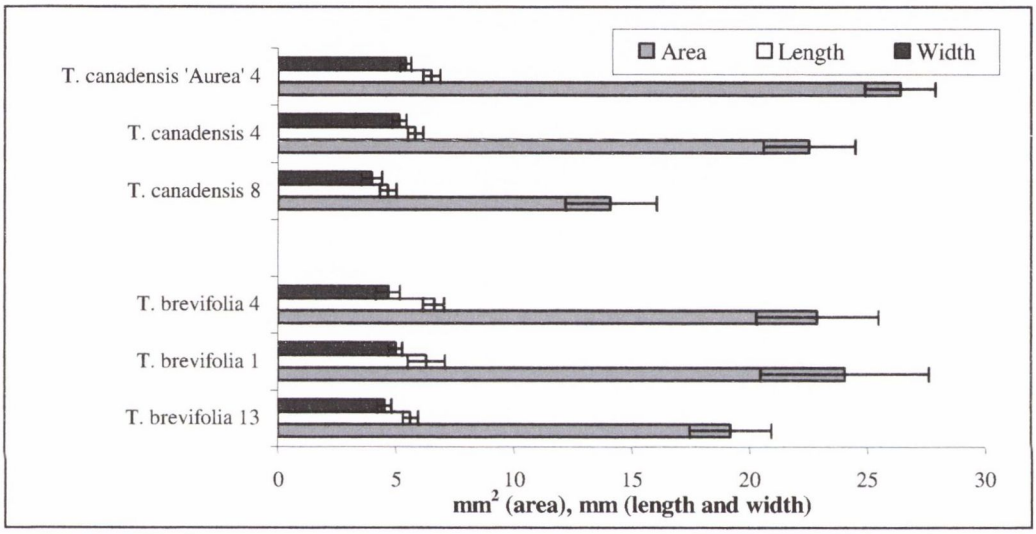


Figure 4.1.3.6 Seed dimensions for *T. brevifolia* and *T. canadensis*

Taxus chinensis* and *Taxus cuspidata

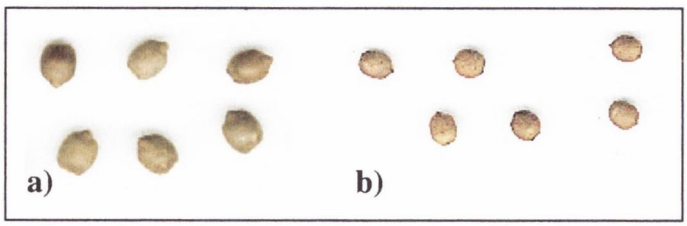


Figure 4.1.3.7 Seeds from a) *T. chinensis* and b) *T. cuspidata*

Two samples of *T. chinensis* and two samples of *T. mairei* were analysed. According to Farjon (Farjon 1998) *T. mairei* is a variety of *T. chinensis*, called *T. chinensis* var. mairei, or according to the nomenclature used in this thesis: *T. chinensis* 'Mairei'. Dallimore and Jackson (1924) reported a seed length of 5.08-6.35 mm and a width of 4.06 mm, whereas here the result was slightly above the expected range (6.64 ± 0.64 x

5.49 ± 0.41 mm). The seeds had a similar shape to those of *T. canadensis*, with a width/length ratio of 0.84 ± 0.02 mm. The *T. cuspidata* seed dimensions were expected to be 6 mm long and 5 mm wide according to Chadwick and Keen (1976), and they found the variety 'Thayerae' to be slightly narrower, having a width of 4 mm. The average range found in this study for *T. cuspidata* was in the lower end of what was expected (5.06 ± 0.24 x 4.25 ± 0.05 mm), and the seeds were quite round in shape, having a width/length ratio of 0.85 ± 0.02. Seeds from the variety 'Capita' were very similar to those of *T. cuspidata*, whereas the 'Thayerae' seeds were both longer and wider than expected (6.18 ± 0.39 x 4.86 ± 0.24mm). They also differed in shape, having a much more oval appearance (width/length ratio of 0.79 ± 0.05).

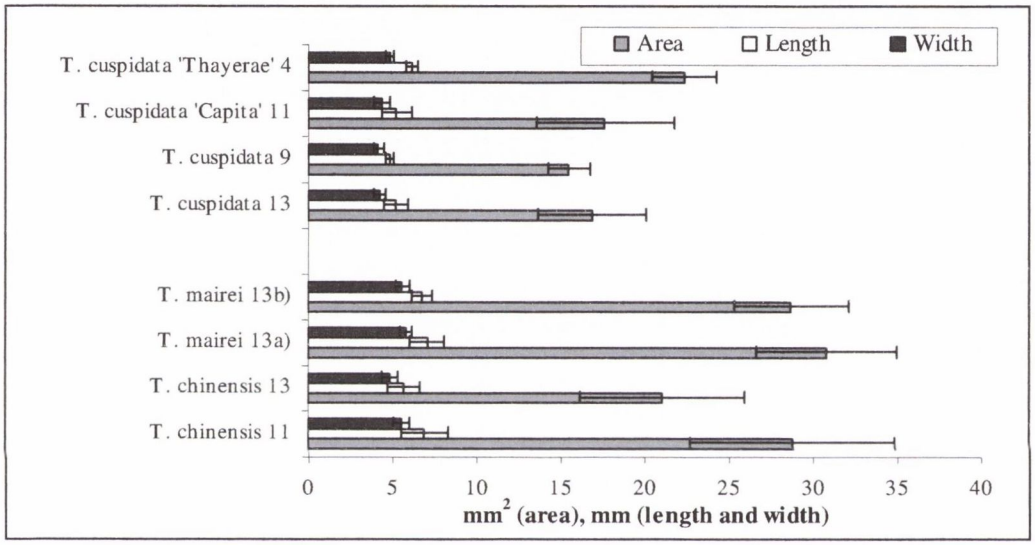


Figure 4.1.3.8 Seed dimensions of *T. chinensis* and *T. cuspidata*

Taxus* X *media

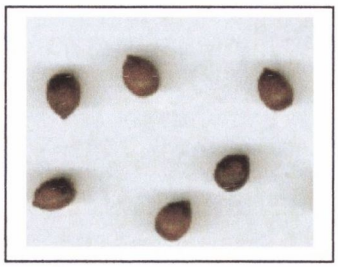


Figure 4.1.3.9 Seeds from *T.* X *media*

The seeds of the hybrid *T.* X *media* (26.05 ± 1.70 mm^2) were somewhat larger than the other varieties 'Hicksii' (21.05 ± 1.50 mm^2) and 'Rozon' (21.76 ± 2.76 mm^2). Size-wise the seeds of this hybrid resemble, to a higher degree, the seeds from *T. baccata* (24.78 ± 2.97 mm^2) than the smaller seeds from the other 'parent plant', *T. cuspidata*

(16.19 ± 0.96 mm^2). This is also true for the seed-shape, where *T.* X *media* seeds were ovate (width/length ratio of 0.77 ± 0.04) like *T. baccata* seeds.

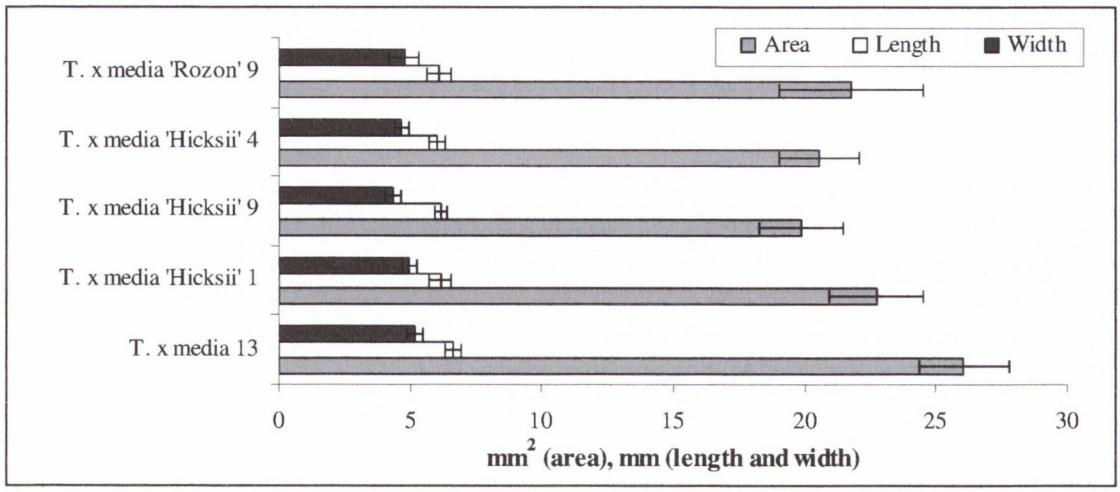


Figure 4.1.3.10 Seed dimensions for *T.* X *media*

4.1.3.2 Discussion of seed examination

The variation in the seed dimensions was mainly found to be inter-specific. The variation within the species was not as obvious as it was in the needle dimensions. Even in the *T. baccata* cultivars the length and width of the samples were similar. The main difference was found to be the actual shape of the seeds. This could bee seen in the width/length ratio. An amalgamation of the seed dimensions is presented in **Table 4.1.3.2**.

The seeds from *T. chinensis* were found to be significantly larger (27.36 ± 4.31 mm^2; $P<0.001$) than the rest. The seeds were longer (6.64 ± 0.64 mm, $P<0.001$), but also wider (5.49 ± 0.41 mm, $P<0.001$) than any of the other species analysed. The *T. baccata* (25.05 ± 2.26 mm^2), *T. baccata* cv. (24.67 ± 3.30 mm^2) and *T.* X *media* (26.05 ± 1.70 mm^2) were also quite large (no significant difference between these three samples ($P>0.05$)), but of a different shape to *T. chinensis*. The smallest seeds analysed were those of the authentic *T. canadensis* sample. They were both significantly shorter (4.67 ± 0.36 mm) and narrower (3.99 ± 0.43 mm) than any of the other seeds ($P<0.001$), although the shape, was not significantly different to that of the other *T. canadensis* seeds analysed, they were smaller.

Table 4.1.3.2 Amalgamated seed dimensions of *Taxus* species and cultivars

Sample	Area (mm^2)	Length (mm)	Width (mm)	Width/Length Ratio	No. of samples
T. baccata	25.02	6.53	5.06	0.79	6
T. baccata cv.	24.67	6.50	5.05	0.78	14
T. brevifolia	22.06	6.19	4.73	0.77	3
T. canadensis *	14.14	4.67	3.99	0.85	1
T. canadensis cv.	24.49	6.19	5.29	0.86	2
T. chinensis	27.36	6.64	5.49	0.84	4
T. cuspidata	16.19	5.06	4.25	0.85	2
T. cuspidata cv.	20.03	5.73	4.64	0.82	2
T. X *media*	26.05	6.64	5.21	0.79	1
T. X *media cv.*	21.23	6.12	4.69	0.77	4

* Authentic *T. canadensis* sample from Canada (8).

From these results we can conclude that the seed characteristics would be more appropriate tools for distinguishing between the various species within the genus *Taxus*. However, overlap between certain species is still a problem, and seed characteristics can not be used on their own in order to identify a specific sample.

4.1.4 Macroscopical appearance of *Taxus* trees used in examination

The appearance of the trees used in the examination has to be taken into consideration when the authentication of a particular tree is in question. Characteristics such as *tree shape*, *foliage colour* and *gender* are presented for *Taxus baccata* trees in **Table 4.1.4.1** and for other *Taxus* species and cultivars in **Table 4.1.4.2**.

Table 4.1.4.1 Characteristics of *Taxus baccata* trees used for analysis

Genus	Species	Cultivar	Source code*	Description of Tree		Gender	Origin**
				Shape	Colour		
Taxus	*baccata*		9L7	Pyramidal, tree	Green	F	1
Taxus	*baccata*			Pyramidal, tree	Green	F	2
Taxus	*baccata*			Pyramidal, tree	Green	M	3a)
Taxus	*baccata*			Pyramidal, tree	Green	F	3b)
Taxus	*baccata*			Pyramidal, tree	Green		5
Taxus	*baccata*			Pyramidal, tree	Green	F	7
Taxus	*baccata*			Pyramidal, tree	Green	M	G
Taxus	*baccata*	'Adpressa'	699296	Bun-shaped, shrub	Green	F	6
Taxus	*baccata*	'Adpressa'	T	Bun-shaped, shrub	Green	F	3
Taxus	*baccata*	'Adpressa'		Bun-shaped, shrub	Green	F	9

*Source code is code given by Botanical gardens for identification of tree **Code of origin is as given in section 4.1.2.3

Table 4.1.4.1-continued Characterisation of *Taxus baccata* trees used for analysis

Genus	Species	Cultivar	Source code*	Description of Tree		Gender	Origin**
				Shape	Colour		
Taxus	*baccata*	'Adpressa Aurea'	071024	Bun-shaped, shrub	Golden	F	6
Taxus	*baccata*	'Adpressa Aurea'		Bun-shaped, shrub	Golden	M	7
Taxus	*baccata*	'Argentea Minor'		Dwarf shrub	Golden	M	7
Taxus	*baccata*	'Aurea Aldenhamensis'		Globose shrub	Golden	M	7
Taxus	*baccata*	'Aurea'		Globose, bush	Golden		9
Taxus	*baccata*	'Aurea'		Globose, bush	Golden	F	3
Taxus	*baccata*	'Aureahoseri'			Golden		9
Taxus	*baccata*	'Barroni'		Bun-shaped shrub	Golden	F	7
Taxus	*baccata*	'Cavendishii'		Low-spreading dwarf shrub	Green	F	7
Taxus	*baccata*	'Compacta'		Globose small shrub	Green	F	7
Taxus	*baccata*	'Contortifolia'		Arching bush, contorted	Green	F	7
Taxus	*baccata*	'Densifolia'	6563	Pyramidal, tree	Green	M	4
Taxus	*baccata*	'Dovastonia'	25	Pyramidal, bush with weeping branchlets	Green	M	3
Taxus	*baccata*	'Dovastonia'		Pyramidal, bush with weeping branchlets	Green	M	2
Taxus	*baccata*	'Dovastonia Aurea'	9L30	Pyramidal, bush with weeping branchlets	Golden	M	1
Taxus	*baccata*	'Dovastonia Aurea'		Pyramidal, bush with weeping branchlets	Golden	F	7
Taxus	*baccata*	'Elegantissima'		Wide-spreading oval shrub	Golden	M	7
Taxus	*baccata*	'Elvastonensis'		Arching shrub	Golden	M	7
Taxus	*baccata*	'Erecta'		Columnar, tree fastigiate branchlets	Green	M	9
Taxus	*baccata*	'Fastigiata'		Columnar, tree fastigiate branchlets	Green	F	7
Taxus	*baccata*	'Fastigiata'		Columnar, tree fastigiate branchlets	Green	F	9
Taxus	*baccata*	'Fastigiata'		Columnar, tree fastigiate branchlets	Green	F	1a)
Taxus	*baccata*	'Fastigiata'		Columnar, tree fastigiate branchlets	Green	F	1b)
Taxus	*baccata*	'Fastigiata'		Columnar, tree fastigiate branchlets	Green	F	1c)
Taxus	*baccata*	'Fastigiata Aureomarginata'		Columnar, tree fastigiate branchlets	Golden	M	7
Taxus	*baccata*	'Glauca'		Oval small tree	Green	M	7

*Source code is code given by Botanical gardens for identification of tree **Code of origin is as given in section 4.1.2.3

Table 4.1.4.1-continued Characterisation of *Taxus baccata* trees used for analysis.

Genus	Species	Cultivar	Source code*	Description of Tree		Gender	Origin**
				Shape	Colour		
Taxus	*baccata*	'Gracilis Pendula'		Tree with weeping branchlets	Green	F	7
Taxus	*baccata*	'Green Mountain'			Golden		9
Taxus	*baccata*	'Imperialis'			Green		9
Taxus	*baccata*	'Jacksonii'	19688018		Green	M	6
Taxus	*baccata*	'Lutea'		Bun-shaped, shrub	Green	F	3
Taxus	*baccata*	'Lutea'		Bun-shaped, shrub	Green	F	2
Taxus	*baccata*	'Neidpathensis'	6569	Pyramidal, tree	Green	M	4
Taxus	*baccata*	'Neidpathensis'		Pyramidal, tree	Green	M	7
Taxus	*baccata*	'Pendula'		Bun-shaped shrub	Green	F	7
Taxus	*baccata*	'Pyramidalis'	6594	Pyramidal, tree	Green	M	4
Taxus	*baccata*	'Repandens'		Arching, low-spreading shrub	Green	F	7a)
Taxus	*baccata*	'Repandens'		Arching, low-spreading shrub	Green	F	7b)
Taxus	*baccata*	'Repandens Aurea'	19688019	Arching, low speading	Green	F	6
Taxus	*baccata*	'Semperaurea'		Broad, globosa shrub	Golden	F	9
Taxus	*baccata*	'Semperaurea'		Broad, globosa shrub	Golden	F	7
Taxus	*baccata*	'Standishii'	19588809	Narrow, columnar, fastigiate	Golden	F	6
Taxus	*baccata*	'Standishii'		Narrow, columnar, fastigiate	Golden	F	7
Taxus	*baccata*	'Standishii'		Narrow, columnar, fastigiate	Golden	F	9
Taxus	*baccata*	'Variegata'		Oval-shaped bush	Golden/ Green	M	7
Taxus	*baccata*	'Washingtonii'		Arching shrub/bush	Golden	F	7
Taxus	*baccata*	'Washingtonii'	19699305	Arching shrub/bush	Golden	F	6

*Source code is code given by Botanical gardens for identification of tree **Code of origin is as given in section 4.1.2.3

Table 4.1.4.2 Characterisation of other *Taxus* species and cultivars analysed

Genus	Species	Cultivar	Source code*	Description of Tree		Gender	Origin**
				Shape	Colour		
Taxus	*brevifolia*		8M34	Pyramidal, tree/bush	Green	F	1a)
Taxus	*brevifolia*		8L33	Pyramidal, tree/bush	Green	F	1b)
Taxus	*brevifolia*		8L35	Pyramidal, tree/bush	Green	F	1c)
Taxus	*brevifolia*			Pyramidal, tree/bush	Green	F	7
Taxus	*brevifolia*		6759	Pyramidal, tree/bush	Green	F	4
Taxus	*brevifolia*			Pyramidal, tree/bush	Green	F	9
Taxus	*canadensis*		8M5	Low-growing/ oval shrub	Green	F	1
Taxus	*canadensis*		6556	Arching bush/tree	Green	F	4
Taxus	*canadensis*		271013	Low-growing/ oval shrub	Green	M	6

*Source code is code given by Botanical gardens for identification of tree **Code of origin is as given in section 4.1.2.3

Table 4.1.4.2-continued Characterisation of other *Taxus* species and cultivars analysed

Genus	Species	Cultivar	Source code*	Description of Tree		Gender	Origin**
				Shape	Colour		
Taxus	*canadensis*		271013	Low-growing/ oval shrub	Green	M	6
Taxus	*canadensis*			Low-growing, wide-spreading shrub	Green	F	8
Taxus	*canadensis*			Low-growing/ bun-shaped bush	Green	M	7
Taxus	*canadensis*	'Aurea' (1)	6549	Low-growing/ oval bush	Golden	M	4a)
Taxus	*canadensis*	'Aurea' (2)	6570	Globose/oval bush	Golden	F	4b)
Taxus	*chinensis*		311011	Pyramidal tree	Golden	M	6
Taxus	*cuspidata*		8M9	Globose tree, speading or ascending branches	Green	M	1
Taxus	*cuspidata*		6601	Globose tree, speading or ascending branches	Green	M	4
Taxus	*cuspidata*			Globose small tree	Green	M	7
Taxus	*cuspidata*			Globose tree, speading or ascending branches	Green		9
Taxus	*cuspidata*	'Contorta'		Bun-shaped, arching shrub	Green	M	7
Taxus	*cuspidata*	'Luteo Baccata'	6571	Globose tree, speading or ascending branches	Green	F	4
Taxus	*cuspidata*	'Nana'		Low-spreading shrub	Green	M	9
Taxus	*cuspidata*	'Nana'		Low-spreading shrub	Green	F	7
Taxus	*cuspidata*	'Thayerae'	6593	Wide-spreading bush	Green	F	4
Taxus	*floridana*		7M49	Shrub/small bushy tree	Green	M	1
Taxus	*sumatrana*		8M8	Oval shaped shrub	Golden	M	1
Taxus	*sumatrana*			Oval shaped shrub	Golden	M	7
Taxus	*wallichia*		19902824	Bushy tree	Green	F	6
Taxus	X *hunnewelliana*			Bun-shaped bush	Green/ Golden	F	7
Taxus	X *media*		8M54	Arching bush/shrub	Green	M	1
Taxus	X *media*			Arching bush/shrub	Green		9
Taxus	X *media*	'Hatfieldi'		Tall, oval-shaped	Green	F	7
Taxus	X *media*	'Hicksii'		Oval to fastigiate tree	Green	F	7
Taxus	X *media*	'Hicksii'	6584	Oval to fastigiate tree	Green	F	4
Taxus	X *media*	'Hicksii'		Oval to fastigiate tree	Green	F	9
Taxus	X *media*	'Rozon'			Green		9
Taxus	X *media*	'Stricta'		Columnar fastigiate	Green	M	9
Taxus	X *media*	'Wojtek'		Columnar, narrow, fastigiate	Green		9

*Source code is code given by Botanical gardens for identification of tree **Code of origin is as given in section 4.1.2.3

The tree shapes and foliage colour presented in **Figure 4.1.4.1** and **4.1.4.2** were compared with pictures from the National Centre for Biotechnology Information (NCBI) Taxonomy Database (NCBI 2005) and found to comply.

4.1.5 Conclusion of morphological examination

From the preliminary experiments done in order to determine a suitable collection protocol for the samples used in the morphological examination it was concluded that height and location on tree affected the needle sizes. Although the difference in needle size was not statistically significant, the trend showed that the needles on the lower-situated branches were longer than the higher ones. In addition two of the four sides of the tree also had slightly longer needles. The effect of drying the samples was also investigated, and it was found that the surface-area decreased and the shape of the needles was distorted. In addition it was observed that the difference in surface-area of the previous year's and this year's growth, which before drying was significant, decreased upon drying. These findings illustrated the necessity of pooling needles from all the eight twigs collected from each tree (two heights and four sides) before randomly selecting the needles used for analysis. Dry and fresh samples cannot be compared to each other and must be discussed separately.

This highlights the problem of the usage of herbarium samples of *Taxus*. Herbarium samples usually consist only of one twig, and the actual location on the tree (distance from the ground etc.) from where this twig was collected is never/seldom included in the description. Because the sample has been pressed and dried, there has been a decrease in surface-area, and the needle shape could also have been distorted. As a result of the findings in our preliminary experiments we can conclude that for correct needle dimensions the samples should be kept fresh for analysis.

From the results of the examination of needle dimensions from both fresh and dried samples of *Taxus baccata* and its cultivars it was obvious that there was a significant variation in sizes (especially area and length) even when the outliers were excluded from the calculations. One trend observed in the results of the needle examinations of *T. baccata* was that younger or more "modern" trees generally had longer needles than the older, larger trees. The seed dimensions obtained for this species were also shown to vary considerably, mainly due to the actual shape of the seed (reflected in the width/length ratio). Although the variation within the species was big, most of the samples of *T. baccata* and its cultivars analysed (both needles and seeds) compared well with literature values obtained for the same samples (Chadwick and Keen 1976,

Dallimore and Jackson 1924, Vidakovic 1991). In the macroscopical examination of the actual trees of this species it was observed that the appearance ranged from tall pyramidal trees, such as *T. baccata* itself, to bun-shaped, low-growing shrubs (e.g. *T. b.* 'Adpressa'). The foliage colour can be either dark green, light green or golden. The variation in the tree shape and foliage colour within this species has lead to a great deal of misidentifications (Farjon 1998), and the question as to whether some of the cultivars are in fact are actual species remains unsolved. The major variations in needle and seed dimensions together with major differences in tree shape and foliage colour suggests that macroscopical characteristics are of dubious value for taxonomic purposes.

There has been some confusion about the right nomenclature of the Pacific yew, *Taxus brevifolia*. Dallimore and Jackson (1924) described samples denoted *Taxus baccata* 'Brevifolia' and *Taxus brevifolia* in their study. The *T. b.* 'Brevifolia' was described as: "A small bush with short and dense branchlets, and small densely arranged leaves, rarely more than ½ in. long..." while their description of *T. brevifolia* was different: "A tree 15-50 ft. high or occasionally taller with slightly pendent branches. Leaves shorter and more abruptly pointed than *T. baccata*, 1/2-3/4 in. long....". According to Farjon (Farjon 1998) the correct name of the pacific yew is *T. brevifolia*. The *T. brevifolia* trees used in this study all had a similar appearance, which would indicate that the nomenclature given by the Botanical Garden of origin was correct. Although the shape and foliage colour of the trees was similar, the needle and seed dimensions obtained showed considerable amount of variation within the species. This could be due to geographical differences, differences in light, nutrition etc., but misidentification of the trees can not be ruled out. In particular the results from the seed examination raise some questions of identity as there was no association between the obtained and literature values (Dallimore and Jackson 1924, Vidakovic 1991).

The correct identification by the Botanical Gardens of the *T. canadensis* trees used in our study has been queried due mainly to the great variation found in needle and seed dimensions. The shape of the trees examined also reflects this uncertainty, where the trees range from low-growing, wide-spreading shrubs to arching bushes or trees. The authentic sample from Canada (8) is a low-growing, wide-spreading shrub, but also in

this case geography, light, soil nutrition and age of the plant has an influence on shape and character.

The modifications of the previous study by Dempsey and Hook (2000) made in the present study where as follows:

- Larger number of yews examined for needle dimensions (n=94)
- Seed dimensions included
- A more sophisticated method of examination (WinSEEDLE^{TM})
- Additional characteristics of the trees (such as tree shape, foliage colour and gender)

The results obtained from morphological examinations performed still tended to support the opinion of Elwes and Henry (Elwes and Henry 1906) that the genus *Taxus* is mono-specific, consisting of a large number of varieties which exhibit enormous diversity in terms of their appearance but which are essentially taxonomically indistinguishable. Of the parameters investigated only the seed dimensions could to a certain extent be used to distinguish between the various species, but even these results were questionable in some instances and can not be used as a method in its own.

A further chemical and molecular investigation is necessary in order to be able make an overall conclusion.

4.2 *Taxus canadensis* – A Case Study

A high level of variation in both morphological characters and chemical constituents was found in the different *T. canadensis* samples analysed. Since an authentic sample of *T. canadensis* was obtained from Canada, this was used for comparison with other so-called *T. canadensis* samples. This section is a case study showing how the various methods used for identification could be used together in order to try to come to a valid conclusion (The chemical analysis data is from the following section 4.3).

4.2.1 Summary of morphological and chemotaxonomic examination

Figure 4.2.1.1 *Taxus canadensis* from National Botanical Gardens, Dublin, Ireland

Figure 4.2.1.2 *Taxus canadensis* (NCBI 2005)

Table 4.2.1.1 Results from morphological and chemical analysis of *T. canadensis*

Sample → / **Parameter ↓**	**8** Canada	**7** Bedgebury	**1a)** New Ross	**6** Edinburgh	**4** Glasnevin	**4a)** Glasnevin	**4b)** Glasnevin
Fresh Needles							
Area	-	-	38.6± 7.8	41.0± 5.6	24.9± 3.9	20.4±5.1	36.3± 6.2
Length	-	-	21.2± 2.6	21.0± 1.7	17.6± 2.0	13.4± 2.2	20.5± 2.3
Widh	-	-	2.4± 0.3	2.5± 0.2	1.8± 0.2	2.1± 0.2	2.3± 0.3
Dried Needles							
Area	27.5± 5.7	42.1± 9.4	38.6± 7.8	41.0± 5.6	24.9± 3.9	20.4± 5.1	36.3± 6.2
Length	16.3± 2.0	21.3± 3.6	21.2± 2.6	21.0± 1.7	17.6± 2.0	13.4± 2.2	20.5± 2.3
Width	2.2 ± 0.3	2.0 ± 0.2	2.4 ± 0.3	2.5 ± 0.2	1.8 ± 0.2	2.1 ± 0.2	2.3 ± 0.3
Seeds							
Area	14.1± 1.9	-	-	-	22.6± 2.0	-	26.4± 1.5
Length	4.7 ± 0.4	-	-	-	5.8 ± 0.3	-	6.5 ± 0.4
Width	4.0 ± 0.4	-	-	-	5.2 ± 0.3	-	5.4 ± 0.2
W/L ratio	0.9 ± 0.1	-	-	-	0.9 ± 0.1	-	0.8 ± 0.1
Taxanes							
Paclitaxel	227.1 ± 20.9	264.3 ± 6.1	57.6 ± 15.0	84.0 ± 2.4	57.6 ± 15.0	148.1 ± 5.1	110.5 ± 3.5
10-DAB III	*NQ	*NQ	148.5 ± 39.1	397.2 ± 32.8	166.1 ± 0.2	*NQ	190.3 ± 31.9
9-DHAB III	720.6 ± 76.3	-	39.2 ± 0.9	-	-	114.2 ± 2.4	-
Cephalomannine	89.3 ± 54.5	129.3 ± 2.3	25.4 ± 14.9	115.0 ± 5.4	27.2 ± 2.8	26.3 ± 0.6	45.6 ± 2.6

Samples: numbers represents samples from origin according to code used in analysis (see section 4.1.2.3 and 4.1.3.1) *NQ= non-quantifiable

4.2.1.1 Needle dimensions

A dried sample from an authentic *T. canadensis* was obtained from Canada (8). The samples analysed fresh were therefore dried and pressed in order to be directly compared to this authentic sample. The authentic *T. canadensis* sample (8) had a needle range of 16.27 ± 2.02 x 2.24 ± 0.32 mm. The needles fell within the needle length given by Dallimore and Jackson (1924), but the needles were slightly wider than expected. The only other samples that fell within the expected range were *T. canadensis* from Glasnevin (4) (higher end of expected range) and *T. canadensis* 'Aurea' 4a) from Glasnevin (lower end of expected range).

T. canadensis needles were expected to fall within the range 12.70-19.05 mm in length and 1.59-2.12 mm in width as proposed by Dallimore and Jackson (1924). Vidakovic

(1991) quoted a range of 13-20 x 0.5-2mm. There was a considerable amount of variation in the areas measured for the five fresh *T. canadensis* samples and the two *T. canadensis* cultivars analysed. The largest areas were found in the *T. canadensis* sample from Edinburgh (6) (41.02 ± 5.61 mm^2) and the sample 1a) from New Ross (38.56 ± 7.83 mm^2). The *T. canadensis* 'Aurea' 4a) sample was found to have a significantly smaller area (20.41 ± 5.13 mm^2) than any of the other samples analysed ($P<0.001$). The average value of the samples analysed in the present study was in the upper end of this range (18.59 ± 2.76 x 2.29 ± 0.27 mm). Most of the samples were in fact wider than the width given by both Dallimore and Jackson (1924) and Vidakovic (1991).

4.2.1.2 Seed dimensions

Vidakovic described the *T. canadensis* seeds as being short and wide, but no dimensions were given. Chadwick and Keen (1976) gave a similar description with a length of 5mm and a width of 4 mm. The dimensions of the seeds from the authentic *T. canadensis* were at the higher end of the expected range (4.67 ± 0.36 x 3.99 ± 0.43 mm). The two other samples analysed, *T. canadensis* (4) and *T. canadensis* 'Aurea' (4b)) were significantly ($P<0.001$) larger than expected. All the *T. canadensis* seeds analysed were considerably rounder (average width/length ratio of 0.85 ± 0.03) than the *T. baccata* and the *T. brevifolia* seeds.

4.2.1.3 Chemical constituents

The chemical analysis (section 4.3) of the eight different *T. canadensis* samples showed great intra-specific variation in the amount of taxanes present in the needles. There was also a difference in what taxanes the different samples contained. The sample from Bedgebury (7) had the highest paclitaxel content (264.27 ± 6.05 mg/kg), significantly higher than any of the other samples analysed ($P<0.001$). However, this sample was collected in July 1997, so because of the late collection and long storage time, this sample could not be directly compared to the other samples. The authentic sample from Canada (8) also had a high level of paclitaxel (227.08 ± 20.89 mg/kg), significantly higher ($P<0.001$) than any of the other samples (if Bedgebury sample was excluded). The lowest level of paclitaxel was found in the two samples from New Ross (1).

The Canadian sample, the *T. canadensis* 'Aurea' 4a) sample from Glasnevin and the Bedgebury sample did not have any quantifiable amounts of 10-DAB III. The rest of the samples analysed contained varying amounts of this taxane.

The highest levels of cephalomannine were present in the sample from Bedgebury (129.26 ± 2.33 mg/kg) and the sample from Edinburgh 114.99 ± 5.41 mg/kg). Both of these samples were collected later in the year than the others analysed. The Canadian sample also had a significantly higher ($P<0.001$) level of cephalomannine than the other samples (89.31 ± 54.56 mg/kg).

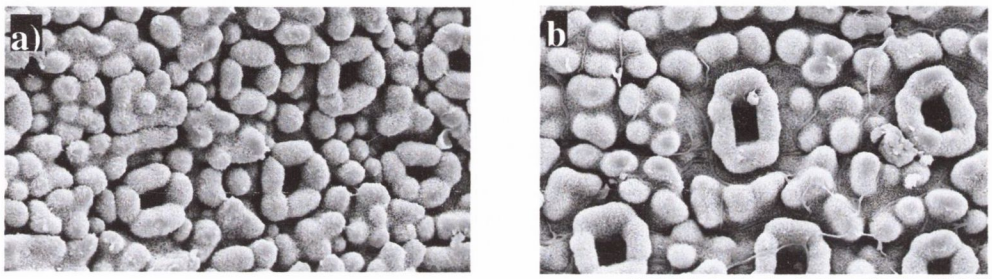
The taxane specific to *T. canadensis* (9-dihydro-13-acetylbaccatin III) was only detected in the *T. canadensis* from Canada (720.59 ± 76.30 mg/kg), *T. canadensis* 'Aurea' a) from Glasnevin (114.15 ± 2.40 mg/kg) and a small amount in the *T. canadensis* from New Ross (39.23 ± 0.87 mg/kg). The concentration of this taxane was 3- times higher than the concentration of its co-metabolite paclitaxel (227.08 mg/kg) in the authentic *T. canadensis*. This was expected since 9-dihydro-13-acetylbaccatin III is found to be the most abundant taxane in the species(Gunawardana et al. 1992, Zamir et al. 1992, Zamir et al. 1995).

Because of the high degree of variation present in the needle and seed- dimensions and the varying chemical constituent concentrations no absolute conclusion could be drawn and a microscopic examination of the *T. canadensis* needles was performed to see if further clarification could be achieved by determining stomatal sizes and number.

4.2.2 Microscopic examination of *Taxus canadensis*

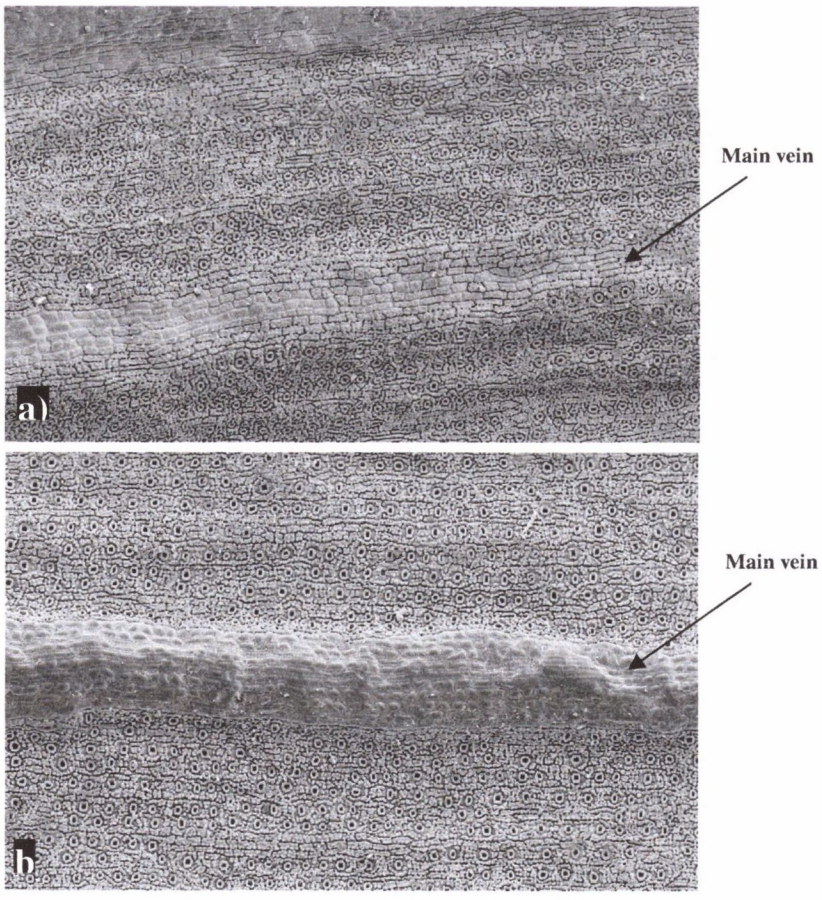
Stomata are tiny pores flanked by specialized epidermal cells called guard cells. In many plants two or more of the cells adjacent to the guard cells appear to be associated functionally with them, and are morphologically distinct from the other epidermal cells. These cells are called subsidiary cells (Esau 1965). In all *Taxus* species and cultivars there are four (sometimes more) enlarged subsidiary cells arranged in a circle around/above the stomatal pore and the guard cells. This arrangement is known as a Florin's ring, and is unique to the stomatal arrangement in *Taxus* (Florin 1931).

Strobel and Hess (Strobel and Hess 1996) found, using a scanning electron microscope, that the four North American species (*T. canadensis, T. brevifolia, T. floridana* and *T. globosa*) had 3-5 rows of stomata on one half of the abaxial leaf surface, whereas the European and Asian species (*T. baccata*, *T. chinensis*, *T. cuspidata*, *T. wallichiana* and *T. sumatrana*) had 7-10 rows of stomata. The stomatal arrangements on the lower epidermis of needles from a) *T. canadensis* from Bedgebury (**8**) and b) *T. baccata* 'Fastigiata' (from Bedgebury are) shown in **Figure 4.2.2.1** and **Figure 4.2.2.2**. A scanning electron microscope was used at magnification x400 and x500, respectively (Dempsey and Hook 2000).



*SEM Unit Trinity College Dublin 5/10/98

Figure 4.2.2.1 Stomata from a) *T. canadensis* and b) *T. baccata* 'Fastigiata'



*SEM Unit Trinity College Dublin 5/10/98

Figure 4.2.2.2 Stomatal rows of a) *T. canadensis* and b) *T. baccata* 'Fastigiata'

In addition to examining the stomatal rows on the needles the number of stomata per mm^2 of six *T. canadensis* samples was analysed, as well as three *T. baccata* samples, including the authentic sample from Herstmonceux church (5). For comparative reasons the Polish *T. cuspidata* sample (9), the sample most similar to values by Dallimore and Jackson (1924) (*T. cuspidata* (9): 16.09-20.83 mm x 1.69-2.69 mm, Dallimore and Jackson: 12.7-25.4 mm x 2.12-3.18 mm) were also included. As **Table 4.2.2.1** and the corresponding **Figures 4.2.2.3** and **Figures 4.2.2.4** show, the stomatal numbers of the samples analysed showed great variation and the numbers of stomatal rows were less variable.

Table 4.2.2.1 Stomatal arrangements in the abaxial surface of *Taxus* needles

Sample (n = 10)	**No. of Stomata / 1mm² of abaxial leaf surface**	**Stomatal rows on one half of abaxial leaf surface**	**Origin**
T. canadensis	121.96 ± 14.48	Not measured	7
T. canadensis	131.25 ± 10.80	6.75 ± 0.70	8
T. canadensis	132.50 ± 9.40	8.05 ± 0.83	1
T. canadensis	143.13 ± 12.40	8.50 ± 0.69	6
T. canadensis	105.00 ± 6.90	7.25 ± 0.89	4
T. canadensis 'Aurea'	150.63 ± 13.50	6.63 ± 0.74	4a)
T. canadensis 'Aurea'	114.38 ± 9.00	8.00 ± 0.76	4b)
T. baccata	106.25 ± 7.40	9.00 ± 0.92	5
T. baccata	108.13 ± 8.90	8.75 ± 1.04	3
T. b. 'Fastigiata'	115.63 ± 10.00	8.38 ± 0.92	9
T. cuspidata	205.63 ± 10.70	10.88 ± 1.13	9

The code for origin is given in section 4.1.2.3

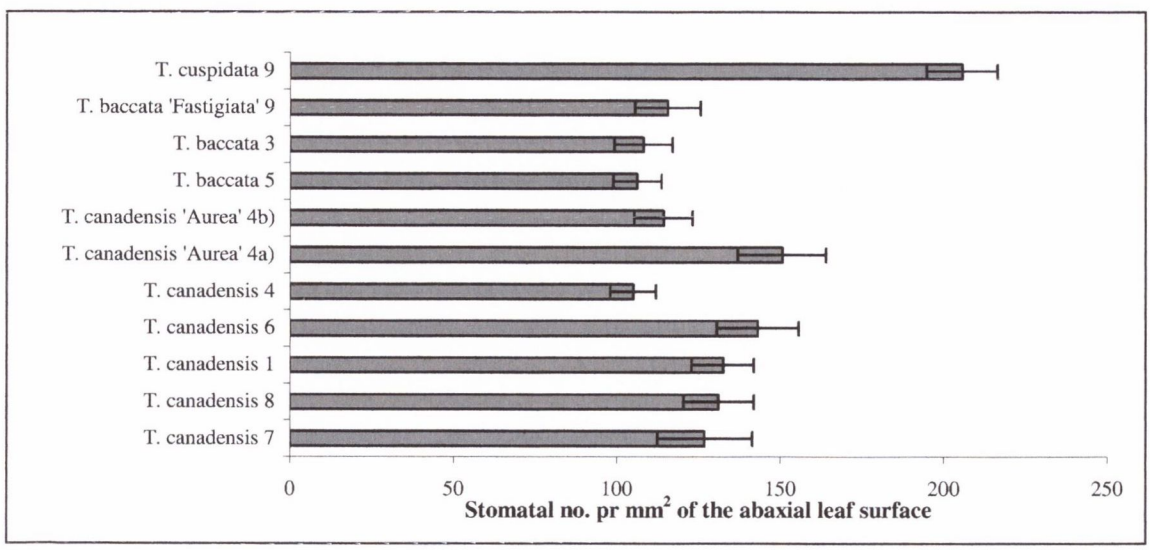


Figure 4.2.2.3 Number of stomata per $1mm^2$ of abaxial leaf surface

The stomatal number showed considerable variation both intra-specific (within the six *T. canadensis* samples) and inter-specific (compared to *T. baccata* and *T. cuspidata*). Each of the individual samples were compared to the authentic *T. canadensis* sample from Canada (8) using the Dunnett Multiple Comparison Test. This showed that the *T. canadensis* samples from Bedgebury (7), New Ross (1) and Edinburgh (6) did not differ significantly from the authentic sample. The *T. canadensis* 'Aurea' 4b) sample only had a slightly smaller number of stomata than the authentic sample ($P<0.05$). But this comparison also showed that there was no significant difference in stomata number between *T. b.* 'Fastigiata' (9) and the Canadian *T. canadensis* sample.

The Dunnett Multiple Comparison Test was also performed using the authentic *T. baccata* sample (5) as control. This analysis showed that there was no significant difference in stomatal number between this sample and the *T. canadensis* (4) and the *T. canadensis* 'Aurea 4b) samples from Glasnevin.

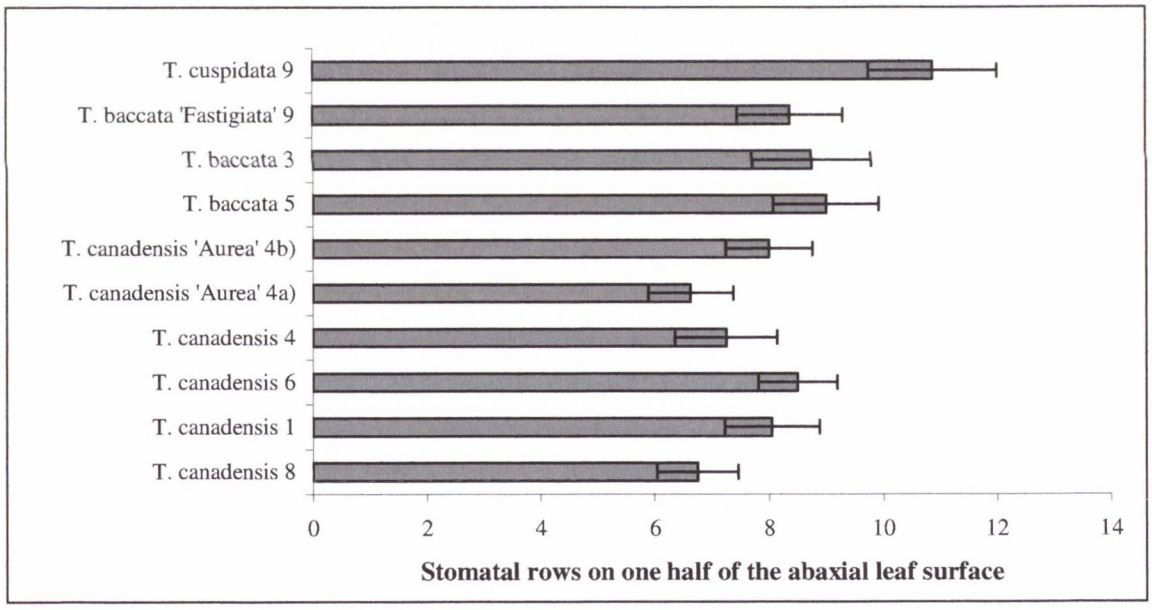


Figure 4.2.2.4 Number of stomatal rows on one half of the abaxial leaf surface

Using the number of stomatal rows as a reference the six *T. canadensis* samples fell into two groups; one group which had 7 rows per half of the abaxial leaf surface, and the other which exhibited 8 rows. Using the Dunnett Multiple Comparison Test, keeping the authentic *T. canadensis* sample as the control sample, the *T. canadensis* (4) and *T. canadensis* 'Aurea' (4a)) samples from Glasnevin did not differ significantly

from the authentic sample. The sample from New Ross (1) and the *T. canadensis* 'Aurea' (4b)) from Glasnevin had only a slightly higher value than the control ($P<0.05$). However, when the authentic *T. baccata* (5) sample was kept as the control the *T. canadensis* from New Ross (1), Edinburgh (6) and the *T. canadensis* 'Aurea' (4b)) from Glasnevin were found to have a statistically similar amount of stomatal rows as the control.

Discussion

The authentic sample of *T. candensis* (8) yielded a stomatal number of 131.25 ± 10.80. The result from the other six *T. canadensis* samples showed the stomatal number for the two samples from Glasnevin (4 and 4a)) to be very different to the rest. *T. canadensis* (4) had a significantly lower number of stomata (105.00 ± 6.90), in fact it resembled the count obtained from *T. baccata*. The variety *T. canadensis* 'Aurea' (4a) had a significantly higher count (150.63 ± 13.50). The other 'Aurea' sample (4b) also differed slightly from the authentic sample by having a lower stomata count (114.38 ± 9.00). However, these results did not correspond to the results found in the stomatal row examination, where the *T. canadensis* (4) (7.25 ± 0.89) and *T. canadensis* 'Aurea' (4a) (6.63 ± 0.74) samples were found to be the most statistically similar to the authentic sample (6.75 ± 0.75).

It is evident from the results that the differences in stomatal number between the authentic sample of *T. canadensis* (8) (131.25 ± 10.80), *T. baccata* (5) (106.25 ± 7.40), and *T. cuspidata* (9) (205.63 ± 10.70) were significant ($P<0.001$). The difference in the stomatal rows were also statistically significant ($P<0.001$), where *T. baccata* had more rows (average 8.71 ± 0.31) than *T. canadensis* (7.53 ± 0.77) and *T. cuspidata* had the most stomatal rows (10.88 ± 1.13). This observation corresponds relatively well with what Strobel and Hess (Strobel and Hess 1996) reported, where they found *T. canadensis* to have 3-5 rows of stomata (slightly lower than our observation), *T. baccata* had around 8 rows of stomata and finally *T. cuspidata* had 9-10 stomatal rows.

A definitive solution to the problem regarding the accurate identification of the *T. canadensis* samples analysed cannot be conclusively drawn from the microscopical examination carried out.

4.2.3 Molecular examination

Since morphological and chemical characteristics were unable to accurately identify *T. canadensis* as a species, it was suggested that molecular analyses could be an alternative approach in order to clarify the relationship within and between the species.

The following molecular systematics work was performed by Lisa Kilmartin, Botany Department, Trinity College Dublin (Kilmartin 2002) as part of the original *Taxus* project (Hook 1999). In her study two plastid DNA regions (*trnL* intron and *trnL-F* intergenic spacer) and one nuclear region (18S-5.8S-26S nrDNA (ITS)) were sequenced from a range of *Taxus* species and cultivars. Amongst the species analysed were three *T. canadensis* samples:

- *T. canadensis*_33: equivalent to *T. canadensis* (4) from Glasnevin in this study.
- *T. canadensis*_aurea_36: equivalent to *T. candensis* 'Aurea' (4a)) from Glasnevin in this study.
- *T. canadensis*_24: equivalent to *T. canadensis* (7) from Bedgebury in this study.

Some of the results generated by the molecular work carried out by Kilmartin (Kilmartin 2002) is shown in **Figure 4.2.3.1** and **Figure 4.2.3.2**.

Phylogenetic glossary:

- Parsimonious trees: the most parsimonious tree is defined to have the fewest character state changes
- Tree-Bisection-Reconnection (TBR): proceeds by breaking a phylogenetic tree into two parts and then reconnecting the two subtrees at all possible branches. If a better tree is found, it is retained and another round of TBR is initiated
- Bootstrap support: an estimation of confidence levels of relationships
- Consistency index (CI): a measure of how well an individual character fits on a phylogenetic tree. If the minimum number of steps is the same as the observed number of steps, then the character will have a CI of 1.0. If a character is not completely compatible with a tree, then it will have a CI value less than 1.0
- Retention index (RI): similar to CI, but maximum value is 1 and minimum is 0
- Tree length: total number of steps required in order to map a dataset onto a phylogenetic tree

In **Figure 4.2.3.1** one of over 10,000 equally parsimonious trees generated after a heuristic search with 500 replicates of random stepwise addition using TBR (Tree-Bisection-Reconnection) branch swapping on the *trnL-F* sequences is shown.

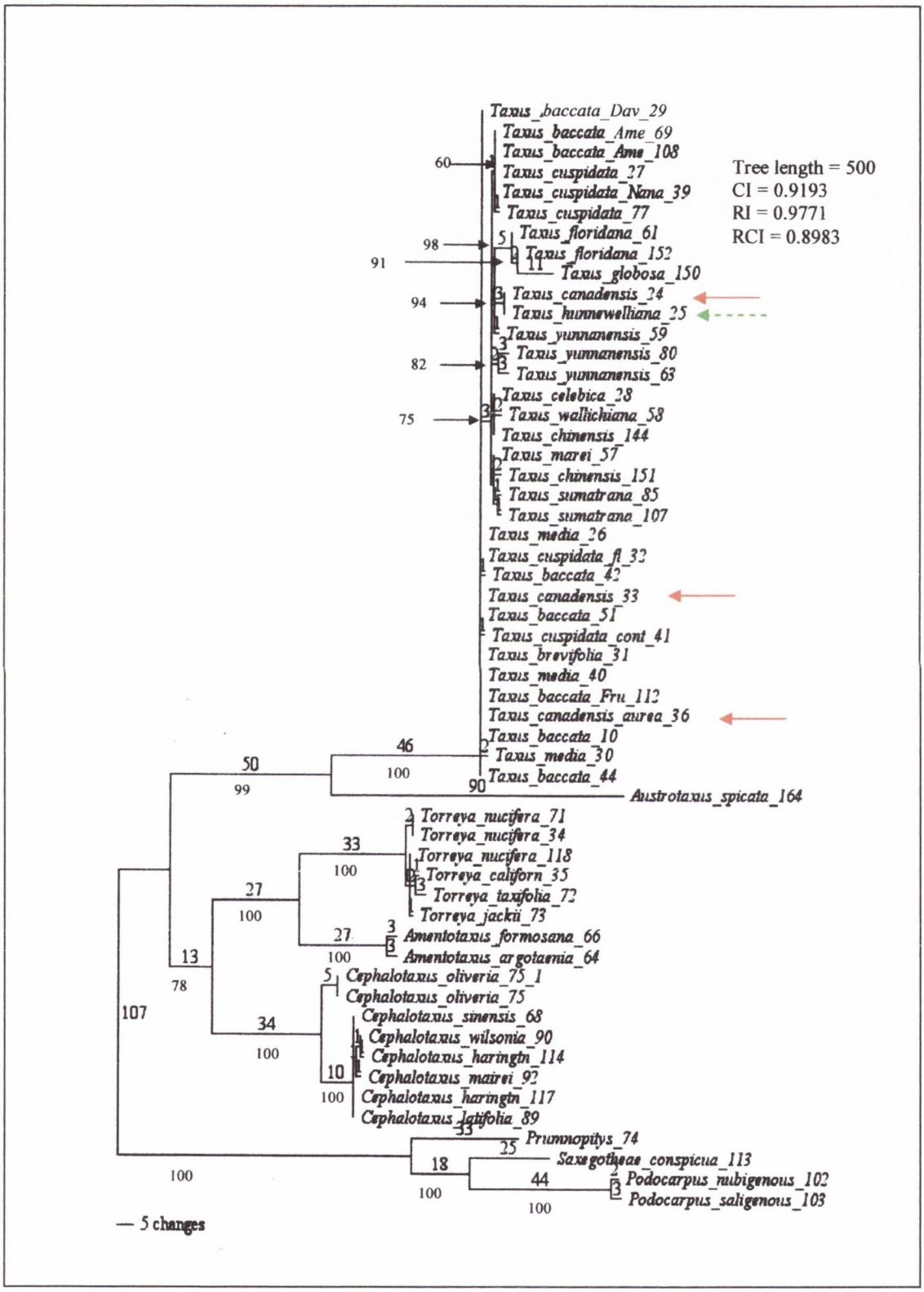


Figure 4.2.3.1 One of over 10,000 trees resulting from a heuristic search using 500 replicates of TBR branch swapping on the *trnL-F* sequences (Kilmartin 2002)

Numbers above the branch represents the branch lengths and numbers below indicate bootstrap support. The consistency index (CI), retention index (RI), rescale consistency index (RCI) and tree length are all shown at the side of the tree, indicating good character similarity. From this it was concluded that the DNA sequencing of the *trnL-F* intron and spacer region was successful in determining relations between genera of Taxaceae. However, within the genus *Taxus* the *trnL-F* intron and intergenetic spacer region did not appear to be variable enough to fully determine the distinctiveness of species. The *T. canadensis* samples analysed did not group together in the molecular analysis and this implies that either the nomenclature or the species status of these taxa needs revision. Interestingly *T.* X. *hunnewelliana* grouped very closely together with the *T. canadensis* sample from Bedgebury (7) (in tree: *T. candensis*_24). *T.* X. *hunnewelliana* is known to be a hybrid of *T. canadensis* and *T. cuspidata*.

In **Figure 4.2.3.2** one of over 5,000 most parsimonious unrooted trees generated from heuristic search using 500 replicates of TBR branch swapping of the Internal Transcribed Spacer Regions (ITS) nrDNA sequences is shown. Again it was found that the intraspecific variation within the *Taxus* species analysed was low. The species were not sufficiently distinct, and the groupings found were not strongly supported. The *T. canadensis* samples analysed did not group together. However, like in **Figure 4.2.3.1** the *T. canadensis* sample from Bedgebury (7) (in tree: *T. candensis*_24) grouped closely with *T.* X. *hunnewelliana*.

From the two phylogenetic trees generated in the molecular work by Kilmartin (Kilmartin 2002) it is clear that the neither the *trnL-F* sequences nor the ITS sequences analysed can give enough information regarding the distinctiveness of the *Taxus* species analysed. Further work is therefore ongoing in trying to find an appropriate approach to the molecular systematics of the genus *Taxus*. One example is the discovery of an adenine-rich microsatellite region in *trnL-F* sequences common to yew species (Kilmartin 2002). A primer was designed, and this primer will be used in order to compare the European *T. canadensis* samples with the authenticated sample from Canada (results are awaited). Collins and co-workers (Collins et al. 2003) addressed the question of species distinctiveness in *T. baccata*, *T. canadensis* and *T. cuspidata* using RAPDs and cpDNA analysis in their work. Using this combined

method they concluded that there was a clear difference between the three species studied.

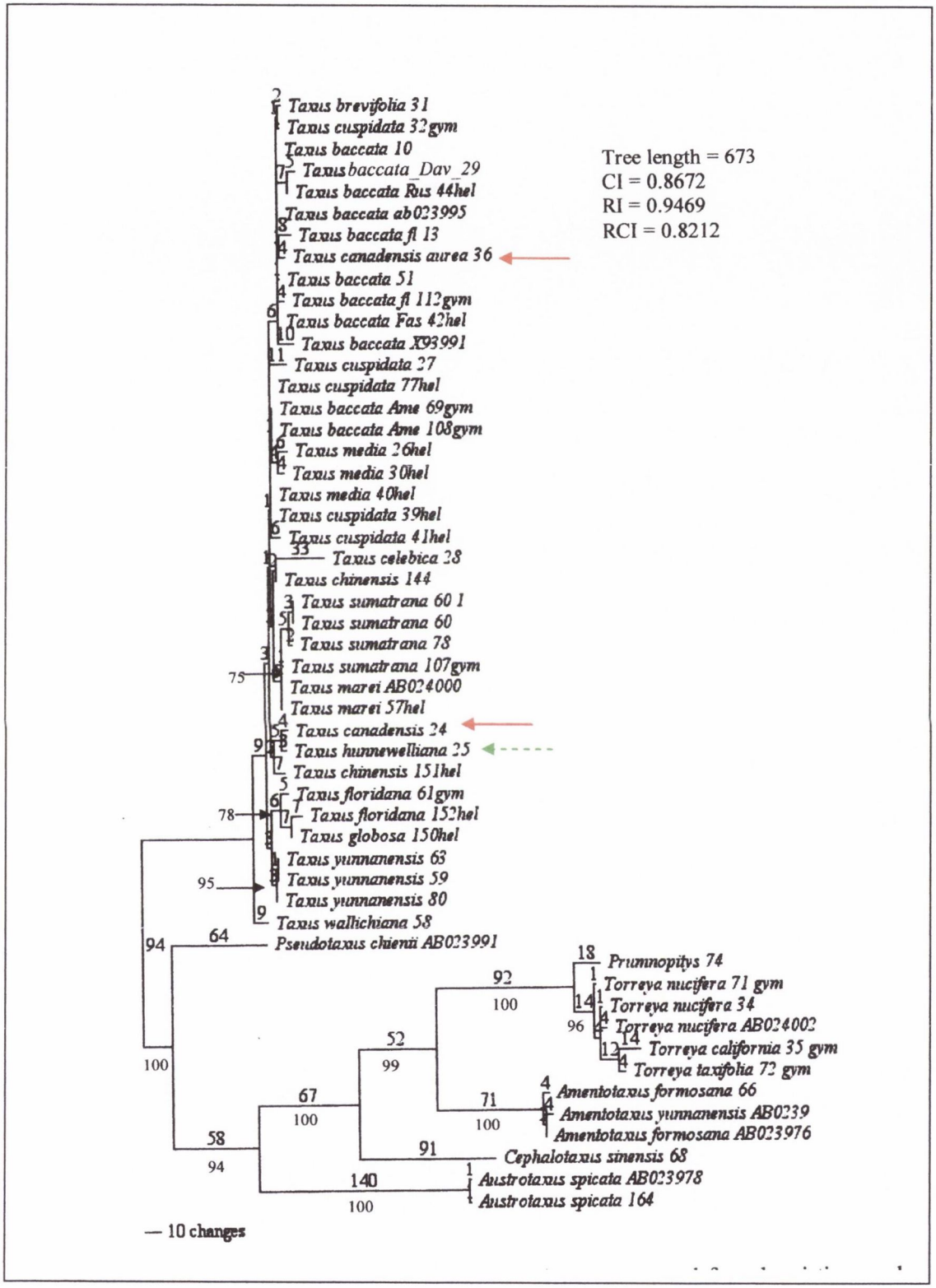


Figure 4.2.3.2 One of over 5,000 most parsimonious unrooted trees generated from heuristic search using 500 replicates of TBR branch swapping of the ITS nrDNA sequences (Kilmartin 2002)

4.2.4 Conclusion

Collective evaluation of results proved that morphological characteristics and chemical profiles, when used in conjunction with molecular methods, were not sufficient in order to identify *T. canadensis* as a species. From the results it is obvious that there are problems concerning the authentication of the *Taxus* species given the great variation not only between species, but as seen here, within the same species. Ambiguous nomenclature and questionable identities in samples supplied as "authenticated" was found.

4.3 Chemotaxonomical Analysis of *Taxus* Species and Cultivars

4.3.1 Paclitaxel and related taxanes in various *Taxus* species and cultivars

4.3.1.1 Chemical examination

As a part of the taxonomic investigation of *Taxus* the needles of a range of species and cultivars were chemically screened for their taxane content. This type of screening has been employed by several research groups (Dempsey 2000, Dempsey and Hook 2000, ElSohly et al. 1995, van Rozendaal et al. 1999, van Rozendaal et al. 2000, Vidensek et al. 1990, Witherup et al. 1990), however, only van Rozendaal et al. and Elsohly et al. screened the needles for related taxanes in addition to paclitaxel. In our study an efficient and taxane specific HPLC method was developed using a Phenomenex Curosil-PFP column, and the aforementioned yew needles were screened for paclitaxel (**1**), 10-deacetylbaccatin III (10-DAB III) (**2**), 9-dihydro-13-acetylbaccatin III (9-DHAB III) (**3**) and cephalomannine (**4**) (**Figure 4.3.1.1**.).

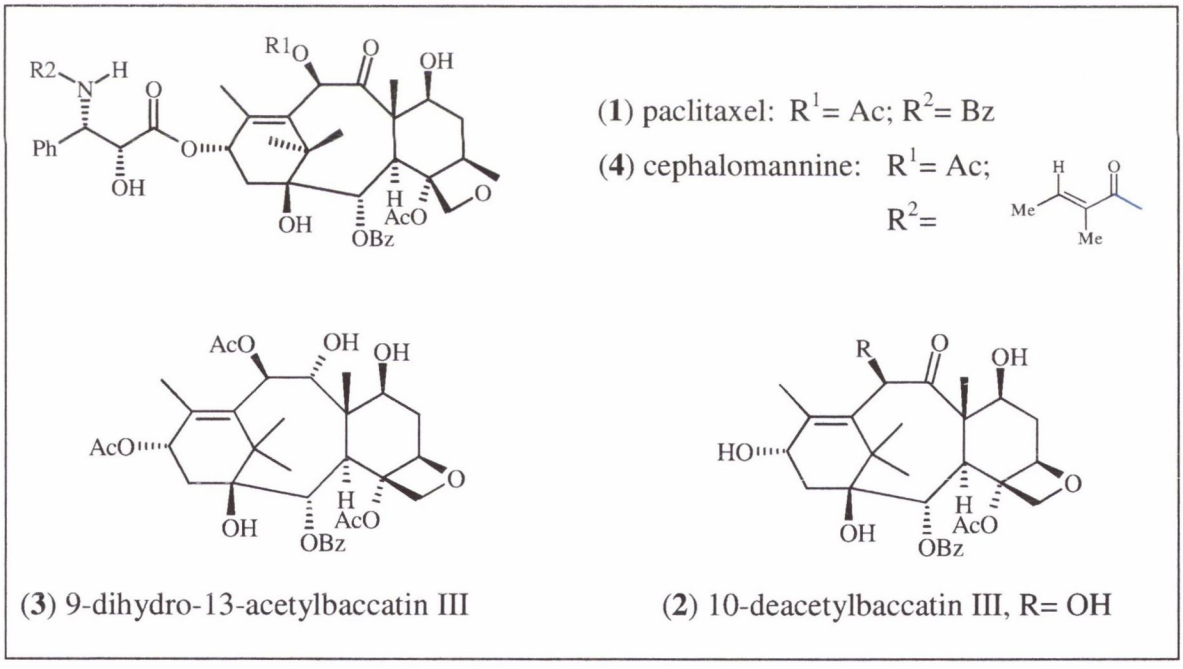


Figure 4.3.1.1 Taxanes identified in screening of *Taxus* needles

The average taxane (**1-4**) content with their respective standard deviation is presented in **Table 4.3.1.1** and this table also gives the origin of the plant samples and the source code (where available).

Table 4.3.1.1 Taxane content in needles of various *Taxus* species and cultivars

Sample	Paclitaxel (1) (mg/kg)	10-DAB III (2) (mg/kg)	9-DHAB III (3) (mg/kg)	Cephalomannine (4) (mg/kg)	Source Code**	Origin
Taxus baccata	350.20 ± 36.70	204.58 ± 1.77	-	48.29 ± 7.77	9L7	1
Taxus baccata	51.58 ± 8.71	124.38 ± 19.18	-	50.08 ± 8.24		2 a)
Taxus baccata	NQ*	NQ*	-	NQ*		2 b)
Taxus baccata	62.77 ± 7.06	147.69 ± 20.72	-	10.16 ± 1.65		3 a)
Taxus baccata	71.52 ± 2.47	134.79 ± 3.65	-	13.24 ± 1.06		3 b)
Taxus baccata	198.43 ± 3.99	180.91 ± 13.97	-	121.80 ± 9.51		5
T. b. 'Adpressa'	40.87 ± 4.12	239.47 ± 4.36	-	11.65 ± 1.65	699296	6
T. b. 'Adpressa'	104.73 ± 4.94	199.49 ± 1.18	-	19.28 ± 0.71	T	3
T. b. 'Adpressa'	106.23 ± 30.94	516.08 ± 59.53	-	72.54 ± 14.59		9
T. b. 'Adpressa Aurea'	23.56 ± 0.59	236.85 ± 11.42	-	43.12 ± 2.35	071024	6
T. b. 'Aurea'	329.25 ± 129.96	*NQ	-	137.64 ± 54.93		9
T. b. 'Aurea'	136.72 ± 1.76	7.81 ± 1.88	-	33.16 ± 0.82		3
T. b. 'Aureahoseri'	216.36 ± 3.64	585.42 ± 50.43	-	46.55 ± 2.82		9
T. b. 'Densifolia'	140.65 ± 13.84	42.21 ± 2.70	-	31.60 ± 3.64	6563	4
T. b. 'Dovastonia'	162.88 ± 59.14	79.32 ± 4.47	-	*NQ	25	3
T. b. 'Dovastonia'	75.17 ± 15.65	83.10 ± 1.64	-	43.45 ± 8.88		2
T. b. 'Dovastonia Aurea'	5.49 ± 0.01	59.94 ± 0.94	-	*NQ	9L30	1
T. b. 'Erecta'	276.94 ± 2.59	570.10 ± 8.59	-	17.72 ± 4.59		9
T. b. 'Fastigiata'	82.74 ± 4.60	223.96 ± 0.00	-	21.58 ± 2.00		9
T. b. 'Fastigiata'§	283.26 ± 58.19	307.53 ± 27.46	-	124.77 ± 16.47	9L4	1 a)
T. b. 'Fastigiata'§	261.96 ± 25.55	325.49 ± 14.66	-	140.45 ± 3.20	9L5	1 b)

* NQ = Not quantifiable § collected in December a), b) and c) show different trees of same species from same origin
** Source code is code given by Botanical gardens for identification of trees

Table 4.3.1.1-cont. Taxane content in needles of various *Taxus* species and cultivars

Sample	Paclitaxel (1) (mg/kg)	10-DAB III (2) (mg/kg)	9-DHAB III (3) (mg/kg)	Cephalomannine (4) (mg/kg)	Source Code**	Origin
T. b. 'Fastigiata'§	186.20 ± 46.26	255.07 ± 49.34	-	116.27 ± 0.84	9L6	1c)
T. b. 'Green Mountain'	179.51 ± 1.06	187.09 ± 5.18	-	90.55 ± 27.68	-	9
T. b. 'Imperialis'	56.45 ± 5.18	*NQ	-	113.15 ± 31.67	-	9
T. b. 'Jacksonii'	185.02 ± 29.81	154.45 ± 1.65	-	28.74 ± 1.77	19688018	6
T. b. 'Lutea'	111.43 ± 2.00	150.46 ± 17.89	-	73.81 ± 4.83	-	3
T. b. 'Lutea'	203.69 ± 16.57	31.75 ± 5.64	-	189.23 ± 59.59	-	2
T. b. 'Neidpathensis'	95.05 ± 0.59	84.13 ± 0.12	-	13.95 ± 2.58	6569	4
T. b. 'Pyramidalis'	179.27 ± 5.16	242.02 ± 48.12	-	18.92 ± 2.35	6594	4
T. b. 'Repandens Aurea'	125.17 ± 1.29	216.23 ± 4.23	-	37.09 ± 10.11	19688019	6
T. b. 'Semperaurea'	334.94 ± 64.27	210.08 ± 6.36	-	50.11 ± 1.41	-	9
T. b. 'Standishii'	39.28 ± 0.47	*NQ	-	46.10 ± 2.35	19588809	6
T. b. 'Standishii'	357.99 ± 45.12	309.50 ± 8.84	-	31.16 ± 2.12	-	9
T. b. 'Washingtonii'	86.58 ± 14.36	168.25 ± 12.13	-	49.62 ± 13.89	19699305	6
T. brevifolia	79.25 ± 4.71	261.23 ± 11.07	-	42.29 ± 11.30	8M34	1a)
T. brevifolia§	132.91 ± 18.87	485.76 ± 59.45	-	58.47 ± 13.04	8L33	1b)
T. brevifolia§	113.97 ± 14.37	398.18 ± 107.24	-	70.02 ± 7.31	8L35	1c)
T. brevifolia	50.22 ± 0.47	436.17 ± 19.05	-	38.74 ± 0.47	6759	4
T. brevifolia	434.09 ± 14.24	1358.54 ± 13.07	-	21.39 ± 0.82	-	9
T. canadensis§	57.56 ± 14.97	148.52 ± 39.13	39.23 ± 0.87	25.39 ± 14.91	8M4	1a)
T. canadensis	27.88 ± 6.24	50.59 ± 0.47	-	*NQ	8M5	1b)
T. canadensis	115.70 ± 10.25	166.09 ± 0.24	-	27.15 ± 2.83	6556	4

* NQ = Not quantifiable § collected in December a), b) and c) show different trees of same species from same origin
** Source code is code given by Botanical gardens for identification of trees

Table 4.3.1.1-cont. Taxane content in needles of various *Taxus* species and cultivars.

Sample	Paclitaxel (1) (mg/kg)	10-DAB III (2) (mg/kg)	9-DHAB III (3) (mg/kg)	Cephalomannine (4) (mg/kg)	Source Code**	Origin
T. canadensis (*T.b.* 'Procumbens')	84.04 ± 2.35	397.15 ± 32.83	-	114.99 ± 5.41	271013	6
T. canadensis	227.08 ± 20.89	*NQ	720.59 ± 76.30	89.31 ± 54.46	-	8
T. canadensis	264.27 ± 6.05	*NQ	-	129.26 ± 2.33	-	7
T. canadensis 'Aurea' (1)	148.14 ± 5.08	*NQ	114.15 ± 2.40	26.32 ± 0.56	6549	4a)
T. canadensis 'Aurea' (2)	110.52 ± 3.52	190.34 ± 31.92	-	45.63 ± 2.58	6570	4b)
T. chinensis	29.51 ± 1.76	*NQ	-	46.29 ± 3.17	311011	6
T. cuspidata	171.64 ± 5.29	511.67 ± 10.35	-	152.17 ± 2.24	8M9	1a)
T. cuspidata§	163.71 ± 10.27	*NQ	-	261.37 ± 31.72	8M10	1b)
T. cuspidata§	215.44 ± 2.63	438.06 ± 63.08	-	199.10 ± 14.31	8M11	1c)
T. cuspidata	30.38 ± 3.76	28.39 ± 0.01	-	2.32 ± 0.01	6601	4
T. cuspidata	43.83 ± 4.12	*NQ	-	126.83 ± 19.61	-	9
T. cuspidata 'Luteo Baccata'	234.12 ± 37.29	378.34 ± 9.29	-	132.41 ± 68.22	6571	4
T. cuspidata 'Nana'	12.66 ± 1.41	128.79 ± 0.00	-		-	9
T. cuspidata 'Thayerae'	173.87 ± 30.14	48.84 ± 3.63	-	44.86 ± 9.26	6593	4
T. floridana	162.87 ± 16.12	184.09 ± 63.32	-	155.21 ± 25.07	7M49	1
T. sumatrana	114.68 ± 6.95	*NQ	-	149.57 ± 4.48	8M8	1
T. wallichiana	193.98 ± 6.27	297.05 ± 6.11	-	139.09 ± 4.50	19902824	6
T. X *media*	248.85 ± 8.48	264.10 ± 32.39	-	148.91 ± 7.77	8M54	1
T. X *media*	172.46 ± 3.22	*NQ	-	28.20 ± 3.22	-	9
T. X *media* 'Hicksii'	124.36 ± 24.82	58.56 ± 11.29	-	22.29 ± 5.65	6584	4

* NQ = Not quantifiable § collected in December a), b) and c) show different trees of same species from same origin
** Source code is code given by Botanical gardens for identification of trees

Table 4.3.1.1-cont. Taxane content in needles of various *Taxus* species and cultivars

Sample	Paclitaxel (1) (mg/kg)	10-DAB III (2) (mg/kg)	9-DHAB III (3) (mg/kg)	Cephalomannine (4) (mg/kg)	Source Code**	Origin
T. X *media* 'Hicksii'	181.50 ± 17.75	130.20 ± 15.99	-	39.16 ± 5.53	-	9
T. X media 'Rozon'	206.44 ± 15.51	1223.86 ± 59.00	-	134.64 ± 13.16	-	9
T. X *media* 'Stricta'	79.61 ± 4.47	279.54 ± 38.31	-	83.26 ± 4.70	-	9
T. X *media* 'Wojtek'	184.25 ± 9.29	*NQ	-	68.63 ± 3.41	-	9

* NQ = Not quantifiable ** Source code is code given by Botanical gardens for identification of trees

The following code indicates the origin of samples used in analyses:

1: The J. F. Kennedy Park Arboretum, New Ross, Co. Wexford, Ireland.

2: Mount Usher Gardens, Ashford, Co. Wicklow, Ireland.

3: Powerscourt Estate and Gardens, Co. Wicklow, Ireland.

4: National Botanical Gardens, Glasnevin, Dublin, Ireland.

5: Herstmonceaux Church, East Sussex, U.K.

6: The Royal Botanical Gardens Edinburgh, Edinburgh, Scotland.

7: Begebury National Pinetum, Kent, UK.

8: Natural Resourses Canada, Fredericton, New Brunswick, Canada.

9: Polska Akademia Nauk, Krónik, Poland.

It is important to emphasise that the samples from The National Botanical Gardens Edinburgh were collected at the end of September, while all the other samples were collected earlier in the year (late April to July). The samples from New Ross marked with **§** were collected 17th of December 2003. Seasonal variation in taxane content has been established previously (Dempsey 2000, ElSohly et al. 1997, Griffin and Hook 1996, Hook et al. 1999, Vance et al. 1994). For the Irish yew, *T. baccata* 'Fastigiata', Hook *et al.* (Hook et al. 1999) found that there was a significant decrease in paclitaxel content in the shoots between the end of April and the end of May. This decrease was thought to be a consequence of the extension growth, which begins in June in *T. baccata* 'Fastigiata' growing in Ireland, and continues until August. The highest levels

of paclitaxel were found present in needles between February and April and the shoots harvested in June had a maximum content of 10-DAB III. During the winter months, from November to January, the basic taxoids (taxines B and total alkaloids) were present in greatest amounts. Interestingly, it was also found that in France the highest yields of basic taxoids could result from clippings taken between August and October, showing that not only has seasonality an effect on chemical constituents in *Taxus*, geographical location also influences the secondary metabolite production (Hook et al. 1999). In the case of *T. brevifolia* Vance *et al.* (Vance et al. 1994) reported that the content of paclitaxel and cephalomannine decreased slightly between March and May. There was a steady increase over the summer months, and then a decline was observed from October. Baccatin III and 10-DAB III had a similar profile, where there was a steady increase over the summer months, and the highest level was present in September. Finally the species-specific taxane brevifoliol was observed at the highest level in August. This taxane had the highest concentration of all the taxanes measured. In *T.* x. *media* cultivars ElSohly et al. (ElSohly et al. 1997) reported that the highest level of taxanes was present in June, while the lowest level was measured in April. They found that it was in fact 10-DAB III concentrations that gave the high level in June, and that the paclitaxel content varied less.

From these studies it is apparent that there is no single month of the year, which is universally ideal for harvesting yew shoots containing maximum amounts of all taxanes. In order to compare the analysis of a range of samples it is therefore crucial that the samples are collected at the same time of the year. Most of our samples were collected between the end of April and the beginning of July. The samples from Edinburgh that were collected at the end of September and the samples from New Ross marked with **§** that were collected in mid December can therefore not be directly compared with the other samples.

The average taxane content in *T. baccata* cultivars, *T. brevifolia* samples and *T. canadensis* cultivars are shown in **Figure 4.3.1.2, 4.3.1.3** and **4.3.1.4,** respectively. **Figure 4.3.1.5** illustrates the taxane content in *T. cuspidata* cultivars, and **Figure 4.3.1.6** accounts for the taxane content in *T.* x. *media* cultivars, the *T. floridana*, *T. sumatrana*, *T. wallichiana* and *T. chinensis* samples collected. The same code of

origin is used in these figures as in **Table 4.3.1.1**. For the taxane analysis 3 g of dried and finely powdered plant material was extracted with methanol under reflux for 2.5 hours. The following residue was further re-dissolved in water and re-extracted with ethyl acetate. The ethyl acetate extract was reconstituted in methanol and analysed by HPLC. Further details can be found in Materials and Methods (section 3.3.2 - 3.3.4).

Taxus baccata

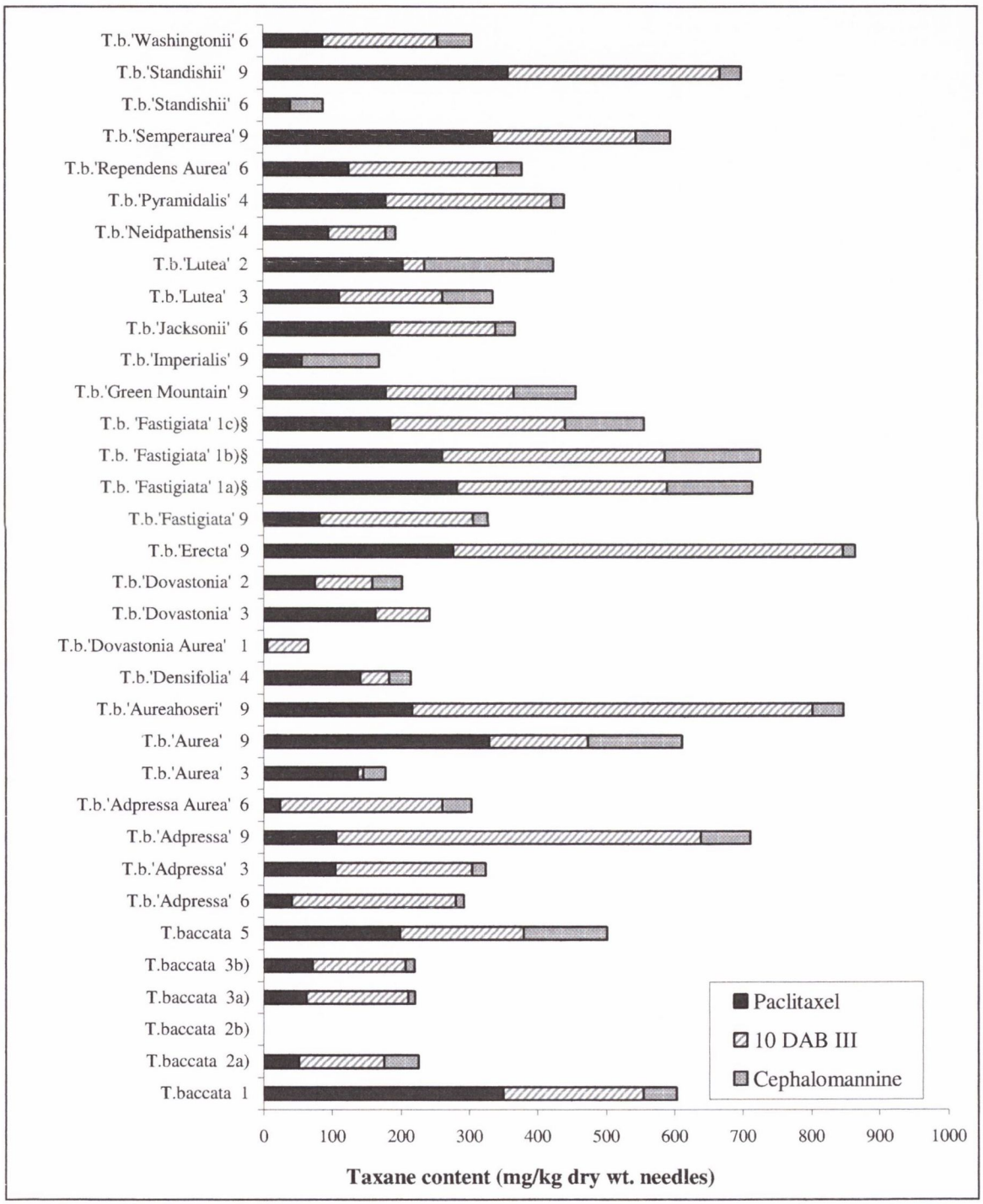


Figure 4.3.1.2 Taxane content of *T. baccata* cultivars

The paclitaxel content of the *T. baccata* samples varied considerably. The highest concentration was present in the sample from New Ross (1) (350.20 ± 36.70 mg/kg). This content was significantly higher than any of the other concentrations measured ($P<0.001$). The amount present in the sample from Herstmonceaux Church (5) was also quite high (198.43 ± 3.99), but as this sample was collected later in the summer (21/07/2002) no direct comparison could be made. No significant difference was found between the samples from Mount Usher (2) and Powerscourt (3) ($P>0.05$). Less variation was seen for the 10-DAB III content. The highest amount was found present in the sample from New Ross (1) (204.58 ± 1.77 mg/kg), and this was only slightly higher ($P<0.05$) than the sample from Herstmonceaux Church (5) (180.91 ± 13.97 mg/kg). There was no significant difference between the samples from Mount Usher (2) and Powerscourt (3). Finally, the cephalomannine content of the sample from Herstmonceaux Church (121.80 ± 9.51 mg/kg) was significantly higher ($P<0.001$) than for any of the other samples analysed. The lowest cephalomannine content was found in the two samples from Powerscourt (3a and b), with only 10.16 ± 1.65 and 13.24 ± 1.06 mg/kg, respectively.

The paclitaxel content in the *T. baccata* cultivars analysed varied considerably and ranged from 357.99 ± 45.12 mg/kg in *T. b.* 'Standishii' from Poland (9) to 5.49 ± 0.01 mg/kg in *T. b.* 'Dovastonia Aurea' from New Ross (1). The highest content of paclitaxel was found in *T. b.* 'Standishii' (9) (357.99 ± 45.12 mg/kg), *T. b.* 'Semperaurea' (9) (334.94 ± 64.27 mg/kg), *T. b.* 'Erecta' (9) (276.94 ± 2.59 mg/kg), *T. b.* 'Aureahoseri' (9) (216.36 ± 3.64 mg/kg) and *T. b.* 'Lutea' (2) (203.69 ± 16.57 mg/kg). There was no significant difference between *T. b.* 'Standishii' (9) and *T. b.* 'Semperaurea' (9). These two samples had a significantly higher paclitaxel content than any of the other samples ($P<0.001$). The lowest paclitaxel content present was in *T. b.* 'Dovastonia Aurea' (1) (5.49 ± 0.00 mg/kg), *T. b.* 'Adpressa Aurea' (6) (23.56 ± 0.59mg/kg), *T. b.* 'Standishii' (6) (39.28 ± 0.47 mg/kg) and *T. b.* 'Adpressa' (6) (40.87 ± 4.12mg/kg), however, the three samples from Edinburgh were collected in late September and can therefore not be directly compared with the other *T. baccata* cultivars. The level of 10-DAB III also showed a great deal of variation between and within the cultivars analysed. The highest level was present in the three polish samples (9): *T. b.* 'Aureahoseri' (585.42 ± 50.43 mg/kg), *T. b.* 'Erecta' (570.10 ± 8.59 mg/kg) and *T. b.* 'Adpressa' (516.08 ± 59.53 mg/kg). The lowest content of this

taxane was found in *T. b.* 'Aurea (3) (7.81 ± 1.88 m g/kg). *T. b.* 'Lutea' (2) (31.75 ± 5.64 mg/kg) and *T. b.* 'Densifolia' (4) (42.21 ± 2.70 mg/kg) also had very low levels of 10-DAB III. Finally, the range for the cephalomannine contents was considerably smaller than for the other two taxanes. The sample with the highest concentration was *T. b.* 'Lutea' (2) (189.23 ± 59.59 mg/kg). *T. b.* 'Aurea' (9) (137.64 ± 54.93 mg/kg) also had a higher level than the other cultivars. The samples with the lowest levels of cephalomannine were *T. b.* 'Adpressa' (6) (11.65 ± 1.65 mg/kg) and *T. b.* 'Neidpathensis' (4) (13.95 ± 2.58 mg/kg). There was no significant difference between these two (P>0.05).

Taxus brevifolia

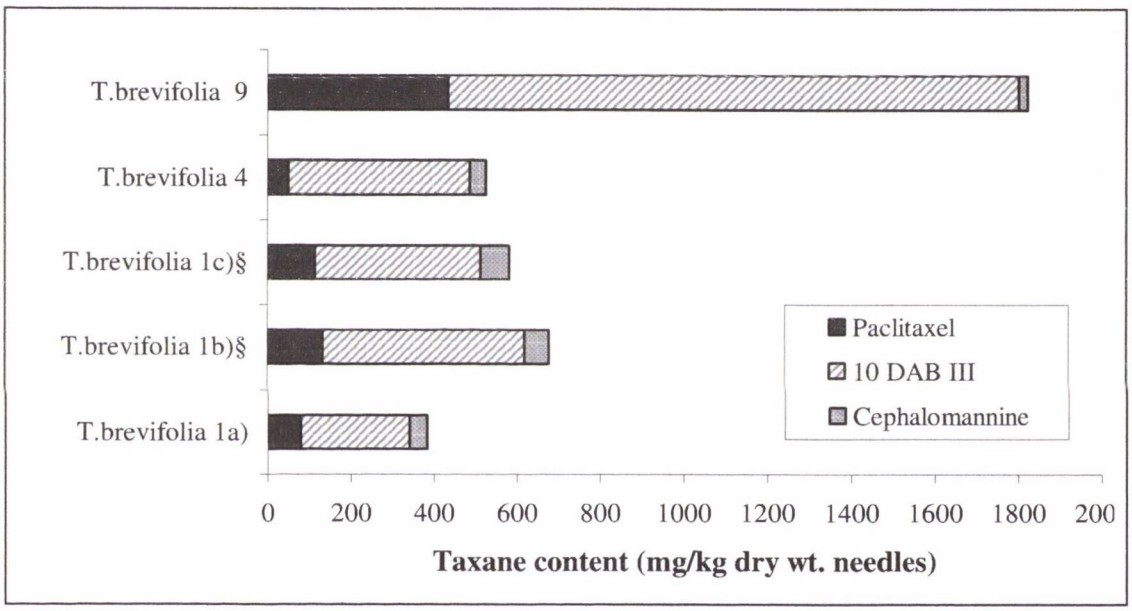


Figure 4.3.1.3 Taxane content of *T. brevifolia* samples

The *T. brevifolia* sample from Poland had a significantly higher (P<0.0001) content of paclitaxel (434.09 ± 14.24 mg/kg) than any of the other *T. brevifolia* samples analysed. If this sample was excluded from mean calculations the average was only 64.74 mg/kg, leaving *T. brevifolia* to be among the species with the lowest paclitaxel content. The Polish *T. brevifolia* sample also had a very high concentration of 10-DAB III present (1358.54 ± 13.07 mg/kg). This was significantly higher (P<0.0001) than the content found in the rest of the *T. brevifolia* samples analysed. If this value was excluded the overall general average 10-DAB III content of *T. brevifolia* was 348.7 mg/kg. The very high content of both paclitaxel and 10-DAB III in the Polish sample compared to

the rest of the *T. brevifolia* samples analysed could be due to a geographical factor, or it could also be a misidentified sample, although the taxane content is higher than any of the other *Taxus* samples analysed in this study. As only one Polish *T. brevifolia* sample was obtainable no certain explanation can be suggested. The sample from Glasnevin had a higher 10-DAB III content than the sample from New Ross. Generally the *T. brevifolia* samples analysed were found to have a low content of cephalomannine. The two samples collected late in the year from New Ross had a higher level present than the others ($P<0.001$). The Polish sample (21.39 ± 0.82 mg/kg) had a significantly lower level than any of the others ($P<0.001$). No difference was present between the other samples.

Taxus canadensis

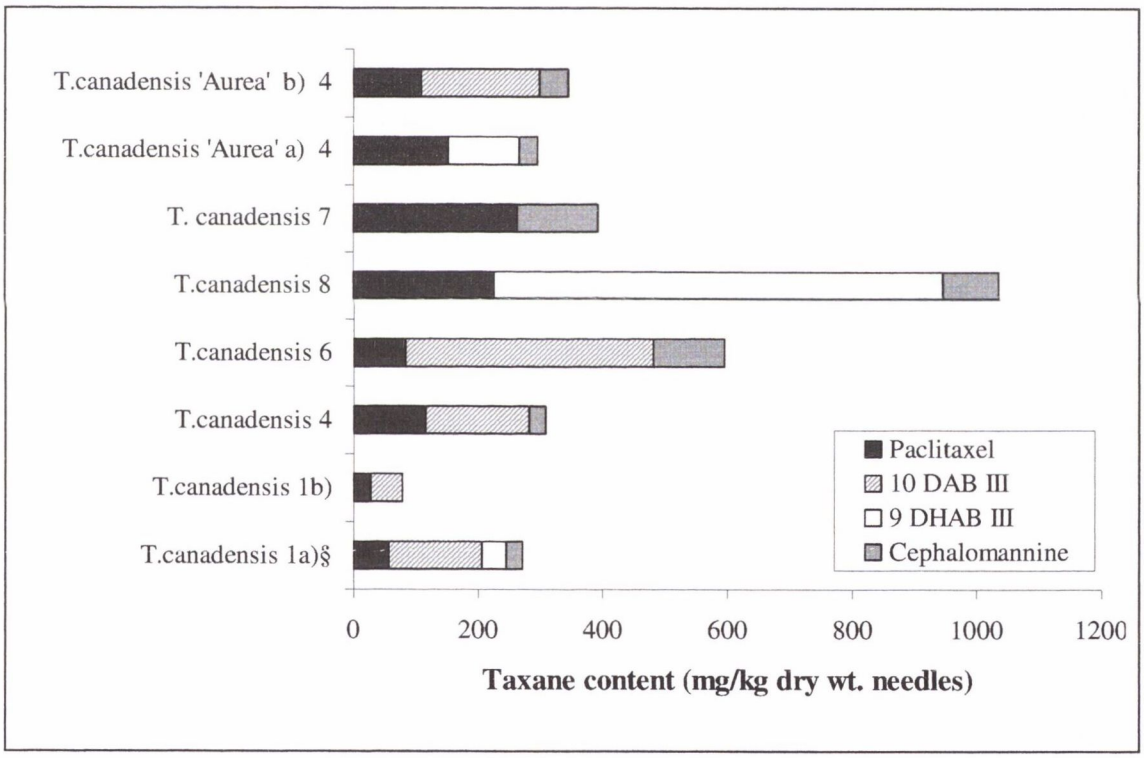


Figure 4.3.1.4 Taxane content in *T. canadensis* cultivars

The chemical analysis of the eight different *T. canadensis* samples showed a variation in the amount of taxanes, and that different taxanes were present in the needles. The paclitaxel content in the *T. canadensis* samples analysed fluctuated widely. The sample from Bedgebury (7) had the highest paclitaxel content (264.27 ± 6.05 mg/kg), significantly higher than any of the other samples analysed ($P<0.001$). However, this

sample was collected in 1997, so because of the risk of change in chemical constituents due to the long storage time before analysis, this sample could not be directly compared to the other samples. The sample from Canada (8) also had a high level of paclitaxel (227.08 ± 20.89 mg/kg). The lowest level of paclitaxel was found in the two samples from New Ross (1a) and 1b)). The sample collected late in the year (1a) had a higher level (57.56 ± 14.97 mg/kg) than the sample collected in May (1b) (27.88 ± 6.24 mg/kg).

The *T. canadensis* sample from Canada (8) contained a large amount of 9-DHAB III (720.59 mg/kg). This amount was in fact 3 times higher than the amount of its co-metabolite paclitaxel (227.08 mg/kg). This was expected since 9-DHAB III is found to be the most abundant taxane in the species (Gunawardana et al. 1992, Zamir et al. 1992, Zamir et al. 1995) and has only been found in trace amounts in the bark of one other yew, *T. chinensis* (Zhang et al. 1992). 9-DHAB III is therefore said to be species-specific to *T. canadensis*. In the *T. canadensis* 'Aurea' (4a) from Glasnevin this taxane was only found in a small amount (114.05 mg/kg), lower than the amount of paclitaxel (152.53 mg/kg).

The Canadian sample (8), the sample from Glasnevin (4a) discussed above and the Bedgebury (7) sample did not have quantifiable amounts of 10-DAB III. The rest of the samples analysed contain various amounts of this taxane. A very high level of 10-DAB III was present in the sample from Edinburgh (6) (397.15 ± 32.83 mg/kg). The highest levels of cephalomannine were present in the sample from Bedgebury (7) (129.26 ± 2.33 mg/kg) and the sample from Edinburgh (6) (114.99 ± 5.41 mg/kg). The Canadian (8) sample also had a significantly higher ($P<0.001$) level of cephalomannine than the other samples (89.31 ± 54.56 mg/kg).

Taxus cuspidata

Eight different *T. cuspidata* species and cultivars were analysed. The highest level of paclitaxel was present in the cultivar *T. cuspidata* 'Luteo Baccata' from Glasnevin (4) (234.12 ± 37.29 mg/kg). This sample had a significantly higher concentration ($P<0.001$) than any of the other samples analysed, with the exception of *T. cuspidata* (1c) from New Ross. But this sample was collected late in the year and can therefore not be directly compared with the rest. The *T. cuspidata* (1a) from New Ross (171.64

± 5.29 mg/kg) and the cultivar *T. cuspidata* 'Thayerae' from Glasnevin (4) (173.87 ± 30.14 mg/kg) were also high yielding samples. The lowest level was found in the Polish cultivar *T. cuspidata* 'Nana' (9) (12.66 ± 1.41).

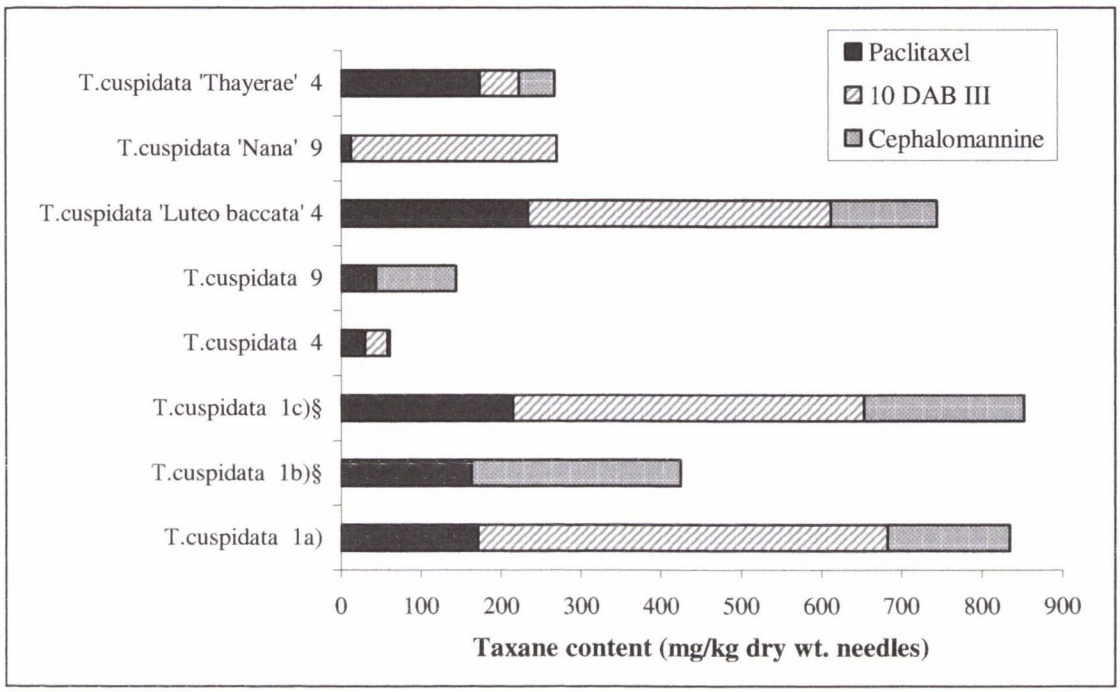


Figure 4.3.1.5 Taxane content of *T. cuspidata* cultivars

The highest overall average value of 10-DAB III in *T. cuspidata* came from the sample from New Ross (511.67 ± 10.35 mg/kg) which was significantly higher than the two other samples analysed. In fact, the sample from Poland did not have any detectible quantities. The *T. cuspidata* sample from New Ross was significantly higher than any of the *T. cuspidata* cultivars analysed, although the *T. cuspidata* 'Luteo Baccata' from Glasnevin also had high concentrations of 10-DAB III (378.34 ± 9.29 mg/kg). The concentration of cephalomannine was highest in the samples collected late in the year (December) from New Ross (b) 261.37 ± 31.72 mg/kg and c) 199.10 ± 14.31 mg/kg). But there was no significant difference between the second sample (1c), the 1a) sample from New Ross (152.17 ± 2.24 mg/kg), the Polish sample (126.83 ± 19.61 mg/kg) and the cultivar *T. cuspidata* 'Luto Baccata' (132.41 ± 68.22 m/kg). Cephalomannine was not present in quantifiable amounts in *T. cuspidata* 'Nana' and only in very small amounts in *T. cuspidata* from Glasnevin (2.32 ± 0.01 mg/kg).

Taxus X media

Seven different *T.* X *media* species and cultivars were analysed, where the two *T.* X *media* samples contained very different amounts of taxanes. The Polish sample (9) had a significantly lower concentration of paclitaxel (172.46 ± 3.22 mg/kg) than that of the sample from New Ross (1) (248.85 ± 8.48 mg/kg), and only non-quantifiable trace amounts of 10-DAB III were present in the Polish sample (9), while the sample from New Ross (1) contained a high concentration of this taxane (264.1 ± 32.39 mg/kg). Finally, the *T.* X *media* sample from New Ross (1) (148.91 ± 7.77 mg/kg) had significantly higher cephalomannine content than the Polish sample (9) (28.20 ± 3.22 mg/kg, $P<0.001$).

With the exception of *T.* X *media* 'Wojtek', the *T.* X *media* samples had a statistically higher ($P<0.001$) content of paclitaxel than the other *T.* X *media* cultivars analysed. The reason for the high mean value of 10-DAB III in the *T.* X *media* cultivars analysed was the high concentration in the Polish cultivar *T.* X *media* 'Rozon' (9). If this value was excluded from the calculations the overall average was only 156.1 mg/kg. No quantifiable amounts of 10-DAB III were present in the Polish cultivar *T.* X *media* 'Wojtek' (9). Of all the cultivars analysed, the Polish cultivar *T.* X *media* 'Rozon' (9) had the highest cephalomannine content (134.64 ± 13.16 mg/kg).

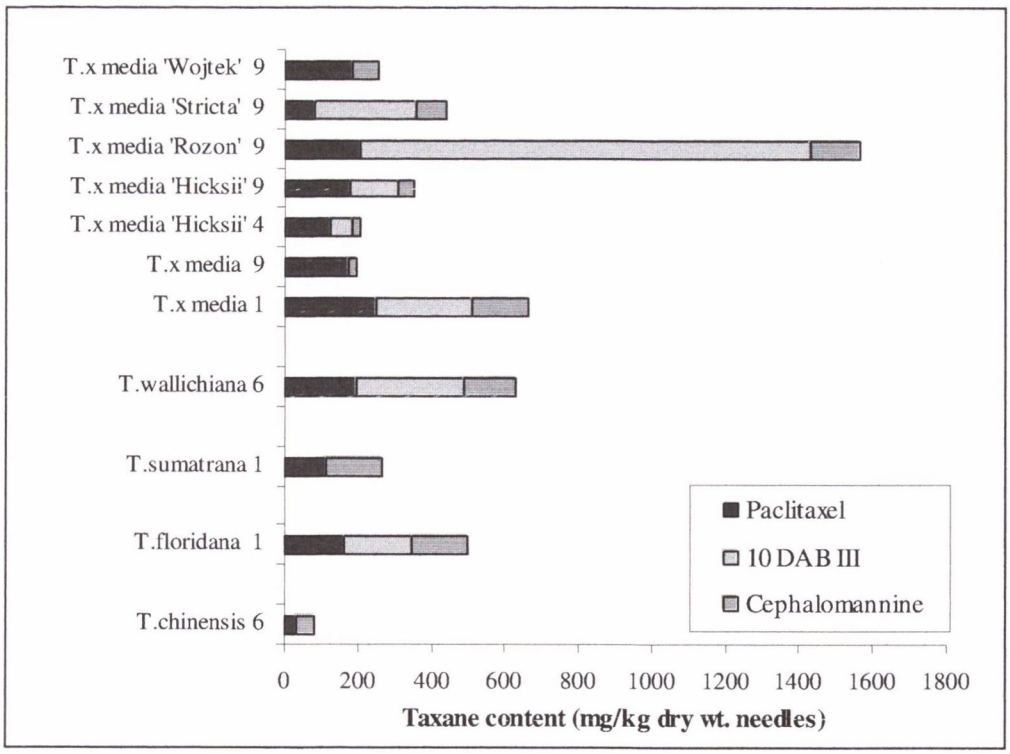


Figure 4.3.1.6 Taxane content in *T.* x. *media* cultivars and other species

Taxus floridana, Taxus chinensis, Taxus sumatrana and Taxus wallichiana

Only one sample of *T. floridana* was analysed and the paclitaxel content present was 162.87 ± 16.12 mg/kg. This value is lower than that found by van Rozendaal et al (van Rozendaal et al. 2000), where *T. floridana* had the highest paclitaxel content of all the samples analysed (516 mg/kg). However, in their study *T. floridana* had the second highest level of 10-DAB III (1689 mg/kg) and an absence of cephalomannine. In our study *T. floridana* had a lower level of 10-DAB III (184.09 ± 63.32 mg/kg) and it did contain cephalomannine (155.21 ± 25.07 mg/kg). The difference in collection time could account for this variation, where their samples were collected between 16th of February and 15th of March, earlier than the samples in the present study.

The taxane content in the Asian yew species *T. chinensis* (Chinese), *T. wallichiana* (Malaysian) and *T. sumatrana* (Japanese) did vary dramatically. *T. chinensis* had a very low content of paclitaxel (29.51 ± 1.76 mg/kg) and cephalomannine (46.29 ± 3.17 mg/kg). No quantifiable amounts of 10-DAB III were present in this species. The paclitaxel concentration in *T. wallichiana* (193.98 ± 6.27 mg/kg) was significantly higher ($P<0.001$) than in *T. sumatrana* (114.68 ± 6.95 mg/kg), but the cephalomannine content in *T. sumatrana* (149.57 ± 4.48 mg/kg) was significantly higher than that in *T. wallichiana* (139.09 ± 4.50 mg/kg). No quantifiable amounts of 10-DAB III were present in *T. sumatrana*, where as the level of this taxane in *T. wallichiana* (297.05 ± 6.11 mg/kg) was quite high. As this sample of *T. wallichiana* was collected in September, no direct comparison with this sample can be done.

4.3.1.2 Discussion of chemical examination

All of the *Taxus* species and cultivars analysed contained the taxane paclitaxel, confirming that they are authentic yew trees. A high level of variation in the taxane content between the different yew species was observed. There was also a large variation in taxane content within each of the species themselves. This could be due to geographical location, climatic factors, or perhaps environmental differences. van Rozendaal et al. (2000) did not find a significant difference in the total taxane content between the five locations of their sampling sites: Waningen, Boskoop, Horst, Hilversum and Bedgebury. They concluded that because different species were

collected from different sites, varying numbers of samples were collected from each site and because of the variation of taxane concentrations both within and between the species the comparison of total taxane content between the sites of collection did not show any significant difference. They could therefore not isolate any geographical site that gave higher taxane content than the others. The same problem was found in the present study, although the trend showed that the samples collected from Poland appeared to have higher average taxane content than the samples collected in Ireland and the UK. This trend could be as a result of climatic or environmental differences, however no conclusion could be made on the basis of the results from this study.

An amalgamation of the results from all the species and cultivars analysed is presented in **Table 4.3.1.2**. Single samples analysed were found to make the overall average calculated significantly higher or significantly lower than what was seen in the general trend, e.g. the Polish *T. brevifolia* (9) had a very high 10-DAB III concentration (1358.54 ± 13.07 mg/kg) resulting in a high overall average concentration of 10-DAB III for this species.

Table 4.3.1.2 Amalgamation of taxane content in *Taxus* species and cultivars

Sample	Paclitaxel (1) (mg/kg)	10-DAB III (2) (mg/kg)	9-DHAB III (3) (mg/kg)	Cephalomannine (4) (mg/kg)	No. of samples
T. baccata	146.9	158.5		48.7	5
T. baccata cv.	166.1	208.0		59.1	17
T. brevifolia	187.9	685.4		34.1	3
T. canadensis	121.1	237.2	379.9	64.2	4
T. canadensis cv.	131.0	190.3	114.1	37.9	2
T. chinensis (Edinburgh)	30.8			48.5	1
T. cuspidata	82.0	270.0		85.0	3
T. cuspidata cv.	140.1	228.3		88.6	3
T. floridana	162.9	184.1		155.2	1
T. sumatrana	114.7			149.6	1
T. wallichiana (Edinburgh)	194.1	297.1		139.1	1
T. X *media*	210.7	264.1		88.6	2
T. X *media* cv.	155.2	423.9		69.6	5

From the amalgamated results (**Table 4.3.1.2**) the highest overall average of paclitaxel concentrations were found in *T.* X *media* (210.7 mg/kg), *T. brevifolia* (187.9 mg/kg),

T. baccata cv. (166.1 mg/kg) and *T. floridana* (162.9 mg/kg). *T. wallichiana* (194.1 mg/kg) had a high concentration of paclitaxel, but this sample was collected at the end of September and therefore cannot be directly compared with the other samples. van Rozendaal and co-workers (van Rozendaal et al. 2000) found that *T. floridana* (516 mg/kg, 1 sample analysed) and *T. globosa* (433 mg/kg, 1 sample analysed) had the highest paclitaxel content. *T. wallichiana* (272 mg/kg, 1 sample analysed), *T.* X *media* cv. (211 mg/kg, 108 samples analysed), and *T. brevifolia* (130 mg/kg, 1 sample analysed) also had quite a high content of paclitaxel. It has to be taken into account that the samples analysed by van Rozendaal et al. were collected between the middle of February and the middle of March. This makes a direct comparison with the samples analysed in their study impossible.

The highest overall average 10-DAB III contents were present in *T. brevifolia* (685.3 mg/kg), *T.* X *media* cv. (423.9 mg/kg), *T .cuspidata* (270.0 mg/kg) and *T.* X *media* (264.1 mg/kg). A high content was also present in *T. wallichiana*, but again this sample can not be compared directly because of the late collection time. van Rozendaal et al (2000) found that *T. canadensis* (2665 mg/kg, 2 samples analysed), *T. floridana* (1689 mg/kg, 1 sample analysed), *T. globosa* (1395 mg/kg, 1 sample analysed) and *T. wallichiana* (1092 mg/kg) had the highest contents of 10-DAB III present.

Three samples stood out as having a high overall average content of cephalomannine. These were *T. floridana* (155.2 ± 25.1 mg/kg), *T. sumatrana* (149.6 ± 4.5mg/kg) and *T. wallichiana* (139.1 ± 4.5 mg/kg). van Rozendaal et al. (van Rozendaal et al. 2000) did not find a presence of cephalomannine at all in *T. floridana* and in *T. wallichiana*. They reported that *T. globosa* (480 mg/kg), *T. canadensis* (289 mg/kg), and *T.* X *media* cv. (131 mg/kg) had the highest content of this taxane.

The taxane specific to *T. canadensis*; 9-DHAB III, was only detected in the *T. canadensis* from Canada (720.59 ± 76.30 mg/kg), *T. canadensis* 'Aurea' a) from Glasnevin (114.15 ± 2.40 mg/kg) and in a small amount in the *T. canadensis* from New Ross (39.23 ± 0.87 mg/kg).

One might conclude that the taxanes analysed are of doubtful taxonomic value as to their usefulness in being markers for specific species within the genus *Taxus*. Some taxanes, however, are species-specific such as brevifoliol (in *T. brevifolia*) and 9-DHAB III (in *T. canadensis*), and these could be used a chemotaxonomic markers for these species. Although no marker has been suggested for *T. baccata* our analysis suggests the presence of such a compound. This finding will be further discussed in the following section (section 4.3.2).

4.3.2 Identification of possible chemotaxonomic marker for *Taxus baccata*

On closer examination of the HPLC chromatograms produced during the chemotaxonomic analysis of various *Taxus* species and cultivars (section 4.3.1), it became evident that a compound eluting with a retention time of 15.6-16.0 minutes was present at a higher relative concentration in *T. baccata* samples than in samples from other species (see **Figure 4.3.2.1**).

For the isolation and structure elucidation of this unknown compound a methanolic extract of dried *T. baccata* needles, collected in Trinity College, Dublin, was originally used and subjected to liquid column chromatography. The stationary phase used was silica gel, and the mobile phase was a gradient of chloroform and methanol. Further details to experimental procedure can be found in Material and Methods (section 3.4.3). However, the unknown compound was retained on the column. Therefore, aliquots from the ethyl acetate (EtOAc) extract (a less complex extract, because of less chlorophylls), which also contained the desired compound, was repeatedly injected into the same HPLC system used for taxane analysis (see section 3.3.4 for further details), and the fraction containing the compound in question was collected and combined. This resulted in a pure fraction containing the unknown compound, however, this method was time consuming, and the resulting residue was very small. Finally, the EtOAc extract was subjected to liquid column chromatography using silica gel yielding a suitable amount of residue for further analysis (40 mg). On re-injection on the HPLC system used previously it was found that the residue contained only the compound eluting with a retention time of 15.6-16.0 minutes (for more details see Materials and Methods, section 3.4.5).

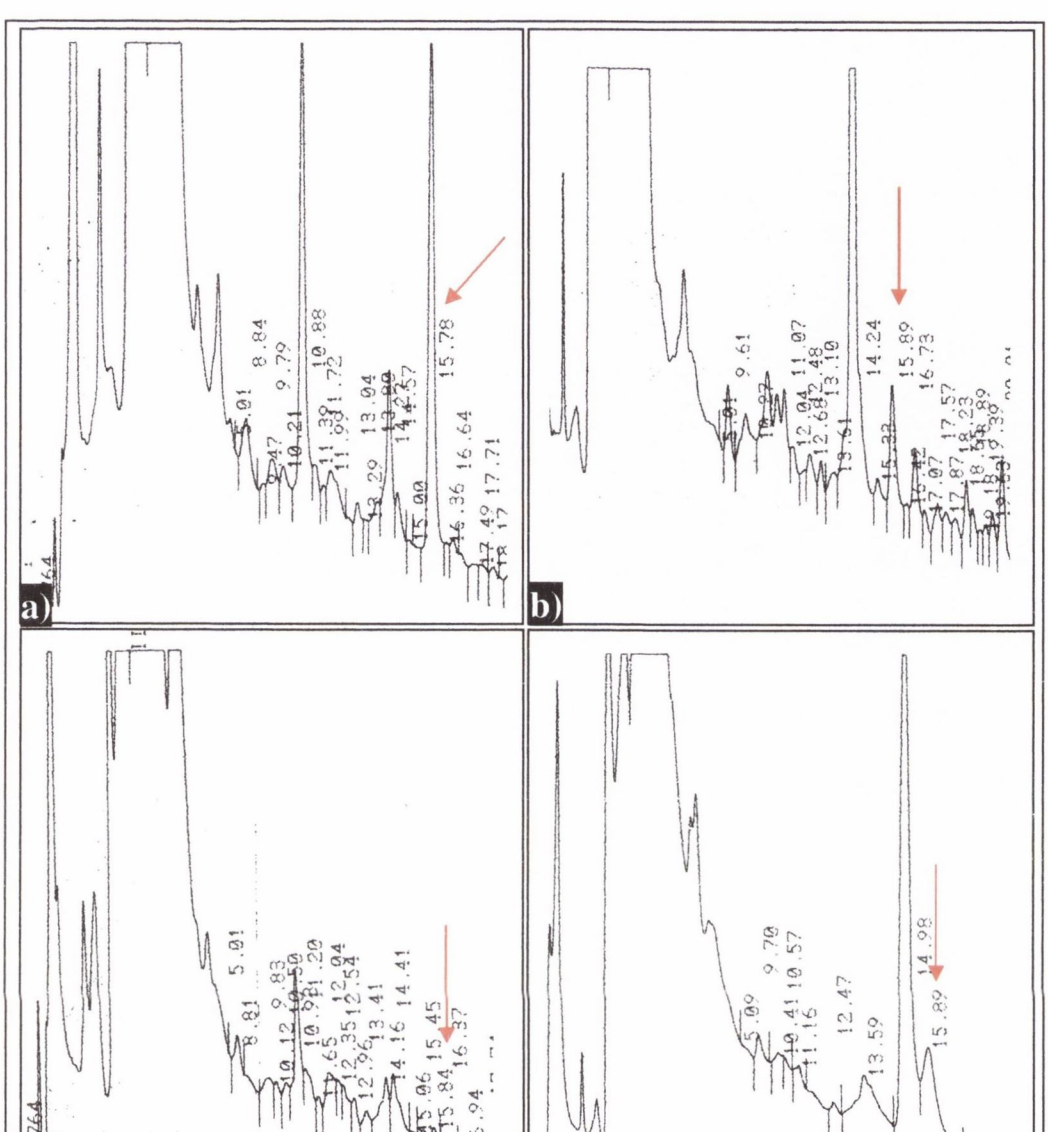


Figure 4.3.2.1 Different relative concentrations of compound eluting with retention time of 15.6-16.0 minutes in a) *T. baccata* (2), b) *T. brevifolia* (4), c) *T. sumatrana* (1) and d) *T. cuspidata* (1)

The pure fraction was further analysed by nuclear magnetic resonance spectroscopy (NMR). The ^{1}H- and ^{13}C-NMR spectra are shown in **Figure 4.3.2.2** and **Figure 4.3.2.3**, respectively. The ^{13}C-NMR spectrum is compared to the DEPT 135 and DEPT 90 spectra in **Figure 4.3.2.4**. Mass spectral analysis yielded a molecular ion of M^{+1} at m/z 154, which in conjunction with the ^{13}C-NMR spectrum suggested a molecular formula of $C_8H_{10}O_3$.

Analysis of the ^{1}H- and ^{13}C-NMR spectra indicated that the compound contained an aromatic moiety, having signals in the aromatic region of ^{13}C spectrum (160 ppm and 90 ppm region) and signals around 6ppm in the ^{1}H spectrum. This was confirmed by the typical m/z value of for an aromatic ring (m/z 77) seen in the mass spectrum. That the aromatic ring was substituted directly with an OH-group was also suggested because of the relative intense signal at m/z 95, which is typical of an aromatic ring substituted with an OH-group. A broad singlet at 5.25 ppm in the ^{1}H-NMR spectrum would also suggest an OH-group on an aromatic ring. A signal for a typical methoxy group was present as a singlet in the ^{1}H spectrum (3.78ppm). Two of the aromatic carbons (C3 and C5) were more downfield than the others, suggesting that they could be substituted by methoxy groups (161.2 ppm). Upon integration the methoxy peak in the ^{1}H spectrum it had 6 protons, suggesting that two methoxy groups were present. The downfield aromatic signals at 157.54 and 161.1 ppm in the ^{13}C-NMR spectra disappears in the DEPT 135 spectrum, indicating that they are quaternary carbons. In the DEPT 90 spectrum the signal at 54.9 ppm also disappears, confirming that this is CH_3 group(s). Because of its downfield position it can be suggested that this is a methoxy group, confirming the suggested signal of methoxy groups in the ^{1}H-NMR spectrum. The summarised spectral data from both ^{1}H-NMR and ^{13}C-NMR are presented in **Table 4.3.2.1**.

Table 4.3.2.1 ^{1}H- and ^{13}C-NMR data for unknown compound isolated (in $CDCl_3$

Proton	**^{1}H (ppm)**	**Carbon**	**^{13}C (ppm)**
1	5.25 (1H, br s)	1	156.9
2,6	6.051 (2H, d, J=2.05Hz)	2	93.8
4	6.1 (1H, t, *J*=2.14 Hz)	3	161.2
7	3.78 (methoxy, s)	4	92.7
8	3.78 (methoxy, s)	5	161.2
		6	93.8
		7	54.9
		8	54.9

s= singlet, br s= broad singlet, m=mltiplett, t=triplett, J= coupling constant

The typical coupling constant for aromatic protons in meta-, para- and ortho-position is J_{meta}=1-3 Hz, J_{para}=6-9 Hz and J_{ortho}=0-1 Hz. The coupling constant for the doublet at 6.051 ppm is 2.05 Hz, and the integration of this signal gives two protons, suggesting that the two aromatic protons are meta to each other.

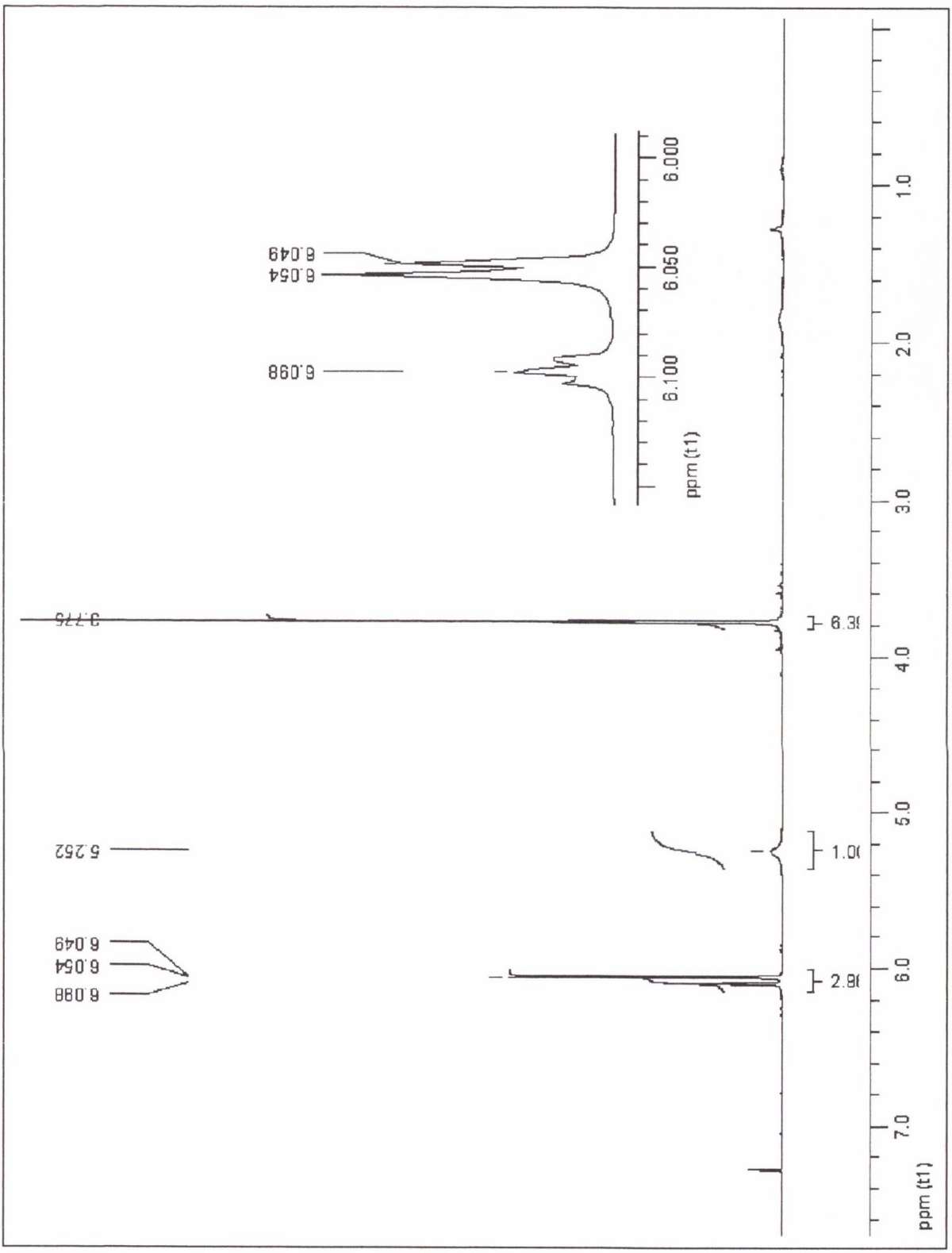


Figure 4.3.2.2 ^{1}H-NMR of unknown (in $CDCl_3$)

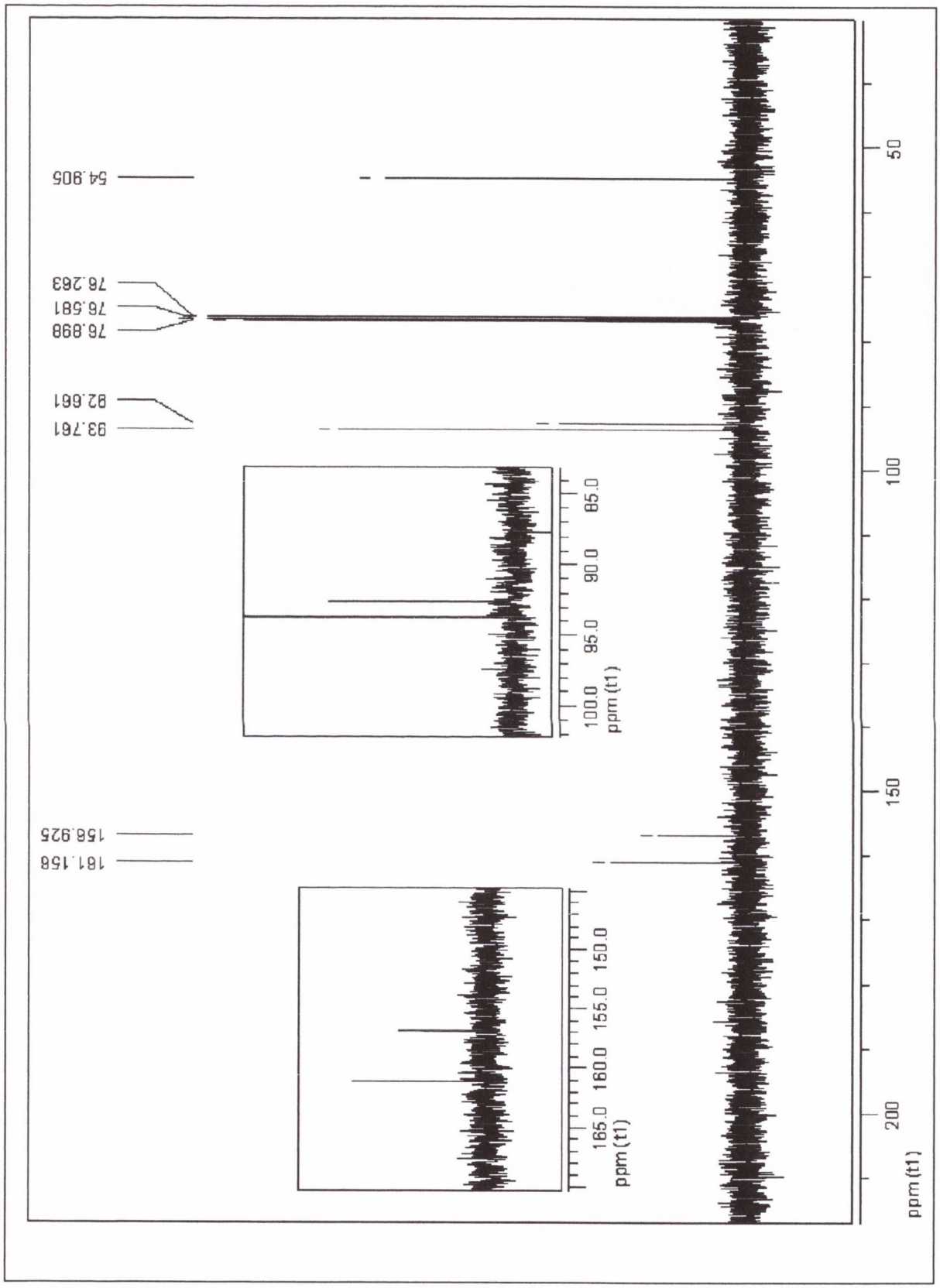


Figure 4.3.2.3 ^{13}C-NMR of unknown (in $CDCl_3$)

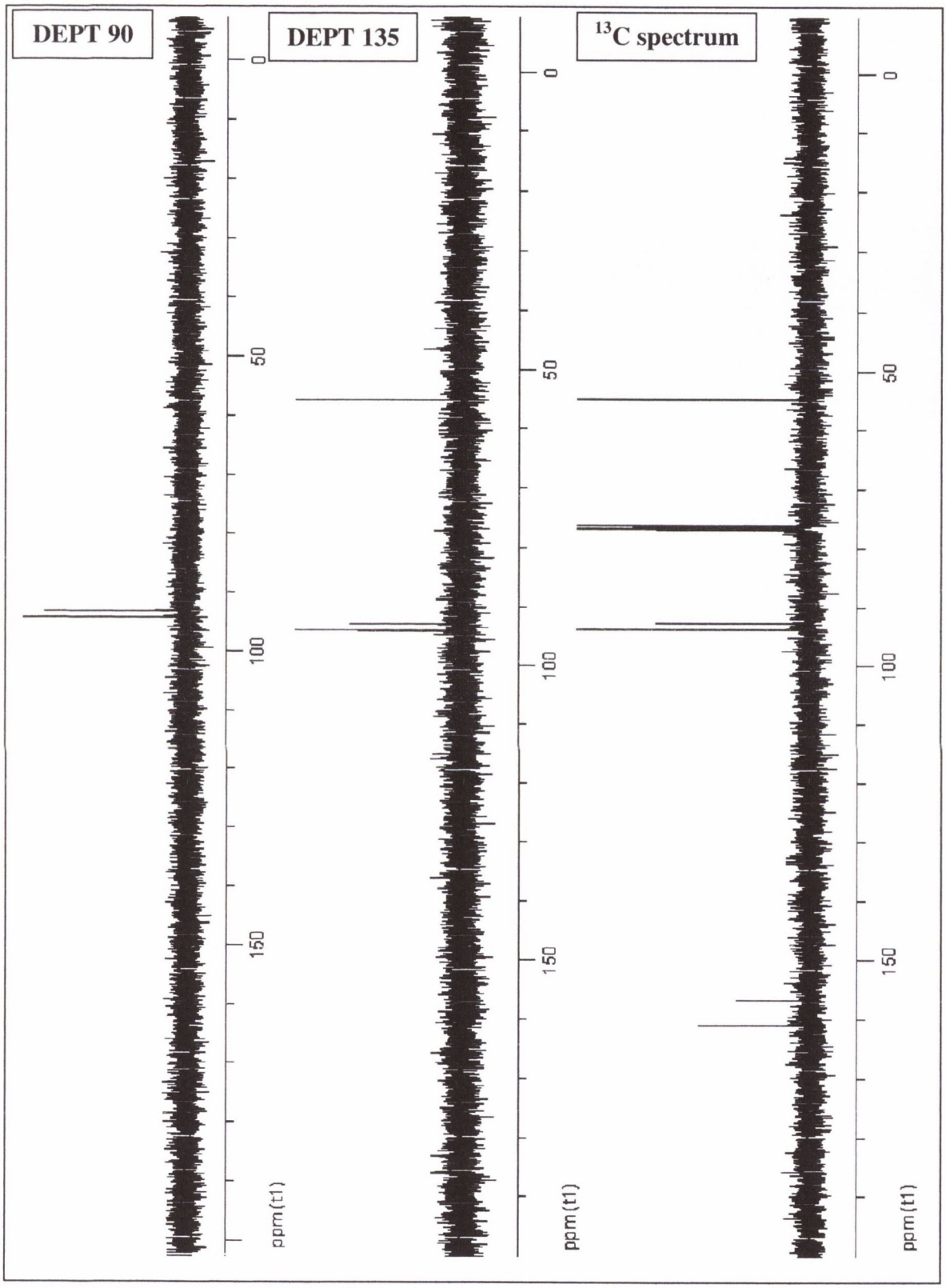


Figure 4.3.2.4 DEPT 90, DEPT 135 and ^{13}C-NMR of unknown (in $CDCl_3$)

These results suggested that the isolated compound was 3,5-dimethoxyphenol (*syn.* phloroglucindimethylether (Mußhoff et al. 1993)). This is the aglycone of taxicatin previously isolated from *T. baccata* (Lefebvre 1907, Merz and Preuss 1941), *T. baccata* 'Fastigiata' (Dempsey 2000) and from *T. canadensis* (Olsen et al. 1998).

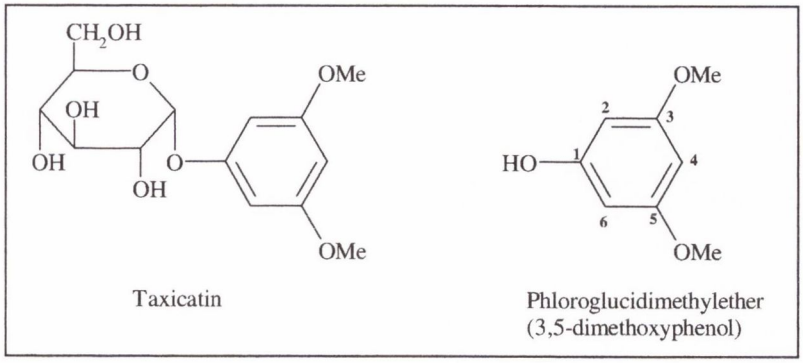


Figure 4.3.2.5 Taxicatin and its aglycon phloroglucindimethylether

The spectral data obtained compared well with literature values, as shown in **Table 4.3.2.2** below.

Table 4.3.2.2 Spectral data from unknown compared with literature values

Unknown in present study ($CDCl_3$)		**SDBS database * ($CDCl_3$)**		**(Ushiyama and Furuya 1989) ** (CD_3OD)**	
C-1	156.9	C-1	157.54	C-1	161.3
C-2	93.8	C-2	94.66	C-2	96.8
C-3	161.2	C-3	161.1	C-3	163.2
C-4	92.7	C-4	93.49	C-4	96.0
C-5	161.2	C-5	161.1	C-5	163.2
C-6	93.8	C-6	94.66	C-6	96.8
OMe	54.9	OMe	55.38	OMe	56.1
H-1	5.25 (1H, br s)	H-1	6.0	H-1	-
H-2,-6	6.051 (2H, d, J=2.05Hz)	H-2,-6	6.03	H-2,-6	6.40 (2H, d, J=2Hz)
H-4	6.1 (1H, t, *J*=2.14 Hz)	H-4	6.07	H-4	6.24 (1H, t, J=2Hz)
OMe	3.78 (s, OMe)	H-7	3.72	OMe	3.83 (s, OMe)

* (SDBS 2005)

** Phloroglucindimethylether part of taxicatin (hence no H-1 value)

In the present study phloroglucindimethylether appeared to be present in a relatively higher concentration in most of the *T. baccata* samples than in any other species analysed, although not all *T. baccata* samples analysed had a presence of this constituent. The *T. baccata* samples that lacked phloroglucindimethylether could have been misidentified by the Botanical Gardens of origin, or the variation could be due to other factors such as location, environmental factors etc. As the samples analysed

were collected at the same time of the year, seasonal changes can be ruled out as a factor for variation. It is important to mention that the peak eluting at retention time 15.6-16.0 min was detected in various concentrations in other species, for example in some *T. canadensis* samples, some *T.* X *media* samples and some *T. brevifolia* samples. However, the concentration of phloroglucindimethylether was found to be highest in *T. baccata*.

It could therefore be suggested that rather than being a chemotaxonomic marker, phloroglucindimethylether could be used as an indicator of the species *T. baccata*, and together with morphology and molecular sytematics it could be of taxonomic value.

That this compound could be used as a marker indicative of *T. baccata* was previously suggested. In a forensic study, after a suicide believed to be a result of yew leaf (*T. baccata* L.) ingestion, phloroglucindimethylether was found by Mußhoff et al (Mußhoff et al. 1993) to be resorbed into the blood. They concluded that this compound could therefore be used as a marker for poisoning from *Taxus baccata*.

4.3.3 Conclusion of chemical analysis

The taxanes analysed (paclitaxel, 10-DAB III and cephalomannine) were found to be present in varying amounts in all the species and cultivars analysed. The fourth taxane, 9-DHAB III, was only found present in various concentrations in some *T. canadensis* samples.

From the results obtained we can conclude that the highest-yielding species of the taxane paclitaxel were found to be *T.* X *media* (210.7 mg/kg), *T. brevifolia* (187.9 mg/kg), *T. baccata* cv. (166.1 mg/kg) and *T. floridana* (162.9 mg/kg), although the concentrations varied considerably within each of these species. This variation made it difficult to point out one particular species that would be best suited for direct isolation of paclitaxel. van Rozendaal and his co-workers (van Rozendaal et al. 2000) reported that the highest paclitaxel concentrations were found in *T. floridana* and *T. globosa*. But because these species are not widespread they suggested that *T.* x *media* 'Hillii' and *T.* x *media* 'Hicksii' were best suited for the isolation of paclitaxel because of the high paclitaxel content, availability and fast growth. However, they did not consider

the high levels of cephalomannine that these hybrids contain. Because cephalomannine is structurally so closely related to paclitaxel, it also has very similar chromatographic properties, which makes separation of the two taxanes difficult. Several chromatographic methods for the separation of paclitaxel and cephalomannine have been published (Cardellina 1991, Richheimer et al. 1992, Wickremesinhe and Arteca 1993, Witherup et al. 1989) but these primarily rely on reversed-phase chromatography or on the use of expensive bonded-phase columns. These methods are therefore not readily adaptable to large-scale operations as required for commercial operations. If paclitaxel is to be directly isolated from the plant it is better to choose a plant with low levels of cephalomannine. The most abundant taxane in all the samples analysed was found to be 10-DAB III. This finding corresponded with what other studies reported (ElSohly et al. 1995, van Rozendaal et al. 2000).

The value of the taxanes analysed as taxonomic markers is rather questionable due to the considerable amount of variation present between the species as well as within each species analysed. The fact that taxane concentrations also vary depending on the season of collection of plant material, and that there seems to be a certain extent of variation due to geographical location and environmental factors etc. would suggest the same. The species-specific taxanes, such as brevifoliol (in *T. brevifolia*) and 9-DHAB III (in *T. canadensis*), can be used a chemotaxonomic markers for these particular species. More work should be done in order to find suitable markers, especially for the more high-yielding species. Our suggestion of phloroglucindimethylether being an indicator of *T. baccata* is one example of this, where this constituent was found in higher concentrations in *T. baccata* than in any other *Taxus* species analysed.

The problem of variations even in the species-specific taxanes (as was seen especially in the case of *T. canadensis*) makes it difficult to only use chemotaxonomic methods in the identification of a particular species. The presence of the specific constituents has to be used in conjunction with morphological and molecular methods in order to be able to draw a conclusion.

5 Conclusion

The necessity of a suitable collection protocol was illustrated by preliminary experiments analysing the differences in needle dimensions from the eight twigs collected from each tree (two heights and four sides), where the trend showed that the needles on the lower-situated branches were longer than the higher ones. In addition two of the four sides of the tree also had slightly longer needles. The effect of drying the needles were also analysed, and it was found that drying caused reduction of needle surface-area and slight distortion of the needle shape. In addition it was observed that the difference in surface area of the previous year's and this year's growth, which before drying was significant, decreased upon drying. These findings illustrated the necessity of pooling needles from all the eight twigs collected from each tree (two heights and four sides) before randomly selecting the needles used for analysis. Dry and fresh samples can not be compared to each other and must be discussed separately. These findings also questions the usage of herbarium samples for identification of species, where the herbarium sample usually consist of one twig and location of the twig on the tree (side, distance from the ground etc) is never or seldom included in description. The preparation of the herbarium sample, such as pressing and drying, will have altered the needle dimensions and shape, making comparison difficult and inaccurate.

The results of the morphological and chemotaxonomical examination of the range of *Taxus* species and cultivars analysed in the present study raises questions regarding the correct identification of species, and also if the species established in the genus *Taxus* really can be considered different enough to be separate species.

The major variations in needle and seed dimensions together with major differences in tree shape and foliage colour from both fresh and dried samples of *Taxus baccata* and its cultivars suggests that the value of morphological characteristics for taxonomic purposes is doubtful. The appearance ranged from tall pyramidal trees, such as *T. baccata* itself, to bun-shaped, low-growing shrubs (e.g. *T. b.* 'Adpressa'). The foliage colour was either dark green, light green or golden. The variation in the tree shape and foliage colour within this species has lead to a great deal of misidentifications (Farjon

1998). The examination of needle dimensions showed that there was a significant variation in sizes (especially area and length) even when the outliers were excluded from the calculations. The seed dimensions obtained for this species also varied considerably, mainly due to the actual shape of the seed (reflected in the width/length ratio). Although the variation within the species was big, most of the samples of *T. baccata* and its cultivars analysed (both needles and seeds) compared well with literature values obtained for the same samples (Chadwick and Keen 1976, Dallimore and Jackson 1924, Vidakovic 1991). Also the taxane content varied considerably within the species *T. baccata*. The *T. baccata* cultivars were found to have the highest overall paclitaxel content of all the *T. baccata* samples analysed.

The *T. brevifolia* trees analysed all had a similar appearance, although the needle and seed dimensions obtained showed considerable amount of variation within the species. This could be due to geographical differences, differences in light, nutrition etc., but misidentification of the trees can not be ruled out. In particular the results from the seed examination raise some questions of identity as there was no association between the obtained and literature values (Dallimore and Jackson 1924, Vidakovic 1991). The taxane content varied between the *T. brevifolia* samples analysed, where especially the Polish sample had a significantly higher paclitaxel and 10-DAB III content than the rest. This could be due to geographical and environmental factors, but because only one sample was supplied the results are only an indication.

The hybrid samples analysed (*T.* X *media* and its cultivars) had generally larger needle-areas than the two "parent plants" *T. cuspidata* and *T. baccata*. The seeds of this hybrid resemble, to a greater degree, the larger and more ovate seeds of *T. baccata* than the smaller seeds from the other 'parent plant', *T. cuspidata*. The appearance of the trees varied from arching bush or shrub, oval to fastigiate tree and columnar, narrow, fastigiate tree. Although the foliage colour remained the same, macroscopical characteristics were found to be of limited value for taxonomic purposes in the case of *T.* X *media* and its cultivars. The taxane content of the hybrid samples was found to be high, with the overall average paclitaxel content being the highest of the samples tested.

Three of the Asian *Taxus* species analysed, *T. chinensis*, *T. sumatrana* and *T. wallichiana*, all had larger needle areas than *T. cuspidata*, mainly due to the width of the needles. *T. cuspidata* has smaller needles and its appearance could easily be confused with *T. canadensis* or *T. baccata*. With the exception of *T. cuspidata* it was found that macroscopical characteristics such as tree shape, needle density on the twigs and foliage colour could distinguish the Asian *Taxus* species from the other species analysed. Especially was the appearance of *T. chinensis* easily distinguishable from other *Taxus* species, where the needle distribution was less dense on the twigs, the foliage colour was golden, and the twigs were not always straight and the needles were wider than average. In the chemotaxonomical examination *T. sumatrana* and *T. wallichiana* stood out from the rest having high cephalomannine contents together with *T. floridana*. *T. chinensis* on the other hand had a very low content of both paclitaxel and cephalomannine. However, in the case of *T. chinensis*, *T. sumatrana* and *T. wallichiana* only one sample was obtainable so the results for both morphology and taxane content are only indicative.

The correct identification by the Botanical Gardens of the *T. canadensis* trees used in our study has been queried due to the great variation found in needle and seed dimensions and because of the absence of the species specific taxane 9-dihydro-13-acetyl baccatin III in some of the samples analysed. The shape of the trees examined reflects the same uncertainty, where the trees range from low-growing, wide-spreading shrubs to arching bushes or trees. The authentic sample from Canada (8) is a low-growing, wide-spreading shrub, but also in this case geography, light, soil nutrition and age of the plant has an influence on shape and character. Further microscopical analysis of both stomatal rows and stomatal number showed variation within the species and molecular sytematics performed on some of the samples concluded that the samples supplied as *T. canadensis* did not show strong relationship with each other.

The variation of taxane content within the species analysed made it difficult to point out one particular species that would be best suited for direct isolation of paclitaxel. The highest-yielding species of the taxane paclitaxel were found to be *T.* X *media* (210.7 mg/kg), *T. brevifolia* (187.9 mg/kg), *T. baccata* cv. (166.1 mg/kg) and *T. floridana* (162.9 mg/kg), although the concentrations varied considerably within each of these species. The most abundant taxane in all the samples analysed was found to

be 10-DAB III. This finding corresponded with what other studies reported (ElSohly et al. 1995, van Rozendaal et al. 2000). The considerable amount of variation present in taxane content between the species as well as within each species analysed suggests that the value of taxanes as taxonomic markers is limited. Taxane concentrations also vary depending on the season of collection of plant material, and that there seems to be a certain extent of variation due to geographical location and environmental factors etc. would suggest the same. Species-specific taxanes, such as brevifoliol (in *T. brevifolia*) and 9-dihydro-13-acetylbaccatin III (in *T. canadensis*), can be used a chemotaxonomic markers for these particular species. In the present study phloroglucindimethylether was isolated and a high concentration of this constituent was suggested as an indicator of *T. baccata*. The problem of variations even in the species-specific taxanes (as was seen especially in the case of *T. canadensis*) makes it difficult to only use chemotaxonomic methods in the identification of a particular species.

The results obtained from morphological examinations and chemotaxonomical analysis performed tended to support the opinion of Elwes and Henry (Elwes and Henry 1906) that the genus *Taxus* is mono-specific, consisting of a large number of varieties which exhibit enormous diversity in terms of their appearance but which are essentially taxonomically indistinguishable. Of the parameters investigated only the seed dimensions could to a certain extent be used to distinguish between the various species, but even these results were questionable in some instances and can not be used as a method on its own. Further work is needed especially in the fields of molecular systematics to determine what relationships exist between the species and in chemotaxonomy to find species-specific chemical markers.

PART II

6 Introduction

6.1 Plant Cell and Tissue Culture – an Outline

6.1.1 History and evolution of the use of plant cell culture

Plant cell cultures are an attractive alternative source to whole plants for the production of high-value secondary metabolites. It was long thought that undifferentiated cells, such as callus or cell suspension cultures were not able to produce secondary compounds, unlike differentiated cells or specialized organs (Krikorian 1969). This was experimentally proved wrong by Zenk (Zenk 1991) when they obtained 2.5 g of anthraquinones per litre of medium from un-differentiated cell cultures of *Morinda citrifolia*. The plant cells were found to be biosynthetically totipotent, meaning that each cell retains its complete genetic information and hence is able to produce the range of chemicals found in the parent plant.

Already in 1902, the Austrian botanist Gottlieb Haberlandt, pioneer in the development of physiological plant anatomy, and the first person to study plant tissue culture, attempted to cultivate isolated plant cells, but cell division was never observed in these cultures (Caponetti et al. 1996). His vision of the totipotency of plant cells represented the actual beginning of tissue culture. The first cultures of plant cells were from carrot roots (Gautheret 1939). In the same year, White (White 1939a, White 1939b) reported the growth and organ formation in culture from the interspecific hybrid *Nicotiana glauca x N. langsdorffii*. Skoog and his colleagues discovered the plant growth substance kinetin, a cytokinin, and developed an important tissue culture medium, the Murashige and Skoog (MS) medium in the late 1950s (Murashige and Skoog 1962). This medium would lead to the commercial application of tissue culture.

By the middle of 1950s, plant tissue culture methods were progressively becoming a major research tool in plant developmental biology. The techniques of sterile culture gave possibilities that the traditional methods such as morphology, anatomy, physiology, biochemistry, cytology and genetics could not give (Caponetti et al. 1996). The prediction that somatic plant cells could undergo embryogenesis was validated by Steward et al (Steward et al. 1958) who showed that somatic cells of carrot would

differentiate into embryos when cultured with a proper nutrient regime. The responsiveness of carrot tissue to growth induction has made it a model system for studies of factors promoting cell division (Steward et al. 1969). This was followed by a period of development of culture media and of cultivation methods (Street 1973). The ease with which the carrot cells can be grown as suspension cultures in liquid media has made it useful for studies of cell growth, including scale-up using bioreactors, secondary product synthesis and mutant selection.

The discovery of restriction endonuclease enzymes (enzymes that cleave DNA molecules at predictable sites and allow removal of specific genes) in the early 1970s changed the direction of plant cell and tissue culture research (Caponetti et al. 1996). The potential commercial value of genetically engineered plants attracted industry and in the late 1970s and early 1980s a number of new plant biotechnology-based industries were created (Caponetti et al. 1996).

6.1.2 Types of plant cell and tissue cultures

6.1.2.1 Undifferentiated cell culture systems

Higher plants depend on the organised allocation of functions to different parts of the plant itself, which in turn becomes differentiated into tissues such as vascular, glandular etc. Undifferentiated growth is seldom found in nature, but occurs frequently *in vitro*. The tissues formed typically lack any recognisable structure and contain only a limited number of differentiated cells found in the intact plant (George 1993).

Callus cultures

A wounded plant will sometimes produce a proliferation of undifferentiated cells (parenchyma cells) at the wound site. This mass of cells is called callus (Seabrook 1980). A similar mass of undifferentiated cells can be initiated *in vitro* by placing small pieces of whole plant (explants) onto growth medium, containing endogenous growth substances or growth regulating hormones, under aseptic conditions. The cells begin active division, and cell differentiation is reversed, giving rise to unspecialised cells (George 1993). Callus cultures can then be induced to differentiate and form organs and embryos (Evans et al. 2003).

Suspension cultures

Undifferentiated plant cells can also be freely dispersed in agitated liquid media, a technique similar to those used for large scale culture of bacteria. Suspension cultures are usually started by placing an inoculum of friable callus in a liquid medium. Under agitation single cells break off and, by division, form cell chains or aggregates of cells. These can again fracture and form individual single cells (Endress 1994). In general, suspension cultures grow more rapidly than callus cultures and are more amenable to experimental manipulation (Evans et al. 2003).

Haploid cultures

The development of *in vitro* techniques to produce haploid plants has been one of the most significant biotechnological advances in commercial plant breeding (Evans et al. 2003). Two procedures are used in the production of haploids: (i) Anther cultures: pollen-producing anthers are collected from flowers, sterilised and cultured on a specific nutrient medium. Callus forms from the individual microspores in the anthers, and haploid plants can be regenerated from the callus either directly by embryogenesis or indirectly by organogenesis (Evans et al. 2003). (ii) Microspore cultures: Microspores can be isolated from the anthers at a specific stage of development. The purified microspores are then cultured in liquid medium. Small embryos develop directly from the microspores and can be transferred to a regeneration medium for development of plants (Reed 1996).

Protoplast cultures

A protoplast consists of the cytoplasm and nucleus of a cell bounded by the plasma membrane, but with the cell walls removed. Protoplast can be isolated from whole plant organs or from plant tissue cultures. Isolated protoplasts can be cultured in a suitable medium to regenerate cell walls and produce callus (Evans et al. 2003).

6.1.2.2 Differentiated cell culture systems

'Organised growth contributes towards the creation or maintenance of a defined structure' (George 1993). An important difference between differentiated cell culture or organ culture and the undifferentiated cell culture (callus and suspension culture) is that the organs grow and develop in a similar manner to that of the parent plant. A wide range of plant organs have been cultured (Endress 1994). Some examples are:

Transformed organ culture

Transformed organ cultures are generated when a plant tissue is transformed with a culture of the bacterium *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens*. After the cell has been infected with the bacteria, the plasmid enters the plant cell and is incorporated into the plant genome. The bacteria themselves are consequently required only as a transport aid for bringing the plasmid into the plant cell. The transformation using *Agrobacterium rhizogenes* results in the induction of roots. The root system developed branches much more frequent than the usual root system of the plant, and is covered with a mass of tiny root hairs. Hairy root cultures can be maintained in liquid medium and produces the secondary metabolites of the parent plant. They have the advantages of being fast growing, genetically and biochemically stable and they can grow in hormone-free media. However, regeneration of whole plants from hairy root cultures is problematical. (Evans et al. 2003, Shanks and Morgan 1999). Plant transformation can be achieved by incubating *Agrobacterium tumefaciens* with plant protoplasts. The bacterium is then killed with an antibiotic, and protoplasts are allowed to regenerate walls and form a tissue culture. The non-transformed cells are killed by addition of kanamycin, and the remaining cells (which survive kanamycin treatment because they have the resistance gene) are used to regenerate plants from tissue culture (Deacon 2005).

Embryo cultures

Fertilized or unfertilized seed embryos (zygotic) are diverted from developing seeds or fruits. They are cultured in vitro until they have grown into seedlings. Embryo culture can assist in the rapid production of seedlings from seeds that have a protracted dormancy period, and it can enable seedlings to be produced when the genotype has low seed/embryo viability (George 1993).

Micropropagation

Micropropagation is a technique for producing large numbers of identical copies of a plant from tissue fragments of a 'mother plant'. The most widely used method for micropropagation is cultures from shoot tips. These cultures are stared from shoot meristems or buds with several leaves primordial and grown on medium containing suitable hormones. The shoots are finally rooted to form plantlets which can be grown

in vivo. These plants are genetically identical, and the result is therefore clonal propagation (Evans et al. 2003, George 1993).

6.1.3 Growth and secondary metabolite optimisation

6.1.3.1 Medium optimisation

Optimisation of the nutritional elements in the cell culture medium is the most fundamental approach to improved growth and productivity. Many reports and patents have been published discussing an optimal medium, but such a medium varies according to plant species. However, common factors in plant cell culture medium, such as the carbon source and growth hormones, shows similar effects in most cell and tissue cultures of different plant species. The preferred carbon sources are sucrose or glucose, although other carbon sources have been used (DiCosmo and Misawa 1995). The concentration of the carbon source have been found to have a major effect on cell growth and the secondary metabolite production (de Piava Neto and Otoni 2003), with one of the factors affecting the cells being the osmotic pressure change in the cell culture medium. Hormone, auxin and kinetin levels have also shown a great effect on the growth and productivity of cells in culture systems. Increased auxin levels usually cause a higher level of dedifferentiation in the cells, resulting in decreasing accumulation of secondary metabolites (DiCosmo and Misawa 1995).

6.1.3.2 Environmental factors

Environmental conditions, such as temperature, light and medium pH, can affect the growth and secondary metabolite production of a cell culture system.

Temperature: A temperature range of 17-25°C is normally used for the induction of callus tissues and growth of cultured cells (Ramachandra Rao and Ravishankar 2002). However, each plant species may favour different temperatures.

Medium pH: The medium pH for plant tissue culture is usually adjusted to around pH 5-6 before autoclaving, and extremes in pH are avoided (Ramachandra Rao and Ravishankar 2002). When callus cultures of *Ipomoea cairica* were grown at medium pH from 4-7.8 it was reported that at the lowest and the highest pH both cell growth and metabolite production was inhibited or reduced (Páska et al. 1999).

Light: Light has been found to influence both the growth and productivity of plant cell cultures differently depending on species. Three qualities of light influence in vitro growth: wavelength, flux density and the duration of light exposure (photoperiod) (George 1993). Effects of light on plant cell cultures differ. There was a marked decrease in the production of paclitaxel and a reduction of growth in *Taxus cuspidata* cells when they were grown in light (Fett-Neto et al. 1993). When cell suspension cultures of *Lithospermum erythrorhizon* were cultivated under blue light the biosynthesis of lithospermic acid B was stimulated, while shikonin production was strongly inhibited (Yamamoto et al. 2002). The biomass of *Panax ginseng* hairy root cultures was greater when they were incubated in red light than in the dark. In the same cultures fluorescent irradiation enhanced the accumulation of ginsenosides (Yu et al. 2005).

6.1.3.3 Precursor feeding

Feeding of a biosynthetic precursor to the culture medium may increase the biosynthesis of a specific secondary metabolite. The concept being that an increased concentration of any intermediate in the biosynthetic pathway of a desired secondary metabolite could result in an increased yield of the final product (Mulabagal and Tsay 2004). One example of a successful use of precursor feeding was the addition of phenylalanine to suspension culture of *Salvia officinalis*, where the biosynthesis of rosmarinic acid was reported to be stimulated and the production time was decreased (Ellis and Towers 1970).

6.1.3.4 Elicitation

A majority of the secondary metabolites synthesised by plants are produced as a defence mechanism against pathogen attack. Pathogens or elicitors are signals that can trigger the formation of these metabolites. The use of elicitors has been one of the most effective approaches to enhanced productivity in plant cell culture technology (Roberts and Shuler 1997). There are two types of elicitors: (i) biotic elicitors: including polysaccharides derived from plant cell walls (pectin or cellulose) and micro-organisms (chitin or glucans), glycoproteins, oligosaccharides of fungal origin, inactivated enzymes etc. (ii) abiotic elicitors: predominantly inorganic salts (e.g.$CuSO_4$, $AgSO_4$ etc.), and physical factors (such as pH) acting as elicitors.

6.2 Taxanes from Plant Cell Cultures of *Taxus*

Plant cell culture technology has become an important and irreplaceable tool for the investigation of plant secondary metabolism. It has provided an insight into secondary metabolite biosynthesis, enzymology, gene regulation, plant defence mechanisms etc. Because the environmental conditions, such as medium composition, temperature, pH, light etc, can be so closely controlled and manipulated using plant cell culture, this technique is increasingly becoming commercially important. It offers potentially unlimited and renewable plant material, making it an environmental friendly method which greatly favours biodiversity. It also allows for the study of biosynthetic routes to the production of commercially important plant metabolites such as taxanes.

The taxane yields from the traditional natural sources, such as trees harvested from wild sources or from trees cultivated in plantations, have been found to be far too low (e.g. 2000-3000 mature yew trees give 9000kg dried inner bark resulting in 1 kg paclitaxel). The production of taxanes from plant cell cultures has become a promising and environmentally acceptable solution to the supply problem. Currently Phyton Catalytic in cooperation with Bristol-Myers Squibb is producing paclitaxel (Taxol®) using cell cultures. However, these companies do not give the actual details of the production methods they are using (Takeya 2003). Most of the published work on *Taxus* plant cell culture to date has used undifferentiated cell culture systems (callus or cell suspension cultures), although a number of differentiated culture types have been investigated for the production of paclitaxel. Some examples of these are listed in **Table 6.2.1**. The secondary metabolite accumulation in *Taxus* plant cell cultures is often low, and the profile of the secondary metabolites can be different to the intact plant. The production may not be stable, and maintaining a continuous productivity is a common problem.

A great deal of research has centred on optimisation of the growth culture medium for *Taxus* cell suspension cultures. Some examples are:

- Fett-Neto et al (Fett-Neto and DiCosmo 1996, Fett-Neto et al. 1992) first studied the effects of nutrients and medium composition on growth and taxane production by *T. cuspidata* suspension and callus cultures.

- The growth medium for maximum biomass production in cell cultures of *T. brevifolia* was found by Ketchum et al (Ketchum et al. 1995).
- Concentration of the carbon source and the effect this factor had on growth and taxane production in *T.* X *media* 'Hicksii' cultures was reported by Wickremesinhe and Arteca (Wickremesinhe and Arteca 1994).

The external conditions such as light, pH of medium and temperature has also been found to have an effect on the growth and production of taxanes:

- Decrease of paclitaxel production by *T. cuspidata* cells under light conditions (Fett-Neto et al. 1995).
- pH has little or no effect on growth or taxane production in various cell cultures of *Taxus* species (Fett-Neto et al. 1993, Wickremesinhe and Arteca 1994).
- Low temperature (12-17°C) was shown to increase the doubling time up to three times in *T.* X. *media* 'Hicksii' cell cultures (Wickremesinhe and Arteca 1994).
- A temperature shift from 24° to 29°C after day 21 in the growth cycle of *T. chinensis* cells resulted in an enhanced production of paclitaxel. Cell growth was highest at 24°C (Choi et al. 2000).

Table 6.2.1 Differentiated culture types for production of paclitaxel

***Taxus* species**	**Culture type**	**Comment**	**Reference**
T. cuspidata	Immobilised cellsuspension culture producing microcalli	High paclitaxel production	(Fett-Neto and DiCosmo 1992)
T. brevifolia	Plant regeneration using somatic embryogenesis	Not evaluated for taxane production	(Chee 1995, Chee 1997)
T. cuspidata	Self-immobilised aggregate culture	Increased paclitaxel production compared to suspension culture	(Xu et al. 1998)
Taxus species.	Transformation using *Agrobacterium tumefaciens*	Galls formation in all stages, harvesting of galls allowed plant regeneration	(Stahlhut 1994) US patent 5,279,953
Taxus species.	Transformation using *Agrobacterium rhizogenes*	Hairy roots culture	(Plaut and Yu 1994) Patent WO9420,606

Finally, optimisation of the taxane production in *Taxus* cell culture systems by elicitation has been given a great deal of attention, given the importance of an alternative commercial taxane supply. Some examples of precursor feeding and elicitation are listed in **Table 6.2.2**. Because of the enormous amount of research dedicated to this area (e.g. from 1997-2000 129 papers were published (Takeya 2003)) only a few selected publications are listed here.

Table 6.2.2 Optimisation of taxane production by precursor feeding and elicitation

***Taxus* Species**	**Precursor**	**Comment**	**Reference**
T .cuspidata	Phenylalanine	Doubled paclitaxel production	(Fett-Neto and DiCosmo 1996)
T. wallichiana	Phenylalanine	Increased paclitaxel production	(Jha et al. 1998)
T. baccata	Phenylalanine	Increased paclitaxel and baccatin III production and increased growth rate	(Cusidó et al. 1999)
***Taxus* Species**	**Elicitor**	**Comment**	**Reference**
T. cuspidata	Gas phase composition	10% O_2, 0.5% CO_2, 5ppm ethylene increased production of paclitaxel	(Mirjalili and Linden 1995)
T. chinensis	Methyl jasmonate (MJ)	Increased taxane production	(Menhard et al. 1998)
T. baccata	Methyl jasmonate (MJ)	Increased production of baccatin III and paclitaxel	(Yukimune et al. 1996)
T. x. *media*	Methyl jasmonate (MJ)	Preferentially induces production of taxanes oxygenated at C-13	(Ketchum et al. 2003)
T. canadensis	Methyl jasmonate (MJ) with chitin and chitosan elicitors	Enhanced paclitaxel synthesis whit MJ and elicitors compared to MJ or elicitors on their own	(Linden and Phisalaphong 2000)
T. baccata	Vanadium sulphate (VSO_4)	Stimulates synthesis and excretion of paclitaxel and baccatin III.	(Cusidó et al. 1999)
T. chinensis	Salicylic acid (SA) and fungal elicitor (F)	Both SA and F treated cells resulted in enhanced paclitaxel production, but SA + F treated cells had highest production	(Yu et al. 2001)
T. yunnanensis	Rare earth element: Lanthanum (La)	Stimulates synthesis and excretion of paclitaxel	(Wu et al. 2001)

Oxidative stress has been found to be an important factor in elicitation of taxanes. Biotic elicitors, such as oligosaccharides of fungal origin, and abiotic elicitors, including metal salts and mechanical stress, have been found to induce oxidative stress in *Taxus* cell suspension cultures, which in turn have led to optimized taxane production (Wang and Wu 2005, Wu et al. 2001, Yu et al. 2002, Yuan et al. 2001, Yuan et al. 2002b, Yuan et al. 2002c, Yuan et al. 2002d).

6.3 Oxidative Stress and Plant Defence Mechanism

There is compelling evidence that oxygenated derivation of C_{18} unsaturated fatty acids (oxylipins) actively participate in plant defence mechanisms, including programmed cell death (hypersensitive response). Researchers believe that the hypersensitive response (HR) is initiated by the excessive formation of H_2O_2 and other oxygen radicals such as the superoxide anion ($O_2^{\bullet -}$). Also low valence transition metals can participate in the Fenton and Haber-Weiss reactions forming toxic hydroxyl radicals.

6.3.1 Active Oxygen Species (AOS)

In normal conditions active oxygen species (AOS) (*syn.* reactive oxygen species (ROS)) appear as inevitable by-products formed as a result of the reduction of molecular O_2. Molecular O_2 is reduced to water in four steps. This reduction results in the generation of several oxygen radical species (Van Breusegem et al. 2001).

$$\mathbf{O_2 \rightarrow (H)O_2^{\bullet -} \rightarrow H_2O_2 \rightarrow OH^{\bullet} + H_2O \rightarrow 2H_2O}$$

The first step requires initiation by NADP oxidase using NADPH. The subsequent steps are exothermic and can occur spontaneously. The hydroperoxyl ($HO_2^{\bullet -}$) and superoxide ($O_2^{\bullet -}$) radicals are short-lived, not readily diffusable and highly reactive compounds (Van Breusegem et al. 2001). The second step generates hydrogen peroxide (H_2O_2). H_2O_2 is a relatively long-lived molecule, which can diffuse some distance from its site of production (Levine et al. 1994). H_2O_2 can damage membranes and proteins, especially through the formation of hydroxyl radicals (Pellinen et al. 1999). The biological toxicity of H_2O_2 through oxidation of SH groups can be enhanced in the presence of metal catalysts through Haber-Weiss or Fenton type reactions (Dat et al. 2000).

$$\mathbf{O_2^{\bullet -} + Fe^{3+} \rightarrow Fe^{2+} + O_2}$$

$$\mathbf{H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^{\bullet}}$$

$$\mathbf{Overall: H_2O_2 + O_2^{\bullet -} \rightarrow OH^- + OH^{\bullet} + O_2}$$

H_2O_2 has both positive and negative effects on the host cell. If the concentration is too high it can trigger the hypersensitive response in the cells, which leads to cell death. If the concentration is right, and the pathogen is sensitive to this concentration, H_2O_2 can kill the pathogen. H_2O_2 signals for oxidative cross-linking of cell wall proteins, protecting the cells. It can also serve as a signal molecule for activating host-defence related genes and enhancing the biosynthesis of secondary metabolites. This is thought to be independent of key defence regulators such as salicylic acid, jasmonates, ethylene etc. (see section 6.3.4) (Mehdy et al. 1996).

The last species generated in the reduction of molecular O_2 is the hydroxyl radical ($OH^{\bullet}$). The hydroxyl radical is believed to be a major AOS responsible for modifications of macromolecules and cell damage. It can initiate radical chain reactions including lipid peroxidation, enzyme inactivation and degradation of nucleic acids (Mehdy 1994).

The levels of AOS inside the cell are kept at their lowest by the relevant protective mechanism using compartmentalised isozymes of catalase, superoxide dismutase (SOD) or peroxidase (Bolwell and Wojtaszek 1997). But during periods of more severe stress there is an increased production of AOS and the scavenging systems may become saturated by this increased rate of radical production (Van Breusegem et al. 2001). This over-production of AOS is termed an oxidative burst and it is a rapid response of plant cells to elicitors, pathogens or other stress situations for the plant (Mehdy et al. 1996). The first observation of AOS generation during plant-pathogen interaction were made in studies of potato tubers infected with the fungus *Phytophthora infestans* (Doke 1983). AOS generation was also associated with the HR in the interactions between tomato and the fungus *Cladosporium fulvum* (Vera-Estrella et al. 1994). AOS have been found to have a central role in the induction of pathogen defence genes, such as genes encoding for pathogenesis-related proteins (PR) (Levine et al. 1994), genes regulating the accumulation of phenylpropanoid compounds (Schenk et al. 2000), and genes encoding AOS detoxifying enzymes (Mittler et al. 1999).

The mechanism linking elicitor-receptor occupation and activation of the AOS production is not well understood. In recent years there has been put a question mark to the methods used to study the mechanisms for AOS production (Bolwell 1999). Several sources are known to exist for the generation of AOS (Bolwell and Wojtaszek 1997). These include NADPH and NADPH oxidases in the plasma membrane, apoplastic peroxidases, amine oxidases, oxalate oxidases and protoplastic sources from mitochondria, chloroplasts and peroxisomes. Of these systems the NADPH oxidase system has received most attention in plant-pathogen interactions. It has been suggested that the signalling pathway in plants share components with one pathway leading to AOS in mammalian neutrophils (Bokoch 1994). A model of this pathway is shown in **Figure 6.3.1.1**.

In mammalian neutrophils, many different ligands bind to cell surface receptors, activating G proteins. The G proteins then in turn activate phospholipase C (PLC). PLC catalyses the production of diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3). Subsequently, IP_3 binds to Ca^{2+} channel receptors resulting in increased cytosolic Ca^{2+}. The increase in diacylglycerol, an activator of protein kinase C, correlates well with the increase in protein kinase C activity. Phosphorylation by protein kinase C or other serine/threonine protein kinases is essential for the assembly of the active NADPH oxidase complex in the plasma membrane (Mehdy et al. 1996). An alternative pathway involving tyrosine phosphorylation and the mitogen-activated protein (MAP) kinase cascade also regulates the oxidative burst in mammalian cells (Bokoch 1994).

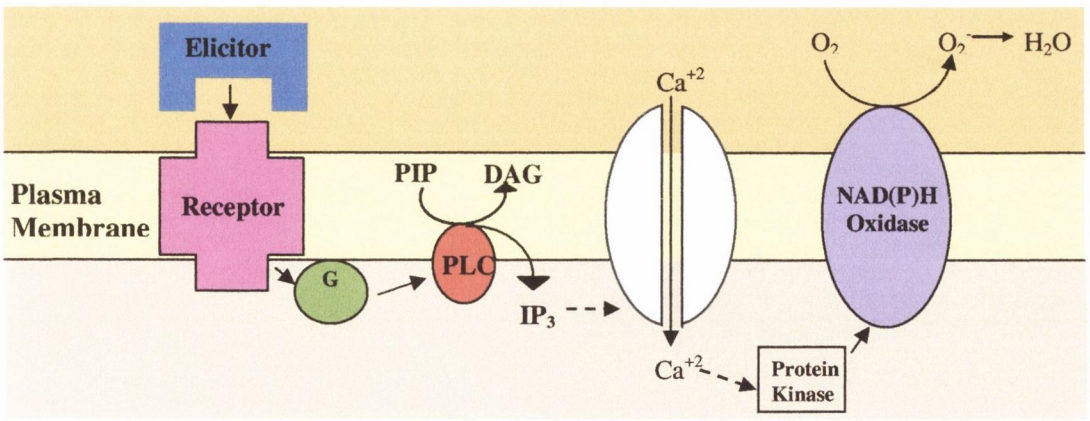


Figure 6.3.1.1 AOS release in mammalian cells (Mehdy et al. 1996)

The oxidative burst in plants is complex, and the full picture is yet to be clarified. **Figure 6.3.1.2** shows a speculative model of the oxidative burst in plant cells (Mehdy 1994).

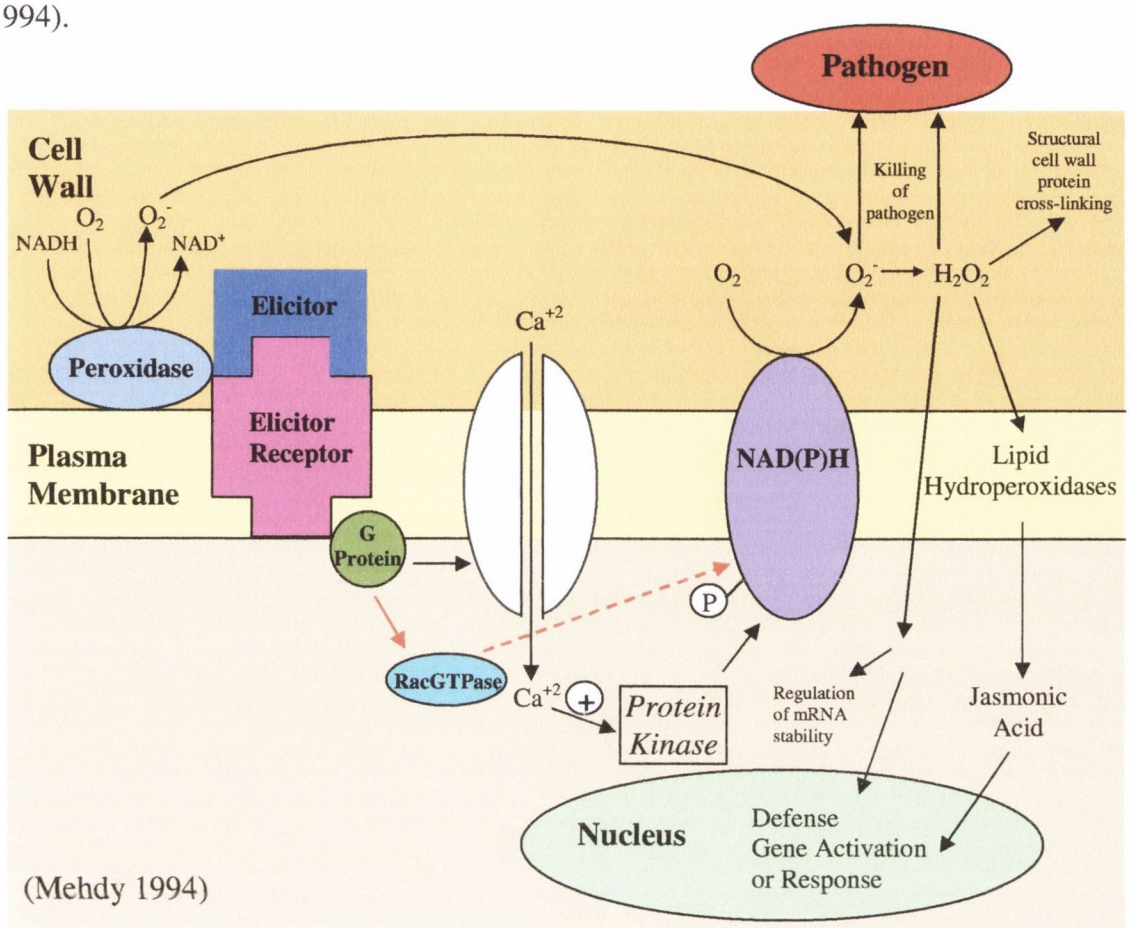


Figure 6.3.1.2 Speculative model of oxidative burst cascade in plant cells

Recent studies have found that the plants and animals use analogous systems to monitor and react to oxidants. It was found early on that G proteins were involved in the AOS generation in plants just like in mammalian cells (Vera-Estrella et al. 1994). Other studies have supported this finding linking G protein signalling with the activation of both Ca^{2+} channels and the membrane bound NADPH oxidase (Aharon et al. 1998). The intrinsic details of how G proteins regulate the Ca^{2+} channels in plants is yet to be concluded (Mahalingam and Fedoroff 2003). There is also evidence that the link in plants between the heterotrimeric G protein and the NADPH oxidase complex is a Rac GTPase as it is in mammalian cells (Suharsono et al. 2002). Receptor and G protein activation has also been found to stimulate the oxidative burst indirectly through phospholipid messengers. As in mammalian cells phospholipase C (PLC) hydrolyses the formation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol

(DAG) (Laxalt and Munnik 2002). Ortega and Perez (Ortega and Pérez 2001) reported that pathogen signalling stimulates IP_3 production and intracellular calcium release. Phosphorylation of DAG results in the production of phosphatidic acid (PA). Pathogen signalling was found to stimulate the PLC-dependent production of PA in plant cells (Laxalt and Munnik 2002). The PLC inhibitors neomycin and U73122 suppress the oxidative burst, whereas PA induces it (Laxalt and Munnik 2002). There is also some evidence that phospholipase A1 and A2, which hydrolyse phospholipids to produce a lyso-phospholipid and a free fatty acid, are activated in the pathogen defence response (Laxalt and Munnik 2002). Fatty acid oxidation by a lipoxygenase is the first step in the synthesis of oxylipin messengers, such as jasmonic acid (Farmer et al. 1998).

6.3.2 Phenylpropanoid pathway

The activation of the phenylpropanoid pathway and the pathogenesis-related (PR) proteins as a response to a wide variety of stress factors has lead to their use a genetic markers for the induction of plant defence responses. Phenylalanine ammonia lyase (PAL) catalyses the first step in the phenylpropanoid biosynthetic pathway (**Figure 6.3.2.1**) (Jones 1984).

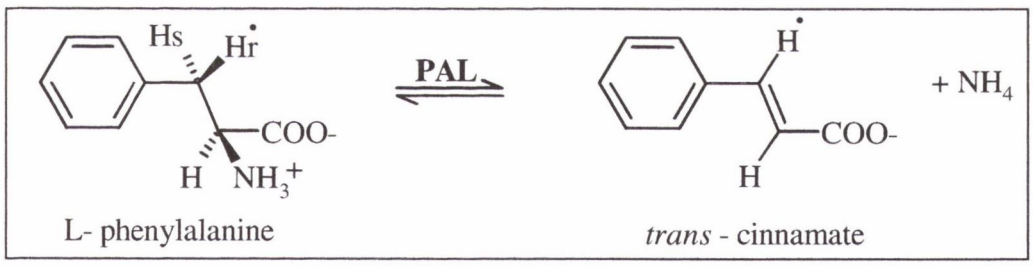


Figure 6.3.2.1 First step in the phenylpropanoid biosynthetic pathway

An increased level of PAL was observed by Yuan and co-workers (Yuan et al. 2002b) after introducing an oligosaccharide from *Fusarium oxysprum* to *Taxus chinensis* cells. They also reported an increase in the accumulation of phenolics. They found that the oligosaccharide induced an oxidative burst generating hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl free radicals ($OH^\bullet$). So in this case the increase in PAL was indicative of the induction of the plant defence mechanism. Recent publications have also found that the elicitor-induced pathways leading to cell death, oxidative burst and expression of defence response genes might be independent, even

if common components such as Ca^{2+} and protein kinases are necessary for the activation of the signal transduction pathway. Sasabe and co-workers (Sasabe et al. 2000) found that inhibition of elicitor-induced AOS generation in tobacco cells did not affect cell death and PAL gene expression. They also reported that chelators or Ca^{2+} channel blockers suppressed elicitor-induced oxidative burst and cell death, whereas they could not affect elicitor-induced PAL gene expression. This indicates that the pathways could be independent. However, very recently Xu and Dong (Xu and Dong 2004) reported that O_2^- from the elicitor-induced oxidative burst was necessary for mediating the elicitor-induced PAL activation and then in turn enhancing the catharanthine production in *Catharanthus roseus* cells. The actual relationship between the different pathways does require further investigation.

Figure 6.3.2.2 shows a general scheme of the phenylpropanoid metabolism, including the major natural product groups associated with it.

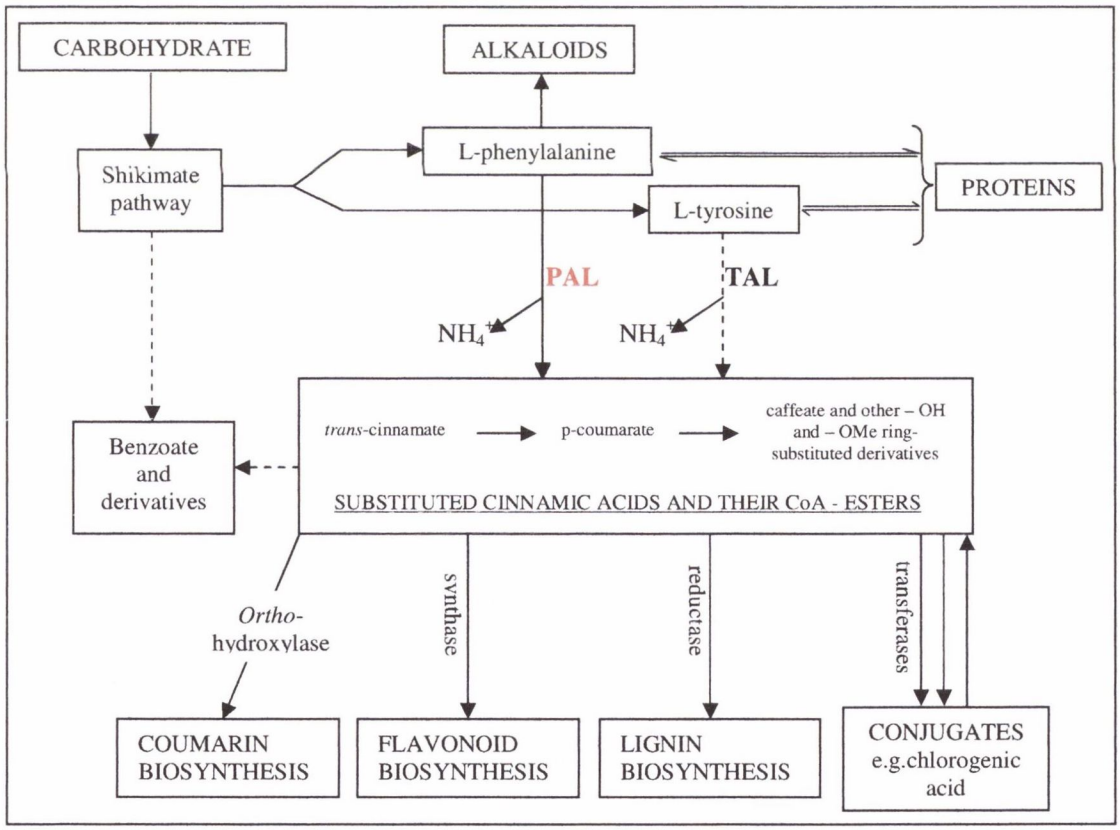
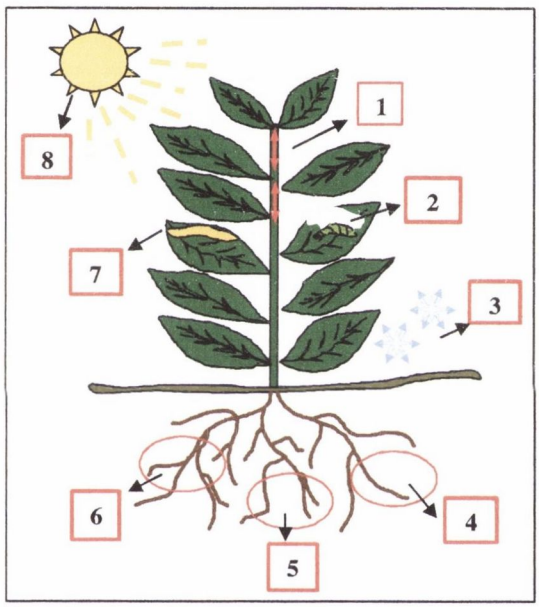


Figure 6.3.2.2 General scheme of phenylpropanoid metabolism (Jones 1984)

Many stress-induced phenylpropanoid compounds are classified as phytoalexins. These antimicrobial compounds are represented by a diverse chemistry and include many classes of secondary metabolites. **Figure 6.3.2.3** below shows some of the most common phytoalexins synthesised in response to various biotic and abiotic stresses in the plant. Salicylic acid (SA) is included in the discussion of phenylpropanoid natural products because of their presumed biosynthetic origin via side-chain shortening of hydrocinnamic acids. But this is only one of the biosynthetic pathways for the generation of salicylic acid. Many questions still remain about this form of defence, and the role which phytoalexins have as signalling compounds in the plant defence mechanism (Dixon et al. 2002).



(**1**) Signalling: salicylic acid, (**2**) Wounding: coumestrol, coumarin, psoralens, chlorogenic acid, ferulate esters, wall bound phenolic acids, lignin and suberin. (**3**) Low temperature: anthocyanins. (**4**) Low iron: phenolic acids. (**5**) Low phosphate: anthocyanins. (**6**) Low nitrogen: flavonoids and isoflavonoids. (**7**) Pathogen attack: pterocarpans, isoflavans, prenylated isoflavonoids and stilbenes, coumarins, furanocoumarins, 3-deoxyanthocyanidins, flavanols and auroenes. (**8**) High light/UV: anthocyanins, flavones, sinapyl esters, isoflavonoids and psoralens.

Figure 6.3.2.3 Examples of stress-induced phenylpropanoids (Dixon and Paiva 1995)

6.3.3 Flavonoids

6.3.3.1 Flavonoid biosynthesis

Flavonoids are a class of low-molecular-weight phenolic compounds that are widely distributed in the plant kingdom. Over 6000 naturally occurring flavonoids have been described, and many of them are common in higher plants (Harborne and Williams 2000). Flavonoids act in plants as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellants and light screening (Pietta 2000).

The flavonoids are built upon a C_6-C_3-C_6 flavone skeleton, in which the three-carbon bridge between the phenyl groups is commonly cyclised with oxygen. Based on the degree of unsaturation and oxidation of the three carbon segment, flavonoids are divided into several classes. The six major subgroups are found in most higher plants: the chalcones, flavones, flavonols, flavandiols, anthocyanins and condensed tannins (or proanthocyanidins). A seventh group, the aurones, is widespread, but not ever-present (Winkel-Shirley 2001). Some plants also synthesise specialised flavonoids, such as the isoflavonoids in legumes, 3-deoxyanthocyanins in sorghum, maize and gloxinia. Flavonoids are synthesized via the phenylpropanoid pathway. **Figure 6.3.3.1** illustrates the biosynthesis of the major classes of flavonoids (Winkel-Shirley 2001).

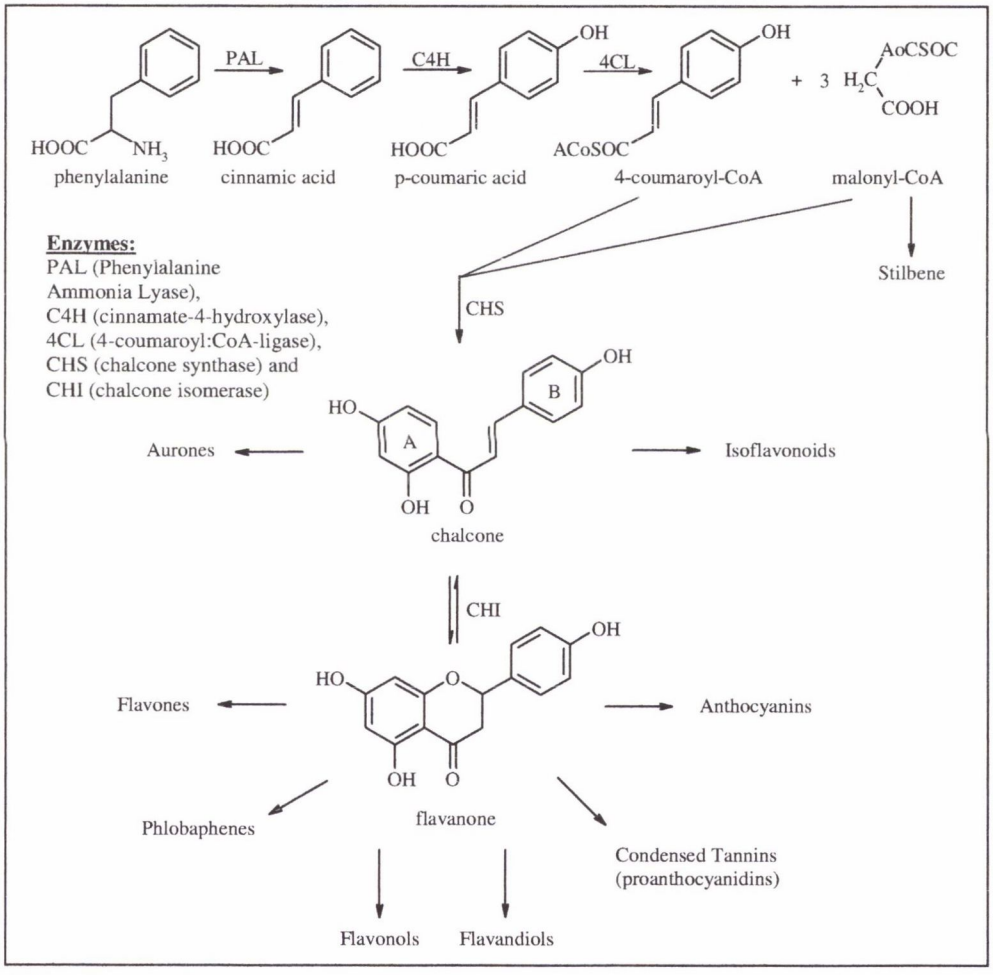


Figure 6.3.3.1 The biosynthesis of the major classes of flavonoid derivatives

Phenylalanine ammonia lyase (PAL) catalyzes the conversion of phenylalanine to cinnamate. The cinnamate 4-hydroxylase (C4H) catalyzes the synthesis of p-hydroxycinnamate from cinnamate and 4-coumarate: CoA ligase (4CL) converts p-coumarate to its coenzyme-A ester, activating it for reaction with malonyl CoA. The

flavonoid biosynthetic pathway starts with the condensation of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA, yielding naringenin chalcone. This reaction is carried out by the enzyme chalcone synthase (CHS). Chalcone is isomerised to a flavanone by the enzyme chalcone flavanone isomerase (CHI). From these central intermediates, the pathway diverges into several side branches, each resulting in a different class of flavonoids (Jaakola 2003). The detailed biosynthesis of some of the common flavonoids themselves is shown in **Figure 6.3.3.2** (Punyasiri et al. 2004).

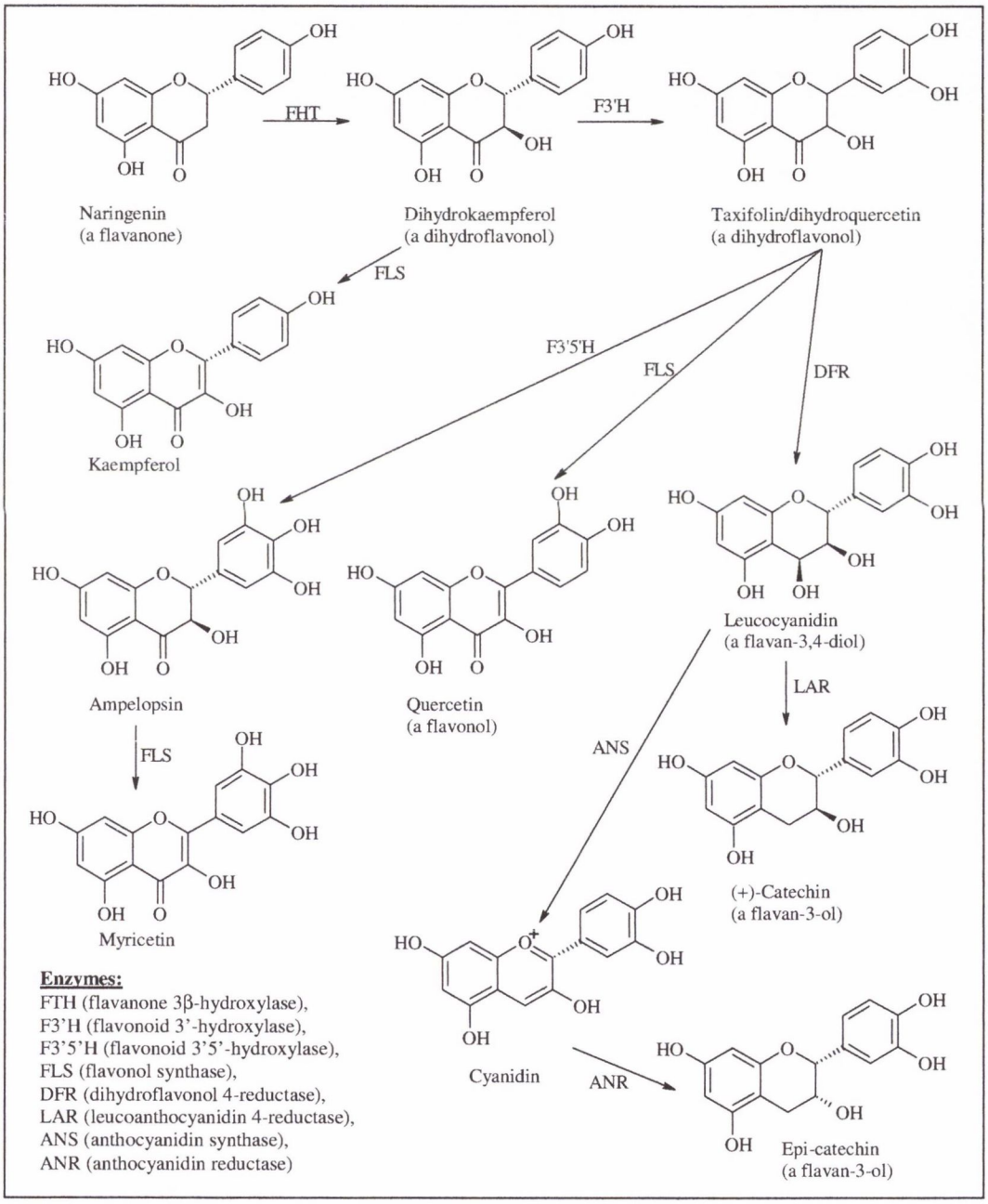


Figure 6.3.3.2 Detailed biosynthesis of common flavonoids including the enzymes

6.3.3.2 Flavonoids as antioxidants

Flavonoids have been found to have beneficial effects in a multitude of disease states, including cancer, cardiovascular diseases and neurodegenerative disorders. Many of these beneficial actions have been attributed to the antioxidant properties of flavonoids (Williams et al. 2004). According to Halliwell and Gutteridge (Halliwell and Gutteridge 1999) the definition of an antioxidant is: 'any substance that, when present at low concentration compared with those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate'. The mechanisms of antioxidant action can include:

- Suppressing reactive oxygen species formation either by inhibition of enzymes or chelating trace elements involved in free radical production.
- Scavenging reactive oxygen/nitrogen species.
- Up-regulating or protecting antioxidant defences.

Flavonoids have been found to inhibit enzymes involved in reactive oxygen species generation such as cyclooxygenase, lipoxygenase, glutathione *S*-transferase, NADPH oxidase, xanthine oxidase and protein kinase C (Middelton et al. 2000). A number of flavonoids efficiently chelate trace metals. Trace metals such as free iron and copper can reduce H_2O_2 to generate the highly reactive hydroxyl radical as shown below:

$$\mathbf{H_2O_2 + Fe^{2+}(Cu^{+}) \rightarrow OH^{\bullet} = OH^{-} + Fe^{3+}(Cu^{2+})}$$ (Pietta 2000)

Another example of the adverse effect of trace metals is the copper-mediated LDL (low-density lipoprotein) oxidation (Browne et al. 1998):

$$\mathbf{LH \rightarrow L^{\bullet} \rightarrow LOO^{\bullet}}$$ (where LH represents LDL) (Pietta 2000)

The proposed binding sites for trace metals to flavonoids are the catechol moiety in ring B, the 3-hydroxyl, 4-oxo groups in the heterocyclic ring, and the 4-oxo, 5-hydroxyl groups between the heterocyclic ring and the A ring (see **Figure 6.3.3.3**). The major contribution to metal chelation, however, has been found to be due to the catechol moiety (van Acker et al. 1996).

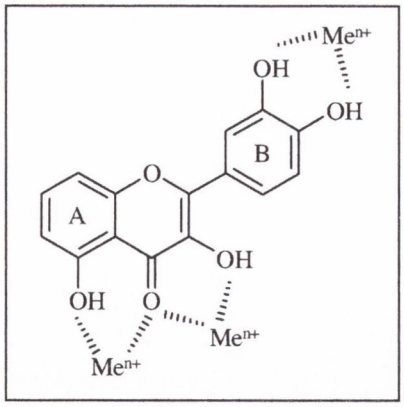


Figure 6.3.3.3 Binding sites for trace metals

Flavonoids function as scavengers of activated oxygen or nitrogen species by rapid donation of a hydrogen atom, as shown in the mechanism in **Figure 6.3.3.4** (Pietta 2000). It has been reported that the catechol moiety in ring B and the hydroxyl group at the 3-position in the flavonoid are necessary for the scavenging effect. Heijnen and co-workers found that if both of these criteria were lacking, the compound would not have be able to effectively scavenge activated oxygen species such as peroxynitrite (Heijnen et al. 2001). The same was reported by Amić et al. in their work to elucidate the relationship between the molecular structures of a series of related flavonoids and their ability to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) free radicals (Amic et al. 2003).

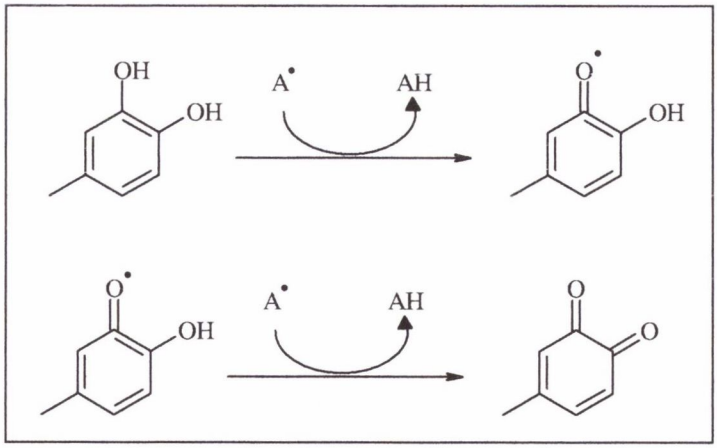


Figure 6.3.3.4 Scavenging of AOS (A$^{\bullet}$) by the catechol moiety in flavonoids

6.3.4 Jasmonates, ethylene and salicylic acid - their role as signals in plant

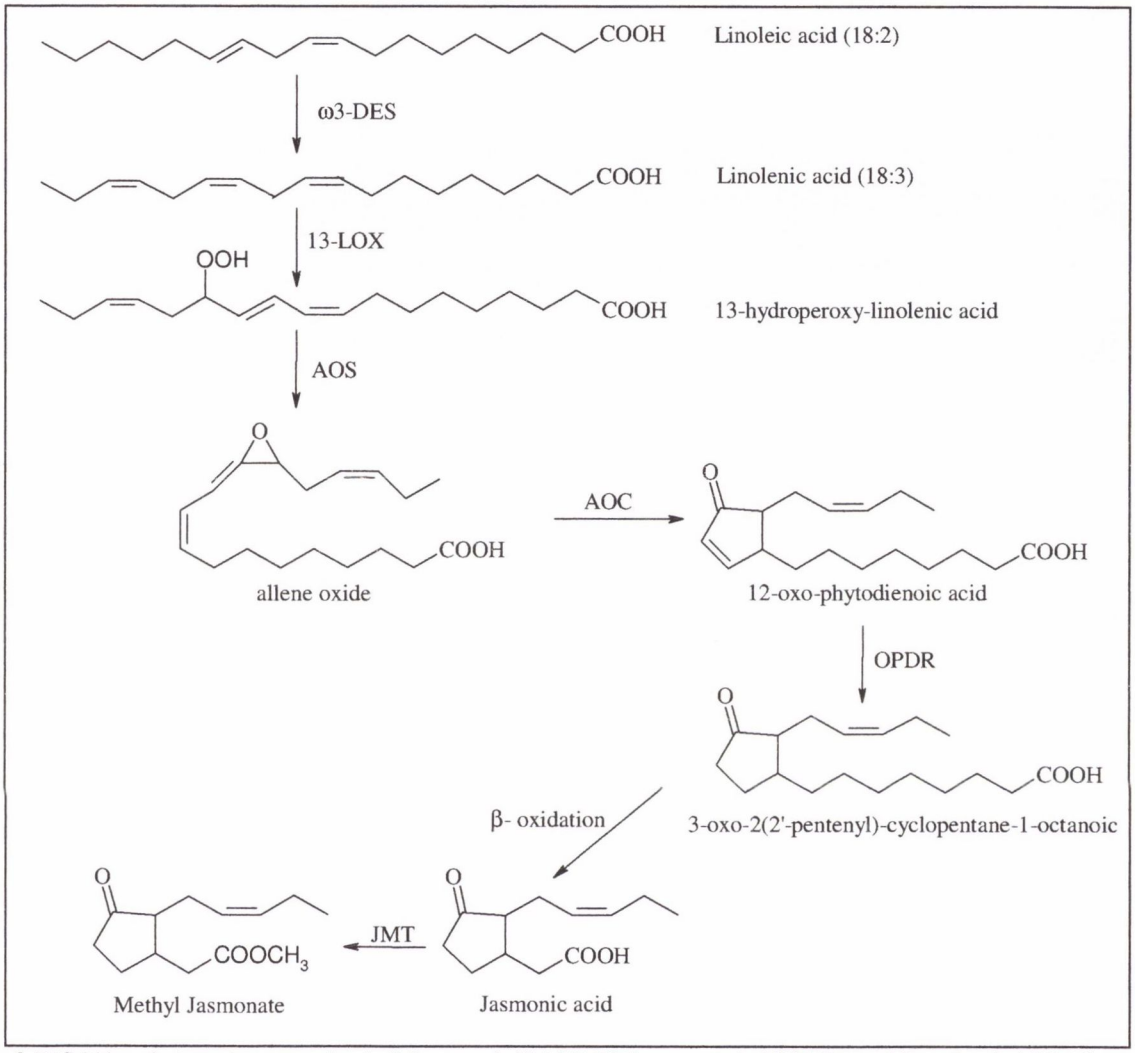
As described plant defence responses are activated on the recognition by plant cell receptors of elicitor molecules derived from the invading microorganisms or pathogens. This recognition response triggers a signal-transduction cascade leading to the production of endogenous signalling compounds in addition to other responses (see **Figure 6.3.1.2**). These signalling compounds are spread to tissues that are distant from the initial infection site, where they activate defence genes (e.g. antimicrobial protein/peptide genes (AMP genes)) (Thomma et al. 1998). Well known signalling compounds in the plant defence mechanism are the jasmonates (jasmonic acid and methyl jasmonate), ethylene and salicylic acid.

6.3.4.1 Jasmonic acid (JA) and methyl jasmonate (MJ)

Jasmonic acid (JA) and its methyl ester (MJ) are fatty-acid derived signals in plants. They affect various processes in the plant such as fruit ripening, production of pollen and root growth. JA signalling is crucial for plant stress responses following wounding, interaction with pathogens, UV light and attack by insects. Methyl jasmonate is a volatile ester and can therefore move in the intracellular spaces within the plant, acting as an interplant communication signal (Weber 2002). JA biosynthesis can also be stimulated in cell cultures as a result of elicitation, and it is therefore thought to be a receptor-induced process (Creelman and Mullet 1997).

The biosynthesis of jasmonic acid occurs via the octadecanoid pathway, starting with linoleic acid and is shown in **Figure 6.3.4.1** (León and Sánchez-Serrano 1999). Linoleic acid (18:2) is found in abundance esterified in glycerolipids and phospholipids in the chloroplast. At least five genes for biosynthetic enzymes are induced in the JA biosynthesis, also targeted to the chloroplast. 13-Lipoxygenase (13-LOX) mediates the essential step in JA biosynthesis, where linolenic acid (18:3) is oxygenated to its hydroperoxy derivative, 13-hydroperoxy-linolenic acid (Turner et al. 2002). Other essential enzymes are allene oxide synthase (AOS), allene oxide cyclase (AOC), and 12-oxo-phytodienoic acid reductase (OPDR), all shown in **Figure 6.3.4.1**. The JA signalling pathway involves a number of signal transduction events, starting with the perception of the primary stimulation, such as wounding, abiotic stresses (including osmotic stress), drought, and exposure to elicitors or pathogens. The

transduction of this signal leads to induction of JA biosynthesis, which in turn is perceived and induces responses (Turner et al. 2002). JA signalling has also been shown to interact in a complex manner with other signalling pathways, such as the salicylic acid pathway and the ethylene pathway (Kunkel and Brooks 2002).



ω3-DES (chloroplast membrane associated ω3 desaturase), 13-LOX (13-lipoxygenase), AOS (allene oxide synthase), AOC (allene oxide cyclase), OPDR (12-oxo-phytodienoic acid reductase), β-oxidation (multienzymatically catalysed), JMT (*S*-adenosyl-*L*-methionine-jasmonic acid carboxyl methyltransferase)

Figure 6.3.4.1 Jasmonic acid biosynthesis (León and Sánchez-Serrano 1999)

6.3.4.2 Ethylene (ET) and salicylic acid (SA)

Ethylene (ET) is a phytohormone that regulates a wide range of processes in the plant, from growth and development to defence responses. The production of ET can be induced by the same types of stresses that induce JA, and their signalling pathways are therefore usually coinciding (Zhao et al. 2005). Evidence that JA and ET regulate

defence genes together was shown in *Arabidopsis thaliana* where half of the genes induced by ET were also induced by JA (Schenk et al. 2000).

Salicylic acid (SA) plays an active role in the plant defence mechanism; it is required for the rapid activation of resistance genes and is especially important for the establishment of systemic acquired resistance (SAR). SAR is an induced state of immunity or heightened defence that is activated throughout the plant following an infection (Thomma et al. 2001). During a pathogen attack or plant hypersensitive reaction, SA quickly accumulates at the site of infection and spreads to other parts of the plant to induce a wide range of defence responses (Zhao et al. 2005). The interactions between JA and SA have in most of the cases been found to be antagonistic. An example is the negative relationship between SA and JA pathways in *cpr*-induced resistance in *Arabidopsis* (Clarke et al. 2000b).

6.3.5 Nitric oxide (NO) as a signal in plants

NO is a small, water and lipid soluble gas. It can exist as three interchangeable species: the radical ($NO^{\bullet}$); the nitrosonium cation (NO^{+}); and the nitroxyl radical (NO^{-}) (Neill et al. 2003). Once produced it can move from one cell to another or within a cell. However, being a reactive free radical, it has a relatively short half-life, in the order of a few seconds. Typically NO rapidly reacts with O_2 to form nitrogen dioxide (NO_2), and rapidly degrades to nitrite and nitrate in aqueous solution (see **Figure 6.3.5.1**)

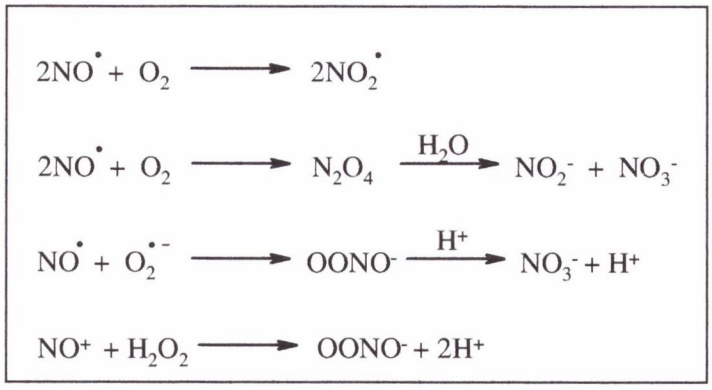


Figure 6.3.5.1 Reactions of the different forms of nitric oxide (Neill et al. 2003)

In mammalian cells NO is formed directly from the guanidino nitrogen of the *L*-arginine by nitric oxide synthase (NOS) through a process that consumes five electrons, and results in the formation of *L*-citrulline (Xu and Liu 1998) as shown in **Figure 6.3.5.2**.

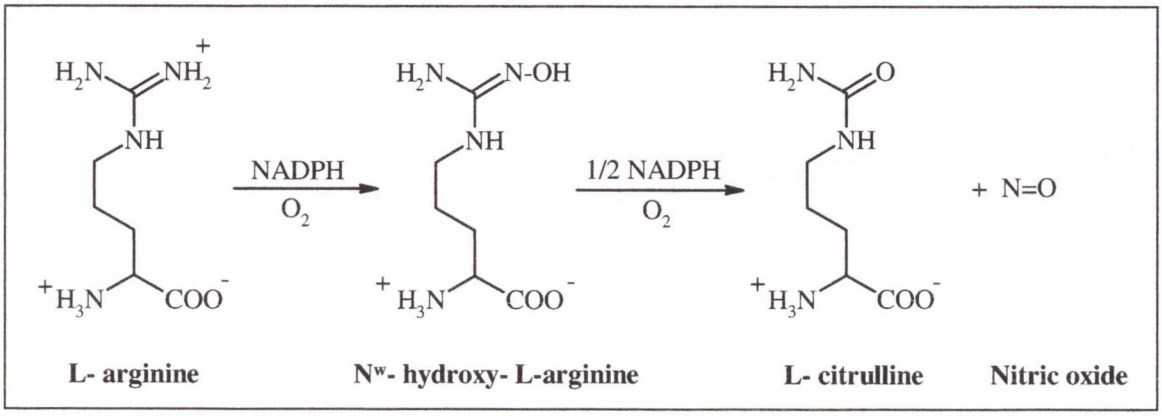


Figure 6.3.5.2 Biosynthesis of NO in mammalian cells

NO is known to be an important signalling molecule in mammalian cells. It can act as a cardiovascular regulator, a modulator of neurotransmission, and is vital in the defence against pathogens (Mayer and Hemmes 1997). However, NO can also have damaging effects on the cells due to its chemical state or the rate of production at the location. The balance between positive and negative effect is fine.

Evidence for the existence of an endogenous pathway for NO synthesis in the plant kingdom has increased in recent years. NOS activity in plant cells, based on the formation of *L*-citrulline from *L*-arginine, was initially observed in extracts of the legume *Mucuna hassjoo* (Ninnemann and Maier 1996). NOS and NO has been localized in the plant cytosol in *Taxus* and maize cells (Pedroso et al. 2000, Ribeiro et al. 1999). Despite growing evidence for the existence of NOS in plants, other groups have reported conflicting data. For example, NOS inhibitors had no effect on NO synthesis in leaf extracts or intact tissues in *Helianthus annuus* or *Spinacia oleracea* plants (Rockel et al. 2002). Clarke and co-workers (Clarke et al. 2000a) found no effects of NOS inhibitors on the release of NO in *Arabidopsis thaliana* cells in response to bacterial challenge. Similarly Sakihama and co-workers (Sakihama et al. 2002) found that NOS inhibitors did not reduce nitrite-dependent NO generation in *Chlamydomonas reinhardtii*, nor did the addition of *L*-arginine induce activity. *L*-

arginine was similarly ineffective as a substrate for NO production in the green alga *Scenedesmus obliqus*, and two NOS inhibitors had no effect (Mallick et al. 2000).

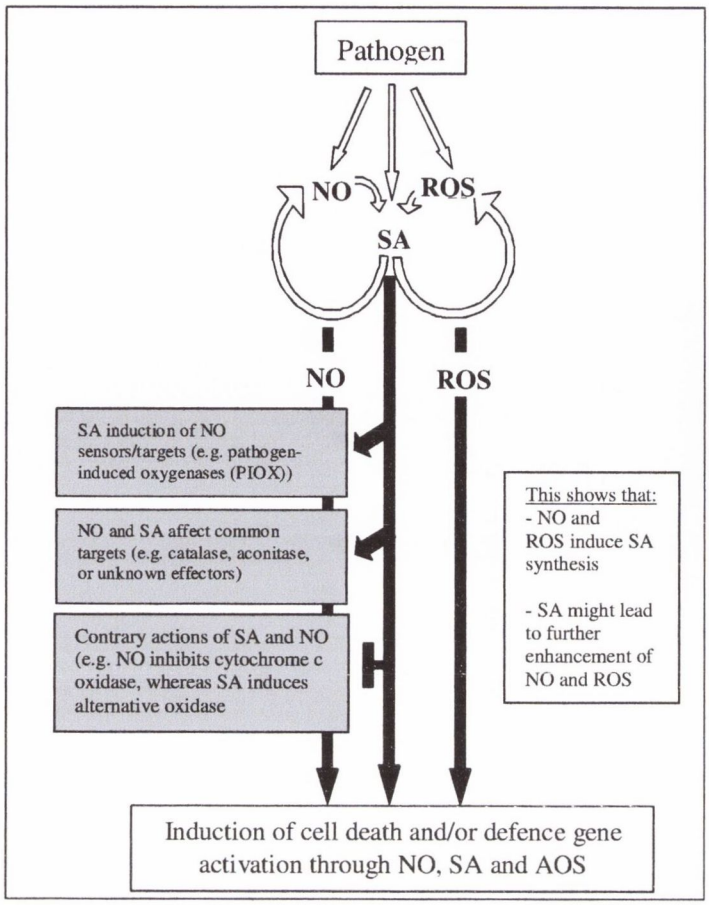
An alternate source of NO production in plants is through the nitrite-dependent enzyme nitrate reductase (NR). Yamasaki and Sakihama (Yamasaki and Sakihama 2000) reported that NR has the capacity to generate NO, O_2^- and peroxynitrite *in vitro*. The same was suggested by Rockel and co-workers (Rockel et al. 2002) *in vivo*. Recently, however, two unrelated groups of NOS-like enzymes have been identified in *Arabidopsis thaliana*. The first ever purification and detailed characterisation of a pathogen-inducible nitric oxide synthase (iNOS) in plants was reported by Chandok and co-workers (Chandok et al. 2003) in May 2003. They revealed that the plant iNOS is a variant of the P protein of the glycine decarboxylase complex (GDC). The P protein shares very little sequence homology with animal NOSs, and this might explain why previous efforts to identify this enzyme have failed. Guo and co-workers (Guo et al. 2003) then identified a hormone-activated nitric oxide synthase gene called AtNOS1. The NOS activity of a mutant line, with a DNA insertion in the first exon of this gene, was 25% that of wild type. That the wild type activity was due to NOS-like enzyme activity was verified by inhibiting this activity using the NOS inhibitor Ng-nitro-*L*-Arg-methyl ester (*L*-NAME). The AtNOS1-knockout mutant also showed reduced growth and fertility, so it is probable that AtNOS1 catalyses NO production in response to a wide range of hormonal and other signals.

The plant defence system against pathogens consists of a wide variety of defence responses that are put in place to prevent pathogen replication and/or movement. The response mechanisms terminate with rapid tissue necrosis at the site of infection. This response is called the hypersensitive response (HR). The hypersensitive response in plants can be compared to the programmed cell death in mammalian cells (Saviani et al. 2002). In animal cells, AOS collaborate with NO to induce apoptosis and to kill invading pathogens. For example, NO reacts with the superoxide anion ($O_2^{\bullet -}$) to form peroxynitrite ($ONOO^-$). Peroxynitrite is thought to be the molecule mediating the programmed cell death (Brüne et al. 1998, Mayer and Hemmes 1997). In contrast it was found that in soybean cells peroxynitrite was not an effective inducer of HR (Delledonne et al. 2001). In plant cells the oxidative burst is necessary but not

sufficient to trigger host cell death, and it has been proven that NO cooperates with AOS in the activation of HR (Clarke et al. 2000a, Delledonne et al. 2002, Durner et al. 1998).

In addition to interacting with AOS, NO appears to activate defence responses through a salicylic acid (SA) -dependent pathway (**Figure 6.3.5.3**). NO was found to induce the expression of the defence gene *PAL* in *Nicotiana tabacum* cells (Durner et al. 1998). *PAL* provides precursors for the phenylpropanoid biosynthesis, hence production of salicylic acid (SA). Micromolar concentrations of NO were found to induce *PR-1* protein and mRNA accumulation effectively. In contrast, H_2O_2, which has been hypothesised to play an important role in the induction of defence genes such as *PR-1*, must be administered in the range of 1mM to 1M to obtain substantial *PR* activity. *PR-1* activity is mediated by SA (Durner et al. 1998).

Recent evidence suggests that NO also plays a role in the wounding/jasmonic acid (JA) signalling pathway. In tomato the NO donors sodium nitroprusside (SNP) and S-nitroso-N-acetyl-penicillamine (SNAP) was found to inhibit both wounding-induced hydrogen peroxide (H_2O_2) synthesis and wounding- or JA-induced expression of defence genes. This inhibition was independent of salicylic acid (SA) so it was suggested that NO may interact with the wounding/JA pathway at a point downstream of JA synthesis but upstream of H_2O_2 generation (Orszco-Cárdenas and Ryan 2002). Jhi and co-workers (Jih et al. 2003) also found that NO-donors delayed or reduced wounding-induced generation of H_2O_2 in sweet potato. It also reduced or delayed that expression of the JA-inducible ipomoelin gene. Recently Huang and co-workers (Huang et al. 2004) demonstrated that wounding induces NO in *Arabidopsis thaliana*. They also reported that although NO activates early JA signalling genes, it is not a key player in the wounding response. In fact they found that SA negatively regulates NO-mediated JA synthesis in wildtype plants. Both Orszco-Cárdenas and Huang suggests that NO might play a role in fine-tuning of wound response or in pathogenesis-related context such as induced systemic resistance.



(Durner and Klessig 1999)

Figure 6.3.5.3 Possible relationship between NO, SA and AOS

To summarise, NO appears to have a bifunctional role in modulating the plant cell defence system. NO can stimulate the process by, in collaboration with ROS, inducing HR or it can inhibit the process by inhibiting the JA pathway at a point upstream of H_2O_2 generation. This bifunctional role of NO has been documented in animal systems previously.

7 Materials and Methods

7.1 Plant Material

Callus cultures were initiated by researchers from Coillte Research Laboratories, Newtownmountkennedy, Co. Wicklow, Ireland, using embryos excised from seeds of *Taxus baccata* 'Fastigiata'. Further maintenance and growth studies were done in Department of Pharmacognosy, School of Pharmacy, Trinity College, Dublin, Ireland (Dempsey 2000). Callus cultures were also initiated from needles of *Taxus baccata* shoots (Hook 1999).

7.1.1 Establishment of callus cultures

Seeds from *Taxus baccata* 'Fastigiata', the Irish yew tree, were collected in August 1997 (Mount Anville School, Dublin, Ireland) (Dempsey 2000). The trees were genetically identical, of the same age (70 years) and same gender (female). Researchers from Coillte Research Laboratories, Newtownmountkennedy, Co. Wicklow, Ireland, initiated the callus cultures in the following manner. The seeds were rinsed in sterile water and then immersed in concentrated sulphuric acid (H_2SO_4) for 5 min. This sterilised the outer seed coat and also softened it, to allow easier removal of the embryo. Embryos were re-sterilised in 70% ethanol after removal of the seed coat and placed on to a Phytagel®-solidified modified *Picea abies* embryogenesis medium (see **Table 7.2.1**) (Gupta and Pullman 1991). The 26 cell lines, successfully developed by Coillte, were subsequently maintained in the Department of Pharmacognosy, Trinity College, Dublin. All cultures were maintained in the dark at a constant temperature of 25°C.

In March 1997 samples of shoots were collected from a hedge of European yew (*T. baccata*) growing in St. Patrick's College, Maynooth, Co. Kildare, Ireland (Dempsey 2000). The needles taken from these were sterilized using a dilute Domestos® (hypochlorite) solution followed by rinsing with sterile water. They were then placed onto Anderson's Stage I medium. Of the original 126 samples sterilized 26 remained sterile. In December 1997 any callus was transferred to a modified McCown's Woody Plant nutrient medium (see formulation in Appendix I) and kept in the dark at 25°C.

Subcultures to fresh medium were done as deemed necessary. Cultures grew very slowly and in June 1998 were transferred to Phytagel™-solidified medium 1902A (formulation in **Table 7.2.1** with 0.3% Phytagel™ incorporated). In August 1999 the cultures began to grow more quickly and were subcultured every 3 months onto fresh medium.

7.1.2 Establishment of cell suspension cultures

Cell suspension cultures of the most productive and best growing cell lines developed from seeds (19, 8 and 3) were established (Dempsey 2000) using the embryo-derived callus as inoculum in November 1998. Callus pieces were aseptically inoculated into 250-ml conical flask containing 100 ml liquid 1902A medium (see **Table 7.2.1**). They were placed on an orbital shaker (Gallenhamp) at 90rpm, at a constant temperature of 25°C and in the dark. The medium was later changed to the more glutamine-rich liquid medium 1902B (see **Table 7.2.1**) (Hook 1999).

In February 2000 callus developed from the needles of *Taxus baccata* was transferred to liquid 1902A medium for the development of suspension cultures of the most productive and best growing cell line H*. The medium was later changed to the more glutamine-rich liquid medium 1902B as above (see **Table 7.2.1**) (Hook 1999).

7.2 Tissue Culture Conditions

The cell line used in the present study was cell line 19, developed from excised embryos from seeds of *Taxus baccata* 'Fastigiata' as described above. Cultures were grown in a 1902B medium (see **Table 7.2.1**). Prior to autoclaving, the pH of the medium was adjusted to a value of 5.7 using a 10% hydrochloric acid (HCl) solution.

For the majority of the experiments approximately 2g (fresh wt) cells were used as inoculum for 100ml liquid medium in 250ml Erlenmeyer flasks. The flasks were capped with non-absorbent cotton wool and a protective cap of aluminium foil. For a minority of the experiments circa 1g (fresh wt) cells were used as inoculum, and grown in 50ml liquid medium in 100ml Erlenmeyer flasks. The cells were subcultured every 35 days, unless otherwise stated.

Table 7.2.1 Media composition

Component	*Picea abies** (mg/L)	1902-A (mg/L)	1902-B (mg/L)	Gamborg's B5 (mg/L)
Macro Elements				
Ammonium nitrate (NH_4NO_3) ♠	206.00	206.00	206.00	
Potassium nitrate (KNO_3) ♠	2340.00	2340.00	2340.00	2500.00
Potassium dihydrogen phosphate (KH_2PO_4) ♠	85.00	85.00	85.00	
Calcium chloride ($CaCl_2$) ♠	220.00	220.00	220.00	113.23
Magnesium sulphate x $7H_2O$ ($MnSO_4$ x $7H_2O$) ♠	185.00	185.00	185.00	121.56
Sodium dihydrogen phosphate (NaH_2PO_4) ♠	-	-	-	130.44
Ammonium sulphate ($(NH_4)_2SO_4$) ♠	-	-	-	134.00
Micro Elements				
Boric acid (H_3BO_3) ♦	3.10	6.20	6.20	3.00
Magnesium sulphate x H_2O ($MnSO_4$ x H_2O) ♠	8.45	16.90	16.90	10.00
Zinc sulphate x $7H_2O$ ($ZnSO_4$) ♦	4.30	8.60	8.60	2.00
Potassium iodide (KI) ♠	0.415	0.83	0.83	0.75
Sodium molybdate (Na_2MoO_4) ♠	0.125	0.25	0.25	0.25
Copper sulphate ($CuSO_4$) ♠	0.0125	0.025	0.025	0.025
Cobalt chloride ($CoCl_2$) ♠	0.0125	0.025	0.025	0.025
Sodium EDTA (FeNaEDTA) ♠	18.63	37.26	37.26	36.70
Ferrous sulphate ($FeSO_4$) ♠	13.93	27.80	27.80	-
Vitamins				
Myo-inositol ($C_6H_{12}O_6$) ♦	1000.00	1000.00	1000.00	100.00
Casein amino acid ♦	500.00	500.00	500.00	-
Nicotinic acid ($C_5H_5NO_2$) ♠	0.50	0.50	0.50	1.00
Pyridoxine hydrochloride ($C_8H_{11}NO_3HCl$) ♠	0.50	0.50	0.50	1.00
Thiamine hydrochloride ($C_{12}H_{17}CIN_4OSHCl$) ♠	1.00	0.10	0.10	10.00
Glycine ($C_2H_5NO_2$) ♠	2.00	-	-	-
L-glutamine ($C_5H_{10}N_2O_3$) ♦	450.00	2.00	450.00	-
2,4-Dichlorophenoxyacetic acid ($C_8H_6Cl_2O_3$) ♠	-	1.10	1.10	2.00
6-Benzylaminopurine ($C_{12}H_{11}N_5$) ♦	-	0.40	0.40	0.10
Kinetin ($C_{10}H_9N_5O$) ♦	-	0.40	0.40	-
Sucrose ($C_{12}H_{22}O_{11}$) ♦	30000.00	30000.00	30000.00	30000.00
pH	5.70	5.70	5.70	5.70

*(Gupta and Pullman 1991), ♠ AnalR, BDH Chemicals Co., UK, ♦ Sigma-Aldrich Chemical Co., UK

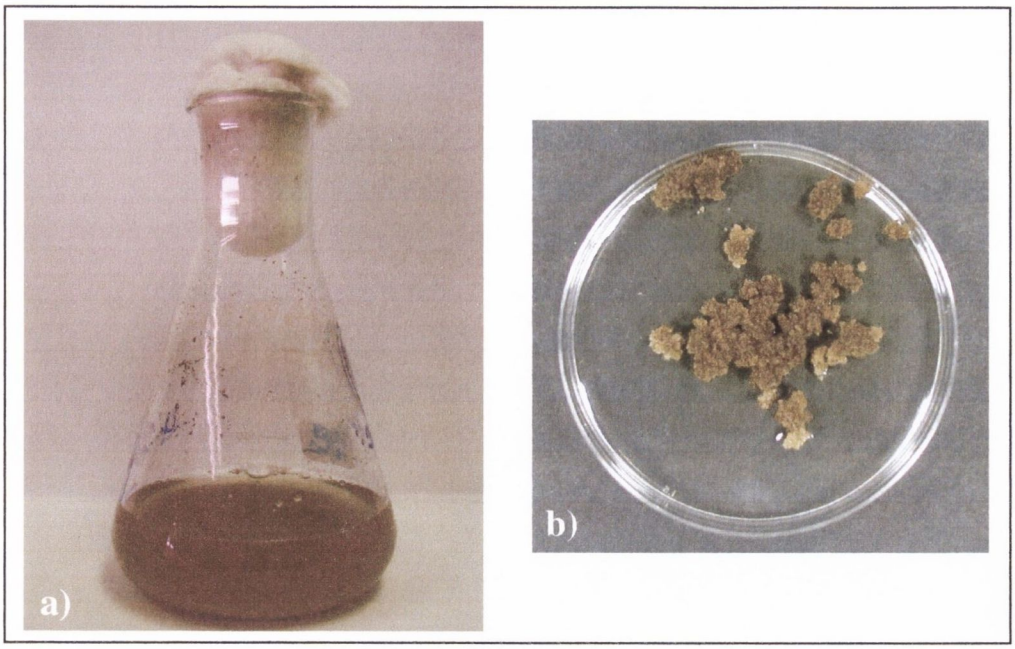


Figure 7.2.1 *Taxus baccata* 'Fastigiata', cell line 19, growing as (a) suspension culture and (b) and static culture on Phytagel™-solidified medium 1902B

The experiments were designed as randomised blocks containing minimum three and maximum four replicates. The conical flasks containing cell material were chosen for each test in each block in a randomised manner in order to safeguard against the introduction of bias in choosing subjects for the different treatment groups. Uncontrolled sources of variation like light, temperature etc. might enter through the pre-existing conditions of the experimental units. Therefore the experimental units were placed on the orbital shaker in a randomised order within their blocks. This would guarantee that the external sources of variation had the same chances of helping or inhibiting the responses produced by the treatments.

The subculture weights of each sample of the four blocks were analysed using Minitab's (Minitab™) One Way ANOVA to make sure that there were no statistical significant difference between them. No statistical significance would guarantee that there was no inherent bias in the beginning of the experiment.

7.3 General Analytical Procedures (Plant Tissues Culture)

7.3.1 Chemicals and solvents

Solvents used for High Performance Liquid Chromatography (HPLC) and phytochemical extraction and isolation were Analar® grade; all other solvents were laboratory grade.

Solvents used for Nuclear Magnetic Resonance (NMR) analysis were:

Dimethylsulphoxide-d_6, 99.8 atom % (Sigma-Aldrich Chemical Co., UK)

Chloroform-d, 99.9 atom %, 0.01% TMS (Sigma-Aldrich Chemical Co., UK)

Reference compounds used for comparative Thin Layer Chromatography (TLC), HPLC, and Gas Chromatography (GC) were:

Paclitaxel	(> 95% purity, Sigma-Aldrich Chemical Co., UK)
10-deacetylbaccatin III	(> 95% purity, Sigma-Aldrich Chemical Co., UK)
Baccatin III	(> 95% purity, Sigma-Aldrich Chemical Co., UK)
Cephalomannine	(National Cancer Institute, USA)
Catechin	(> 95% purity, Sigma-Aldrich Chemical Co., UK)
Epi-catechin	(> 95% purity, Sigma-Aldrich Chemical Co., UK)
Taxifolin	(HPLC grade, Extrasynthese SA, France)
Myrcetin	(HPLC grade, Extrasynthese SA, France)
Quercetin	(>95% purity, Sigma-Aldrich Chemical Co., UK)
Kaempferol	(HPLC grade, Extrasynthese SA, France)
Amentoflavone	(HPLC grade, Extrasynthese SA, France)
Methyl laurate	(Sigma-Aldrich Chemical Co., UK)
Methyl myristate	(99%, Sigma-Aldrich Chemical Co., UK)
Methyl palmitate	(99%, Sigma-Aldrich Chemical Co., UK)
Methyl stearate	(99%, Sigma-Aldrich Chemical Co., UK)
Methyl oleate	(99%, Sigma-Aldrich Chemical Co., UK)
Methyl linoleate	(99%, Sigma-Aldrich Chemical Co., UK)
Methyl linolenate	(98%, Sigma-Aldrich Chemical Co., UK)
Methyl γ-linolenate	(99%, Sigma-Aldrich Chemical Co., UK)

Compounds used for elicitation in cell culture experiments were:

Methyl jasmonate	(>95% purity, Sigma Aldrich Chemical Co., UK)
L-arginine	(Sigma-Aldrich Chemical Co., UK)
Sodium Molybdate	(AnalR, BDH Chemicals Co., UK)
Inulin	(Cosucra Groupe Warcoing SA, Belgium)
Tert-butyl hydroperoxide	(Sigma-Aldrich Chemical Co., UK)
Hematin (porcine)	(Sigma-Aldrich Chemical Co., UK)

Polymers used for adsorbent experiments and column chromatography were:

Silica gel 60 (particle size 0.040-0.063mm)	(Merck KGaA, Darmstadt, Germany)
Silica gel 60 PF254	(Merck KgaA, Darmstadt, Germany)
Amberlite XAD-2 (20-60 mesh)	(Sigma-Aldrich Chemical Co., UK)
Sephadex LH-20 (bead size 25-100μ)	(Sigma-Aldrich Chemical Co., UK)

Other chemicals used were:

di-Sodium hydrogen o-phosphate (Na_2HPO_4)	(AnalR, BDH Chemicals Co., UK)
Pyridine (C_5H_5N)	(Sigma-Aldrich Chemical Co., UK)
Anhydrous sodium sulphate (Na_2SO_4)	(Scharlau Chemie S.A., Spain)
Vanillin ($C_8H_8O_3$)	(AnalR, BDH Chemicals Co., UK)
Hydrochloric acid (HCl)	(Reidel-de Haën®, Germany)
Acetic anhydride ($(CH_3)_2(CO)_2O$)	(Reidel-de Haën®, Germany)
Sulphuric acid (H_2SO_4)	(Reidel-de Haën®, Germany)
Formic acid (CH_3COOH)	(Merck KGaA, Darmstadt, Germany)

7.3.2 Extraction procedure

After harvesting, the cells were dried in a fan-assisted oven (Memmert™) at <30°C for approximately 72 hours. The following parameters were recorded:

- Inoculum weight at subculture (day 0)
- Fresh weight (after filtration at harvest)
- Dry weight
- pH of medium after harvest
- mV (ms) of medium at harvest

The dry cells were powdered using a mortar and pestle, and extracted under reflux with 50ml methanol (MeOH) for two and a half hours. The crude extract was then cooled, filtered, and evaporated to dryness *in vacuo*. The resulting residue was partitioned between 20ml ethyl acetate (EtOAc) and 20ml distilled water (3-4 times). The EtOAc extracts were combined, dried over anhydrous sodium sulphate (Na_2SO_4) and finally evaporated to dryness *in vacuo*. The combined filtrates from the flasks (medium) were extracted using ethyl acetate (EtOAc) in a similar manner to that of the cells (above method).

7.3.3 Analysis by thin layer chromatography (TLC)

Thin layer chromatography was carried out on aluminium-backed pre-coated silica gel $60F_{254}$ plates (Merck KGaA, Darmstadt). The residues produced as above (section 7.3.2) were re-dissolved in an appropriate solvent, applied as spots (10µl) and run against appropriate standards. The plates were developed over 10 cm, unless otherwise stated. Solvent systems used were (Stahl 1969, Wagner and Bladt 1996):

1. Chloroform-methanol ($CHCl_3$ – MeOH) (10:1)
2. Chloroform-methanol ($CHCl_3$ – MeOH) (100:1)
3. Chloroform-methanol-water ($CHCl_3$ – MeOH – H_2O) (75:23:2)
4. Chloroform-methanol-water ($CHCl_3$ – MeOH – H_2O) (75:30:2)
5. Chloroform-methanol-water ($CHCl_3$ – MeOH – H_2O) (75:46:4)
6. Chloroform-methanol-water ($CHCl_3$ – MeOH – H_2O) (50:46:4)
7. Hexane-ethyl acetate (C_6H_{14}-EtOAc) (6:2)

Visualisation was achieved using UV_{254} or spraying with 1% vanillin-sulphuric acid solution followed by heating at 110°C for 10 minutes.

7.3.4 Analysis by high performance liquid chromatography (HPLC)

For the quantification of taxanes as well as flavonoids, analysis by high performance liquid chromatography (HPLC) was employed. The residue was re-dissolved in 0.5- or 1ml of HPLC grade methanol (MeOH) immediately prior to analysis.

7.3.4.1 Instrumentation

Analysis by HPLC was carried out using a Waters™ Multisolvent Delivery System consisting of a 600 Gradient Controller, a 2487 Dual λ Absorbance Detector, and a 746 Data Module Integrator.

7.3.4.2 Columns and chromatographic conditions

The analytical column used for the identification of **taxanes** from the cell cultures and the media was the taxane-specific Phenomenex Curosil®-PFP (pentafluorophenyl) (5μm, 250 x 4.6mm) column with the appropriate Phenomenex Curosil®-PFP (5μm, 3.9 x 20mm) guard column. The mobile phase used was a gradient of acetonitrile-water (from 25:75 to 80:20 over a period of 45 min) as illustrated in **Table 7.3.4.1**.

Table 7.3.4.1 Gradient table for taxane analysis by HPLC

Time	Flow (ml/min)	Solvent A (%)	Solvent B (%)	Curve
Initial	1	25	75	
45	1	80	20	Linearly increasing
50	1	25	75	Linearly decreasing
55	1	25	75	

The injection volume was 7.5 μl for both standards and samples, and the flow rate was 1ml/min. Detection was performed at 228nm. All analyses were carried out at ambient temperature and each extract was analysed in duplicate.

The analytical column used for the identification of **flavonoids** was a Machery-Nagel Nucleosil 120-5 C_{18} (5μm, 250 x 4mm) column with a Machery-Nagel Nucleosil 120-5 C_{18} (CC 5μm, 8x4 mm) guard column. The mobile phase used was a gradient of (a) phosphate buffer (20mM; pH 3) and (b) methanol (MeOH) (from 95:5 to 0:100 over a period of 35 min) as illustrated in **Table 7.3.4.2** (Baczek et al. 2001).

Table 7.3.4.2 Gradient table for flavonoid analysis by HPLC

Time	Flow (ml/min)	Solvent A (%)	Solvent B (%)	Curve
Initial	1	95	5	
35	1	0	100	Linearly increasing
40	1	95	5	Linearly decreasing
45	1	95	5	

The injection volume was, as above, 7.5 µl for both standards and samples, and the flow rate was 1ml/min. Detection was performed at 280nm. All analyses were carried out at ambient temperature and each extract was analysed in duplicate. The adjustment of the pH of the phosphate buffer was carried out using a 10% hydrochloric acid (HCl) solution.

7.3.4.3 Validation of HPLC methods

The HPLC method used for quantification of taxanes was developed according to the manufacturer's guidelines (Phenomenex, UK), with slight modifications in order to obtain a suitable resolution of the principal analytes. It was validated previously in section 3.3.4. The HPLC method used for quantification of flavonoids was developed according to Baczek and co-workers (Baczek et al. 2001) and validated as follows:

The validation of the HPLC method used for the analysis of flavonoid from the plant tissue cultures was carried out in accordance with the specifications outlined in the United States Pharmacopoeia <1225> (USP26 2003), the ICH guidelines (ICH 1996), and guidelines given by the United States Food and Drug Administration (FDA 2001). As before (section 3.3.4) the validation parameters used were:

1. Specificity/Selectivity
2. Precision
3. Linearity
4. Accuracy
5. Range

1. Specificity/Selectivity

Optimum resolution is essential for the separation of the flavonoids from other compounds in the extracts being analysed. The ability of the selected chromatographic methods to resolve the analytes can be measured by determining the resolution factor, R, which, according to the USP 26, should be >1.

The resolution factor, R, is given by the following equation:

$$R = \frac{2(t_2-t_1)}{W_1+W_2}$$

Where t_1 and t_2 are the retention times of the two components and W_1 and W_2 are the corresponding widths at the base of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline. In the chromatographic method developed each of the analytes were separated with resolution values >6. As for the method validated in section 3.3.4 the resolution factor together with comparison to reference standards (a sample spiked with reference standards of the flavonoids analysed) showed that for the purpose of the analysis in the present study the method was found selective.

2. Precision

The precision is calculated as coefficient of variation (C.V.), i.e., relative standard deviation (RSD). According to FDA, repeatability should be tested by the analysis of a minimum of five determinations at three different concentrations (low, medium and high) in the range of expected concentrations (FDA 2001). However, according to the ICH (ICH 1996) repeatability could be measured by the analysis of three determinations at three different concentrations or through six determinations at 100% of the test concentration. The RSD should not exceed 2% for the method developed. The procedure of the ICH was followed, and a calculated average of no more than 1.3% indicated that the system was precise.

3. Linearity

Linearity should be established across the range of the analytical procedure, and is demonstrated by injecting a series of dilutions of the standard and plotting the recorded response versus the concentration. The degree of linearity is evaluated by analysing the regression line for fit to the following equation:

$$y_1 = ax_1+b$$

Where y_1 is the measured response, **a** is the slope of the line, x_1 is the concentration and **b** is the intercept. The range of concentrations should span 80-120% of the expected test concentrations (ICH 1996). The concentrations used for the flavonoid

quantification were: 0.05mg/ml, 0.1mg/ml, 0.2mg/ml, 0.4mg/ml, and 0.6mg/ml. The correlation coefficient of r=0.9983 indicated that the method was linear (see **Figure 7.3.4.1**).

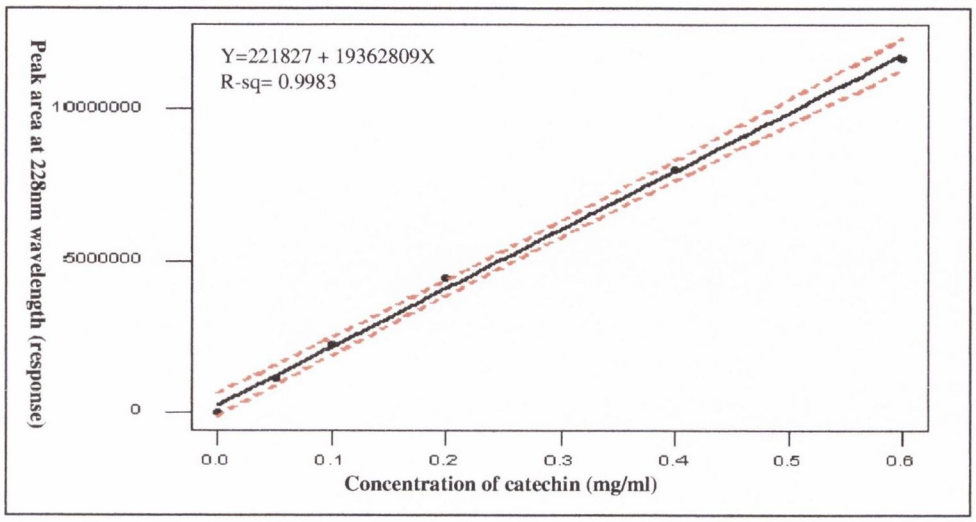


Figure 7.3.4.1 Representative standard calibration curve for flavonoids (catechin)

As before a good linear correlation coefficient alone does not necessarily indicate a linear standard curve, because the standards in the lowest range can deviate from the linearity although r is high (Bildlingmeyer 1993). Instead the linear coefficient should be accompanied with a graph were the response/concentration is plotted versus the logarithmic sample concentrations and the deviation in the y-axis should not exceed 5%. This was done (see **Figure 7.3.4.2**) and the highest deviation in the y-axis was 3.64%. So the results of both of these tests indicate that the methods were linear.

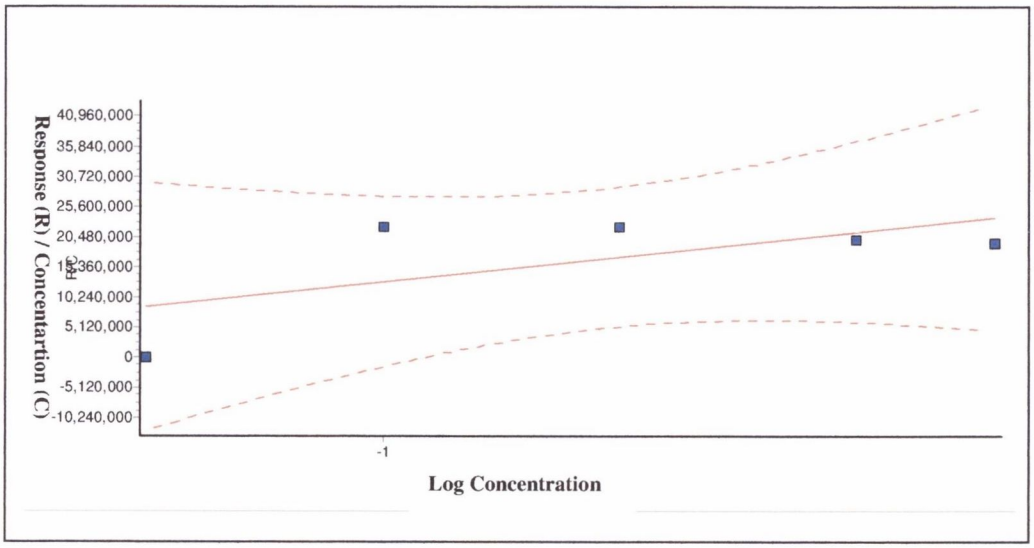


Figure 7.3.4.2 Linearity of response/concentration vs. Log of concentration (<5%)

4. Accuracy

The chromatographic method has been found to be precise, selective and linear, therefore it can also be assumed to be accurate.

5. Range

Range is defined by the ICH guidelines (ICH 1996) as 'the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity'. The precision and linearity data support the use of this method over the concentration range of 0.05mg/ml, 0.1mg/ml, 0.2mg/ml, 0.4mg/ml, and 0.6mg/ml for the flavonoids analysed.

7.3.5 Quantification and statistical analysis

For both the taxane analysis and the flavonoid analysis quantification was carried out by reference to a three-point calibration curve prepared on a daily basis with dilutions of various standards. The results were expressed in milligram per kilogram of the extracted dry weight plant material.

The standards used for the quantification of taxanes were: 10-deacetyl baccatin III, baccatin III, cephalomannine, and paclitaxel (>95% purity, Sigma) (see **Table 7.3.5.1**). The standards used for the quantification of flavonoids were: catechin, *epi*-catechin (>95% purity, Sigma), taxifolin, myrcetin, quercetin, kaempferol, and amentoflavone (>95% purity, Extrasynthese) (see **Table 7.3.5.1**).

Table 7.3.5.1 Concentration of standards used for quantification

	Standards	**Dilution 1**	**Dilution 2**	**Dilution 3**
Taxanes	Paclitaxel, cephalomannine, baccatin III, 10-deacetyl baccatin III	0.33 mg/ml	0.22 mg/ml	0.11 mg/ml
Flavonoids	Catechin, epi-catcehin, taxifolin, myrcetin, quercetin, kaempferol, amentoflanone	0.60 mg/ml	0.40 mg/ml	0.20 mg/ml

Statistical analyses were performed using Instat®Statistical Software (Graph Pad, San Diego, USA) or Minitab Inc Statistical Software. All data was analysed by one-way ANOVA (Analysis Of Variance). The critical value was calculated using Tukey's "Honestly Significant Model" at a familiar significance level of 0.05. This was more appropriate than using an individual significance level of 0.05, as multiple comparisons of means were being made. The results of ANOVA were expressed as p-values. A result was significant if its p-vlaue was less than the significance level (e.i. $P<0.05$). ANOVA was used only if the residuals of the data were normally distributed and had a constant variance, which was not dependent on run order or any one factor. Where many samples were compared to a control the Dunnett's Multiple Comparison Test was used. Dunnett's Multiple Comparison Test is conducted by computing a t-test between each experimental group and the control group.

7.4 Influence of Carbon Sources in Culture Medium on Taxane Yields

7.4.1 Experimental design

The experiment was designed with three "factors".

- A control: standard growth medium (3% sucrose).
- A test 1: modified growth medium (3% inulin instead of sucrose).
- A test 2: modified growth medium (1% inulin instead of sucrose).

The experiment was arranged in a randomised form where each "factor" had four blocks, and the experimental units were placed on the orbital shaker in a randomised order within their blocks.

The conical flask containing cell material were chosen for each test in each block in a randomised manner in order to safeguard against the introduction of bias in choosing subjects for the different treatment groups. Uncontrolled sources of variation might enter through the pre-existing conditions of the experimental units so the experimental units were placed on the orbital shaker in a randomised order within their blocks in order to guarantee that the external sources of variation have the same chances of helping or inhibiting the responses produced by the treatments.

7.4.2 Elicitation procedure

Approximately 2g of cells (fresh wt.) from cell line 19 were inoculated into 100 ml of the three types of variations of the liquid 1902B medium; being the control 1902B medium, test 1 type where 3% sucrose was replaced with for 3% inulin, and test 2 type where 3% sucrose was replaced with 1% inulin.

The cells were grown for 35 days under standard conditions (see section 7.2) before harvesting by suction-filtration. The cells were dried in a fan-assisted oven and extracted. Extracts of both the culture medium and the cells were analysed for taxanes and flavonoids as outlined in section 7.3.4.

7.5 Elicitation of Constituents by Induction of Oxidative Stress

7.5.1 Effect of molybdenum (Mo) on taxanes

7.5.1.1 Experimental design

The experiment was designed with three "factors". Each "factor" had four blocks. The experimental units within the four blocks were arranged in a randomised form on the orbital shaker.

- A control: standard growth medium (no additional concentration)
- A test 1: an addition of 1mg sodium molybdate (Na_2MoO_4 x $2H_2O$) per 100 ml medium
- A test 2: an addition of 5mg sodium molybdate per 100 ml medium

7.5.1.2 Elicitation procedure

Approximately 2g of cells (fresh wt.) from cell line 19 were inoculated into 100ml of the three types of variations of the liquid 1902B medium, where the control medium contained 1.03μM of molybdenum, the test 1 medium contained 42.36μM of molybdenum, and the test 2 medium contained 207.69μM of molybdenum.

The cells were grown for 40 days under standard conditions (see section 7.2) before harvesting by filtration. The cells were dried and extracted. Extracts of the cells were analysed for taxanes as outlined in section 7.3.4.

7.5.2 Effect of *L*-arginine on taxanes

7.5.2.1 Experimental design

The experiment was designed with four "factors". Each "factor" had four blocks. The experimental units within the four blocks were arranged in a randomised form on the orbital shaker.

- A control: standard growth medium (no arginine)
- A test 1: 2.5mM arginine
- A test 2: 1mM arginine
- A test 3: 0.25mM arginine

7.5.2.2 Elicitation procedure

Approximately 1g of cells (fresh wt.) from cell line 19 were inoculated into 50 ml of the three types of variations of the liquid 1902B medium; being the control 1902B medium, test 1 type containing 2.5mM arginine, test 2 type containing 1mM arginine, and test type 3 containing 0.25mM arginine.

The cells were grown for 35 days under standard conditions (see section 7.2) before harvesting by filtration. The cells were dried and extracted. Extracts of the cells were analysed for taxanes as outlined in section 7.3.4.

7.5.3 Effect of methyl jasmonate (MJ) on taxanes and flavonoids

7.5.3.1 Experimental design

The experiment was designed as a randomised, three factorial block experiment with four replications of each treatment. The following factors were included:

- A control: cells in growth medium (1902B).
- A control with ethanol: an addition of the equivalent amount of ethanol to that used in the test.
- A test: an addition of methyl jasmonate (MJ) dissolved in ethanol.

7.5.3.2 Elicitation procedure

Approximately 2g of cells (fresh wt.) from cell line 19 were inoculated into 100 ml of liquid 1902B medium. The cells were cultivated under standard conditions (see section 7.2) for 21 days before addition of the elicitor.

A solution of 44.86mg/10ml of MJ was made up using 96% ethanol (EtOH). 1ml of this solution was added to each of the flasks with test cells giving a final MJ concentration of 200 µM/100 ml media. In order to monitor the effect of ethanol on the cells a control with equal amounts of ethanol (1ml) was included in the study. The addition of MJ and solvent was made using sterile 0.22µm filters (Pall Gelman Laboratory 25mm Acrodisk®) under laminar flow (Envair Ltd.). The cells were grown for 30 days under standard conditions (see section 7.2) before harvesting by suction-filtration. The cells were dried and extracted. Extracts of both the culture medium and the cells were analysed for taxanes and flavonoids as outlined in section 7.3.4.

7.5.4 Effect of *tert*-butyl hydroperoxide (tBH)

7.5.4.1 Experiment 1and 2 - preliminary experiments

The organic peroxide *tert*-butyl hydroperoxide (t-BuOOH) in combination with hematin ($C_{34}H_{33}FeN_4O_5$) was used (Akaike et al. 1992).

Experimental design of experiment 1

The experiment was designed with three "factors". Each "factor" had four blocks. The experimental units within the four blocks were arranged in a randomised form on the orbital shaker.

- A control: standard growth medium.
- A test 1: addition of 10mM tBH and 100µM hematin.
- A test 2: addition of 30mM tBH and 300µM hematin.

Elicitation procedure for experiment 1

Approximately 1.6g of cells (fresh wt.) from cell line 19 were inoculated into 100 ml of liquid 1902B medium. The cells were grown for 29 days under standard conditions before addition of the tBH and hematin solution. The tBH was weighed out and

dissolved in sterile water (10mM and 30mM). The hematin was dissolved in ethanol (100µM and 300µM). The addition was done through sterile 0.22µm filters (Pall Gelman Laboratory 25mm Acrodisk®) under laminar flow (Envair Ltd.). Equal amounts of sterile water and ethanol were added to the control cells. The cells were harvested by suction-filtration after 24 hrs. The cells were dried and extracted. Extracts of the cells and the medium were analysed for taxanes as outlined in section 7.3.4.

Experimental design of experiment 2

The experiment was designed with three "factors". Each "factor" had four blocks. The experimental units within the four blocks were arranged in a randomised form on the orbital shaker.

- A control: standard growth medium.
- A test 1: addition of 30mM tBH and 300µM hematin at day 14.
- A test 2: addition of 30mM tBH and 300µM hematin at day 28.

Elicitation procedure for experiment 2

Approximately 2.5g of cells (fresh wt.) from cell line 19 were inoculated into 100 ml of liquid 1902B medium. The cells were grown for 14 days under standard conditions before addition of the tBH and hematin solution to the T1 cells, and for 28 days before addition to the T2 cells. The tBH and the hematin solutions were made up as described above. The cells were harvested by filtration on day 29. The cells were dried and extracted. Extracts of the cells and the medium were analysed for taxanes as outlined in section 7.3.4.

7.5.4.2 Experiment 3 - "Long time course"

Experimental design

The experiment was designed as a 2x4 factorial design, where the factors are two concentrations at four different harvest times. The two concentrations are the controls, with no addition of tBH and hematin, and the tests with 20mM tBH and 200µM hematin. The controls were only added an equivalent amount of DMSO and water to that of the tests. The addition was done on day 30 for all the factors. The harvest times are shown below:

- An overall control: standard growth medium, harvest on day 34.
- A test 6hrs (and respective control): harvest after 6 hours.
- A test 12hrs (and respective control): harvest after 12 hours.
- A test 24hrs (and respective control): harvest after 24 hours.
- A test 72hrs (and respective control): harvest after 72 hours.

Each "factor" had four blocks. The experimental units within the four blocks were arranged in a randomised form on the orbital shaker.

Elicitation procedure

Approximately 2.5g of cells (fresh wt.) from cell line 19 were inoculated into 100 ml of liquid 1902B medium. The cells were grown for 30 days under standard conditions before addition of the tBH and hematin solution to the test cells, and addition of water and DMSO to the respective control cells. The tBH and the hematin solutions were made up as described in section 7.5.4.2 with the difference, that instead of ethanol, DMSO was used to dissolve the hematin. The cells were harvested by filtration at the respective times. The cells were then dried and extracted. Extracts of the cells and the medium were analysed for taxanes and flavonoids as outlined in section 7.3.4.

7.5.4.3 Experiment 4 - "Long time course"

Experimental design

The concentration of the tests was changed from 20mM tBH and 200µM hematin to 10mM tBH and 100µM hematin. As before equivalent amounts of DMSO and water to that of the tests were added to the controls. Each of the following factors had four blocks. The experimental units within the four blocks were arranged in a randomised form on the orbital shaker.

- An overall control: standard growth medium, harvest on day 32.
- A test 24hrs (and respective control): harvest after 24 hours.
- A test 48hrs (and respective control): harvest after 48 hours.
- A test 72hrs (and respective control): harvest after 72 hours.

The experiment was designed as a factorial experiment using Minitab (Minitab^{TM}). Because there is no interaction between the different concentrations of the elicitor, and because there is one factor with two levels (concentration) and one factor with four levels (time), the design was decided to be a 2x4 instead of 2^4 factorial design. The factors and combinations used are described in **Table 7.5.4.1** below. These were randomised by block using Minitab, and the samples were organised accordingly. Explanations of concentrations in **Table 7.5.4.1**: 1 = no tBH and hematin, and 2 = addition of 10mM in correlation with 100µM hematin. Explanations of time: 1= harvest of cells without any addition, 2 = harvest 24 hours after addition, 3 = harvest 48 hours after addition and 4 = harvest 72 hours after addition.

Table 7.5.4.1 Factorial design of Experiment 4

Combination	**Factors**	
	Concentration	**Time**
Control	1	1
C24	1	2
T24	2	2
C48	1	3
T48	2	3
C72	1	4
T72	2	4

Elicitation procedure

The elicitation procedure was the same as for Experiment 3 in section 7.5.4.2, but the addition was done on day 28 instead of on day 30. The cells were harvested by suction-filtration at the respective times. The cells were then dried and extracted. Extracts of the cells and the medium were analysed for taxanes and flavonoids as outlined in section 7.3.4.

7.5.4.4 Experiment 5 - "Short time course"

Experimental design

Each of the following factors had four blocks. The experimental units within the four blocks were arranged in a randomised form on the orbital shaker. The concentrations of the tests were kept as in Experiment 4 to 10mM tBH and 100µM hematin. As

before equivalent amounts of DMSO and water to that of the tests were added to the controls. The harvest times are shown below:

- An overall control: standard growth medium, harvest on day 28.
- A test 10 min (and respective control): harvest after 10 minutes.
- A test 30 min (and respective control): harvest after 30 minutes.
- A test 60 min (and respective control): harvest after 60 minutes.

The experiment was designed as a factorial experiment using Minitab (Minitab^{TM}). Also in this experiment the design was decided to be a 2x4 factorial design. The factors and combinations used are described in **Table 7.5.4.2** below. These were randomised by block using Minitab, and the samples were organised accordingly. Explanations of concentrations used in **Table 7.5.4.2**: 1 = no tBH and hematin, and 2 = addition of 10mM in correlation with 100µM hematin. Explanations of time used in **Table 7.5.4.2**: 1= harvest of cells without any addition, 2 = harvest 10 minutes after addition, 3 = harvest 30 minutes after addition and 4 = harvest 60 minutes after addition.

Table 7.5.4.2 Factorial design

Combination	**Factors**	
	Concentration	**Time**
Control	1	1
C10	1	2
T10	2	2
C30	1	3
T30	2	3
C60	1	4
T60	2	4

Elicitation procedure

Approximately 1g of cells (fresh wt.) from cell line 19 were inoculated into 50 ml of liquid 1902B medium. The elicitation procedure was the same as for Experiment 4 in section 7.5.4.3. The cells were harvested by filtration at the respective times. The cells were dried and extracted. Extracts of the cells and the medium were analysed for taxanes and flavonoids as outlined in section 7.3.4.

7.6 Influence of a Liquid-solid Culture System on Constituent Yields

The adsorbent used in the following experiments was Amberlite® XAD-2 (Aldrich-Sigma). This is a non-ionic polymeric adsorbent; it is a 20-60 mesh polystyrene resin.

To prepare the Amberlite resin for use it was washed with methanol (MeOH) in a conical flask for 24hrs on an orbital shaker at 130 rpm. The MeOH was then removed by filtration and the resin was washed repeatedly with deionised water. Subsequently the resin was filtered under light vacuum using a Buchner funnel to remove excess water (Williams et al. 1992).

7.6.1 Preliminary amberlite experiment

7.6.1.1 Experimental design

The experiment was designed with five "factors". Each "factor" had four blocks. The experimental units within the four blocks were arranged in a randomised form on the orbital shaker. The following 'units' were extracted and analysed:

- control cells (no Miracloth ® or resin present)
- cells with loose resin (loose resin mixed with the cells)
- cells with control bag (empty Miracloth® bag present)
- control bag (empty Miracloth® bag itself)
- cells with resin bag (resin-filled Miracloth® bag present)
- resin bag (resin-filled Miracloth® bag itself)

7.6.1.2 Experimental procedure

Approximately 2g of cells (fresh wt.) from cell line 19 were inoculated into 100 ml liquid 1902B medium. The cells were grown for 9 days under standard conditions (see section 7.2) before addition of the Amberlite resin either in bags or in loose form.

In each case 1g of prepared and suction-filtered Amberlite was weighed out. The resin was enclosed in a 2x2 cm square of porous Miracloth (pore size of 22-25μm; composed of rayon polyester with an acrylic binder; Calbiochem, La Jolla, CA). The material was closed using a nylon thread after being filled with the resin to form a resin bag. Empty bags were made to match the filled bags in order to simulate the

same effect on the cells. Excess material was cut away in order to minimise the shearing effect possibly produced by the bags. Each of the bags was weighed, wetted, and autoclaved prior to addition. The loose resin was also weighed and autoclaved prior to addition.

The cells were grown for 30 days under standard conditions (see section 7.2) before harvesting by filtration. The cells were then dried and extracted. The resin bags were cut open prior to extraction, and the resin and bag material was extracted in the same way as the cells. The extracts were analysed for taxanes and flavonoids as outlined in section 7.3.4.

7.6.2 Final Amberlite experiment

The same procedures were followed as in the preliminary experiment, but no loose amberlite resin was used and the bags were made from another non-woven porous material consisting of a man-made non-cellulosic filament (pore size 22-25μm) instead of the Miracloth used in the preliminary experiment. This change was necessary to prevent leakage of resin from the cloth bags. The experiment therefore had four "factors". The following 'units' were extracted and analysed:

- control cells (no bag or resin present)
- cells with control bag (empty bag present)
- cells with resin bag (resin-filled bag present)
- resin bag (resin-filled bag itself)

The cells were grown for only 7 days before the addition of the bags. The cells were then cultured under standard conditions for a further 36 days before harvesting by filtration. The cells were dried and extracted as before. Extracts of the culture medium, the cells and the resin bags were analysed for taxanes and flavonoids as outlined in section 7.3.4.

7.7 Phytochemical Analysis of *Taxus baccata* 'Fastigiata' Tissue Culture

7.7.1 Extraction Procedure

Harvested cells from cell line 19 were stored at -20°C until time of use. The bulked and dried cell material was ground to a fine powder using a mortar and pestle immediately prior to extraction. The powdered cell material (107.83g) was then macerated in cold HPLC-grade methanol (MeOH) for 2 days. The solution was filtered and evaporated to dryness *in vacuo* using a rotary evaporator. The remaining cell material was extracted with HPLC-grade methanol (MeOH) under reflux in a Soxhlet Apparatus until the extract was colourless (72 hrs). The extract was filtered and evaporated to dryness *in vacuo*. The two resulting residues were analysed by TLC as described in section 7.3.3 using solvent system 1 and 3. The two extracts were found to be the same, and were therefore combined for further investigation. The combined methanol extract was reconstituted in water and extracted successively with HPLC-grade hexane (C_6H_{14}), HPLC-grade ethyl acetate (EtOAc) and HPLC-grade butanol ($C_4H_{10}O$). The three extracts were all analysed by TLC and compared using the same solvent systems and visualisation as above. The EtOAc and butanol extracts were found to be similar.

7.7.2 Preparative thick layer chromatography (prep-TLC)

Thick-layer glass-backed silica gel plates were prepared as follows. The silica gel (Silica gel 60 PF254, Merck KgaA, Darmstadt) was wetted (120g silica gel to 240 ml water), and the solution was vigorously shaken. Finally the mixture was spread over 5 glass plates using a plate spreader (Shandon Southern Unoplan®). The thickness of the silica gel was set to 2mm. The plates were dried for 12 hrs in air, and then stored in a desiccator. Prior to use the plates were activated at 110°C in an oven for 1 hour.

Some of the EtOAc extract (0.228g) was dissolved in methanol (4ml) and this solution was applied to 4 activated thick layer chromatography plates prepared as above. The plates were developed twice in solvent system 3 (see section 7.3.3). Visualisation was by UV light at 254nm. The bands were scraped off the plates and combined. The compounds were re-extracted from the silica gel using HPLC-grade MeOH and

sonication. The extract was evaporated to dryness *in vacuo* using a rotary evaporator. Two bands (R_f ~0.55 and ~0.45) were isolated and analysed by TLC followed by nuclear magnetic resonance spectroscopy (NMR).

7.7.3 Liquid column chromatography (LCC) of the ethyl acetate extract

A sample of 2g of the ethyl acetate (EtOAc) extract was reconstituted in MeOH and transferred to a round-bottomed flask. Silica gel was added (1:10 extract-silica) for pre-adsorption. The solvent was evaporated under reduced pressure using a rotary evaporator yielding a free flowing powder. This was introduced to a solvent head ($CHCl_3$) on a wet packed silica gel column (Silica gel 60 (0.04-0.063 mm) Merck KGaA, Darmstadt). The column dimension was 25cm x 3.5 cm.

Table 7.7.3.1 Mobile phase system used

Fractions	**Solvent system**
First 500 ml	100% $CHCl_3$
Test tube: 1-73	70:7:0.7 $CHCl_3$-MeOH-H_2O
Test tube: 74-120	75:23:2 $CHCl_3$-MeOH-H_2O
Test tube: 121-180	75:46:4 $CHCl_3$-MeOH-H_2O
Test tube: 181-200	50:46:4 $CHCl_3$-MeOH-H_2O

The fractions collected were analysed by TLC using solvent system (1), (3), and (5) (see section 7.3.3). On the basis of this the fractions were pooled and combined. Combined fractions: 1-5, 6-13, 14-31, 32-46, 47-89, 90-94, 95-101, 102-106, 107-113, 114-117, 118-123, 124-129, 130-142, 143-147, 148-152, 153-163, 166-200.

The combined fraction 130-142 was further investigated by various TLC systems for possible separation. TLC systems used was:

- Aluminium-backed pre-coated silica gel 60F254 plates (E.Merck, Darmstadt).
- Plastic-backed Cellulose (DC) plates (with fluorescent indicator, E.Merck, Darmstadt).
- Cellulose Chromatography Paper (Whatman®, grade 20Chr (thickness: 0.17mm and flow rate: 85mm in 130min)

The following solvent systems were used in this investigation (Stahl 1969, Wagner and Bladt 1996):

1. BAW: Butanol-acetic acid-water ($BuOH-C_2H_4O_2-H_2O$) (4:1:5) (upper layer)
2. Toluene-chloroform-acetone ($CH_3C_6H_5-CHCl_3-Ac_2O$) (40:25:35)
3. Ethyl acetate-butanone-formic acid-water ($EtOAc-C_4H_8O-CH_2O_2-H_2O$) (50:30:10:10)
4. Toluene-ethyl acetate-formic acid ($CH_3C_6H_5$-EtOAc- CH_2O_2) (50:40:10)

7.7.4 Preparative high performance liquid chromatography (prep-HPLC)

Preparative-HPLC was employed for the possible separation of the three components in the combined fraction 130-142.

7.7.4.1 Instrumentation

Analysis by HPLC was carried out using a Waters™ Multisolvent Delivery System consisting of a 600 Gradient Controller, a 2996 photodiodearray (PDA) detector, a 717 plus autosampler, a fraction collector III , and a computer with the Empower™ software.

7.7.4.2 Columns and chromatographic conditions

The preparative column used for the separation was a Phenomenex Luna 100Å $C_{18}(2)$ (5µm, 250 x 10mm) column with a Phenomenex Luna 100Å $C_{18}(2)$ (5µm, 10x10 mm) guard column. The mobile phase used was a gradient of a) 1% TFA (CF_3COOH) and b) methanol (MeOH) (from 80:20 to 0:100 over a period of 25min) as illustrated in **Table 7.7.4.1**.

Table 7.7.4.1 Gradient table for flavonoid analysis by HPLC

Time	Flow (ml/min)	Solvent A (%)	Solvent B (%)	Curve
Initial	4	80	20	
20	4	20	80	Linearly increasing
25	4	0	100	Linearly decreasing
35	4	80	20	

The injection volume was 50µl, and the flow rate was 4ml/min. Detection was performed at 200-400nm (1.2nm res.). All analyses were carried out at ambient temperature. Each peak eluting was collected manually.

7.7.5 Acetylation of ethyl acetate extract

Some of the ethyl acetate extract (0.9488g) was heated under reflux for 4hrs in acetic anhydride ($(CH_3)_2(CO)_2O$) and 2 drops of pyridine (C_5H_5N). The reaction mixture was then poured over ice and stirred until the ice melted. The aqueous mixture was then extracted with EtOAc (3x50ml). The combined EtOAc extracts were washed with dilute HCl (10%) (3x50ml), and finally the combined EtOAc extracts were washed with H_2O (3x50ml). The EtOAc extract was dried over anhydrous sodium sulphate (Na_2SO_4) and evaporated to dryness *in vacuo*, yielding a yellow/brown oil (0.7g).

7.7.6 Liquid column chromatography (LCC) of the acetylated EtOAc extract

For this column a sample of 0.7g of the acetylated ethyl acetate extract was used. The column was made up as described in section 7.7.3. The column dimension was as before 25cm x 3.5 cm. The mobile phase used for eluting the whole column was 0.5% MeOH in $CHCl_3$. The fractions collected were analysed by TLC using solvent system 2 (see section 7.3.3). On the basis of this the fractions were pooled and combined. Combined fractions: 1-11, 12-15, 16-17, 18, 19, 20-22, 23-29, 30-34, 35-39, 40-51.

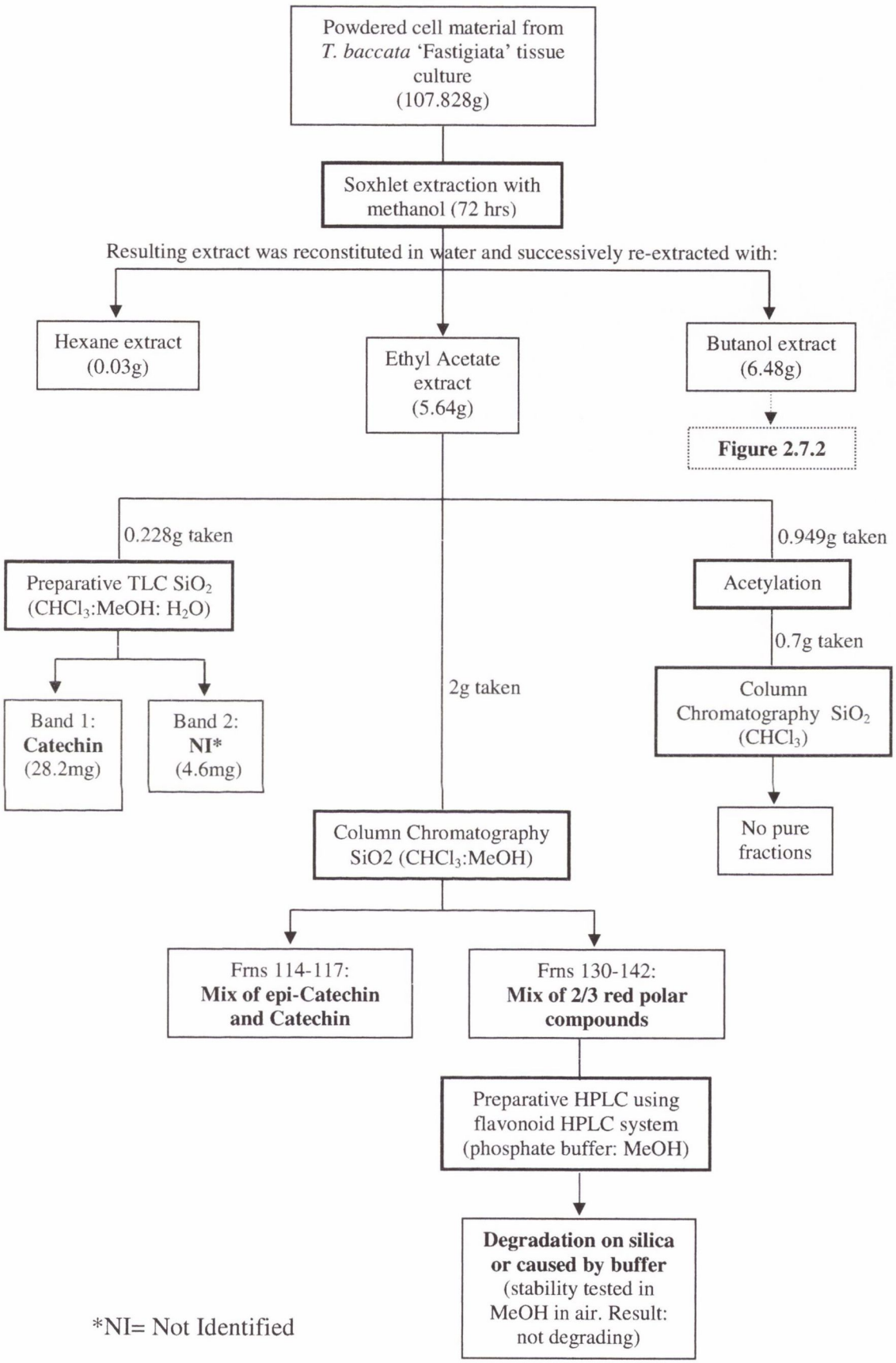


Figure 7.7.6.1 Flow diagram of phytochemical analysis: ethyl acetate extract

7.7.7 Hydrolysis of butanol extract from *Taxus baccata* 'Fastigiata' cell culture

A sample of the butanol extract (1.2432g) was dissolved in dilute HCl (10%) and heated under reflux for 2 hours. The reaction mixture was extracted using EtOAc (3x50ml). The EtOAc extracts were combined, dried over anhydrous Na_2SO_4, and the solvent was then evaporated under reduced pressure using a rotary evaporator, yielding a brown residue (76.2mg). The residue was re-dissolved in the minimum amount of MeOH and spread over four prep-TLC plates. The plates were run twice and four bands were isolated. Band 4 (major band) was further analysed by NMR and gas chromatography-mass spectrometry (GC-MS).

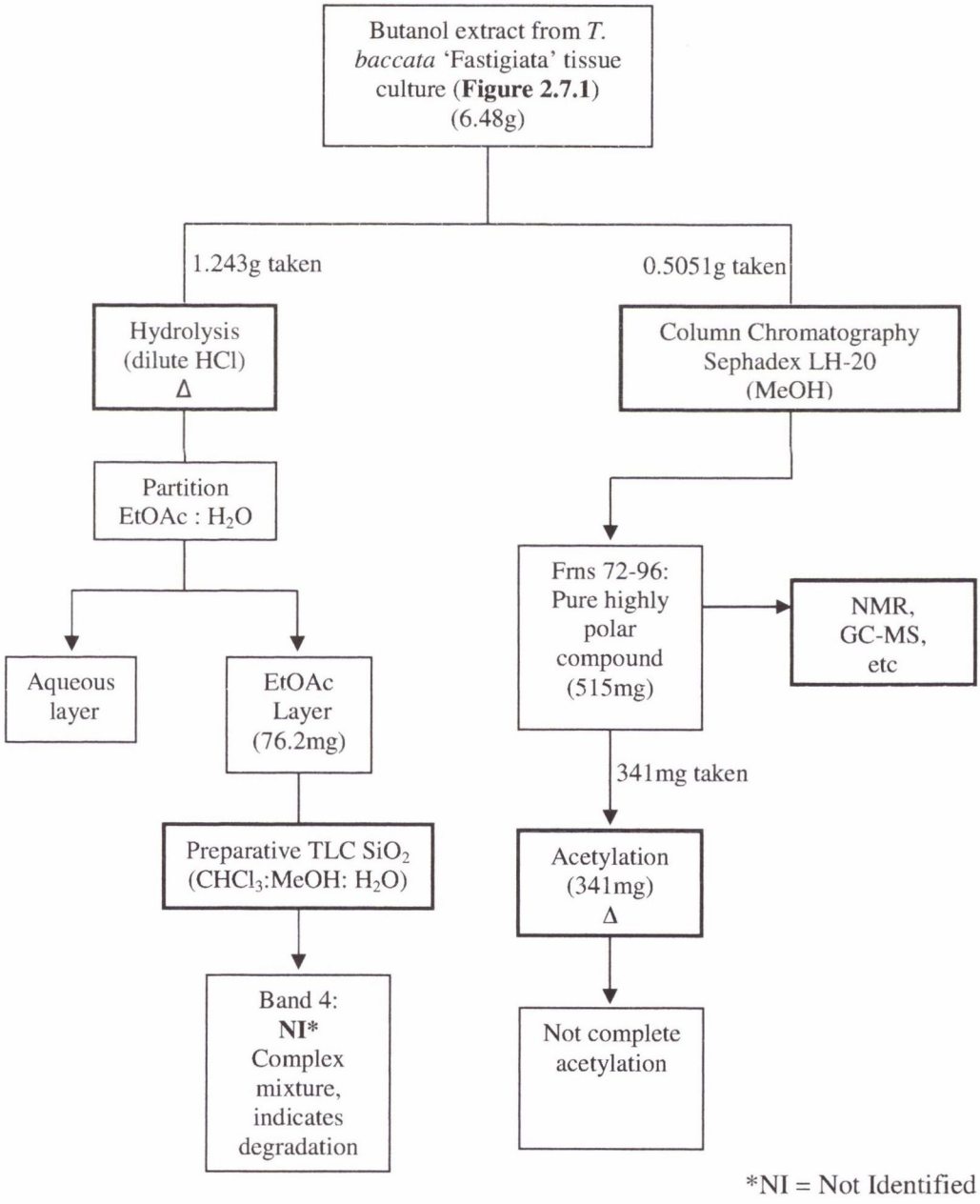


Figure 7.7.7.1 Flow diagram of phytochemical analysis: Butanol extract

7.7.8 Gel filtration chromatography using Sephadex LH-20

Sephadex LH-20 (Sigma-Aldrich) is supplied as a dry powder and must be swollen before use. An excess volume of MeOH was added to the Sephadex, and this mixture was left to swell at room temperature over night. Stirring was avoided as it could break the beads. The excess MeOH was then decanted off, and the slurry was poured into the column used (30cm x 3.5 cm) in one continuous motion. A solvent head of MeOH was added, and the Sephadex was left to settle. The excess MeOH was eluted through the column without the use of positive pressure.

A sample of the butanol extract (0.5051g) was dissolved in the minimum amount of MeOH and added to the top of the column. Only MeOH was used for the elution, and fractions of 5ml were collected without stop until everything had been passed through the Sephadex.

The fractions collected were analysed by TLC using solvent system 1 and 3 (see section 7.3.3). On the basis of this the fractions were pooled and combined. Combined fractions: 1-21, 22-28, 29-39, 40-42, 43-49, 50-66, 67-71, 72-96.

Combined fractions 72-96 was further analysed by nuclear magnetic resonance spectroscopy (NMR) and high-resolution mass spectrometry (high-res. MS). The fraction was also analysed at various temperatures by NMR: at 21, 0, -20, -40 and -80°C. A sample of this fraction (341mg) was acetylated following the procedure given in section 7.7.5. The acetylated fraction (200mg) was further analysed by NMR and GC-MS.

7.7.9 Hydrolysis of combined fractions 72-96 by 5%HCl in *tert*-butanol

Some of the combined fractions 72-96 (1 mg) was dissolved in MeOH and placed in a thick-walled, screw-topped, glass tube with a Teflon lined cap. 5% HCl in *tert*-butanol (2ml) was added and the solution was heated on a boiling water bath for 1 hour (Foo and Lu 1999). The resulting solution was compared with cyanidin by TLC (solvent system 1 and 3, section 7.3.3) and HPLC using mobile phase systems used for flavonoids (section 7.3.4).

7.7.10 Spectroscopy

7.7.10.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

Bruker DPX-400 instrument, at 400.13 MHz for proton (^{1}H) magnetic resonance and 100.61 MHz for carbon (^{13}C) magnetic resonance (NMR analysis performed by John O'Brien in School of Chemistry, Trinity College). Spectral analysis was performed using Bruker WIN-NMR Software or MestRec NMR Software.

For the temperature experiment by NMR: A Bruker Avance 400 instrument, at 400.13MHz for proton (^{1}H) magnetic resonance at a temperature of 21, 0, -20, -40 and -80°C (NMR analysis performed by Maurice Burke in Faculty of Science and Health, Dublin City University). Spectral analysis was performed using MestRec NMR Software.

7.7.10.2 Gas Chromatography-Mass Spectrometry (GC-MS)

Low resolution GC-MS: Saturn GC/MS 2000 (CP-3800 Gas Chromatograph).
Oven temperature: initial 100°C, increasing at 15°C per minute to 300°C.
Run time: 25 minutes.
Column flow rate: 1 ml/min.
Column pressure: 10 psi.
Column: FactFour™, capillary column, VF-Xms (30m, 0.25mm, 0.25μm)

7.7.10.3 High resolution mass spectroscopy (MS)

Bruker Esquire 3000 LC-MS instrument with Ion Trap mass analyser. Ionisation Source: ESI in positive mode. (MS analysis performed by Maurice Burke in Faculty of Science and Health, Dublin City University).

7.8 Fatty Acid Profile of *Taxus* Tissue Culture Compared to Needles and Seeds

7.8.1 Plant material

For this analysis samples from each of the suspension culture cell lines cultivated were used. These include cell lines 19, 8, 3, and cell line H*. The three first cell lines were initiated from seeds of *Taxus baccata* 'Fastigiata' (see section 7.1). The fourth cell line (H*) was initiated from the needles of *Taxus baccata* 'Fastigiata' (see section 7.1) (Hook 1999).

7.8.2 Extraction procedure

The extraction procedure followed was first developed by Browse and co-workers (Browse et al. 1986). This method was further used by Mongrand and co-workers for analysis of fatty acids in leaves from Gymnospermae including *Taxus* species (Mongrand et al. 2001).

Samples (250mg) of each of the cell lines cultivated were placed in screw-capped tubes for extraction. The same amount of chopped needles (fresh) and ground seeds (dried) from *Taxus baccata* 'Fastigiata', collected from Powerscourt Estate and Gardens, Co. Wicklow, was also placed in screw-capped tubes for extraction (for comparison). After the addition of 1ml methanolic sulphuric acid reagent (2.5% H_2SO_4 $^{v}/_{v}$ in MeOH), the tubes were heated to 80°C for one hour. The samples were cooled and 500µl hexane and 1ml water was added. The fatty acid methyl esters (FAMEs) were extracted into the hexane layer by vigorous shaking. The tubes were centrifuged at 1500g for 10 minutes to break any emulsion formed and to completely separate the two phases. A sample (4µl) was taken directly from the hexane phase and used for gas chromatography (GC) analysis.

7.8.3 Analysis of FAMEs by gas chromatography (GC)

7.8.3.1 Instrumentation and column

Analyses were preformed on a Perkin Elmer 8700 gas chromatograph fitted with a BPX70-70% cyanopropyl (Eqiv) polysilphenylene-siloxane (50m x 0.22mm; 0.25µm film thickness) column (SEG, Europe Ltd). The carrier gas was helium (He) set at a

constant pressure of 30kPa. Injections were performed with a split rate of 17:1. Detection was carried out using a flame ionisation detector (FID).

7.8.3.2 Chromatographic conditions

The temperature program used for the analyses was developed according to the manufacturer's guidelines (SEG, Europe Ltd) and is shown in **Figure 7.8.3.1.** An initial oven temperature of 60°C was increased at a rate of 50°C/min to 170°C. This temperature was held for 5 min. Then the temperature was increased at a rate of 4°C/min to 200°C. This temperature was held for 5 min. Finally the temperature was increased at a rate of 50°C/min to 255°C, and this temperature was held for another 4.5 min. The injector temperature was 250°C and the detector temperature was 270°C. In all cases an injection volume of 4µl was used.

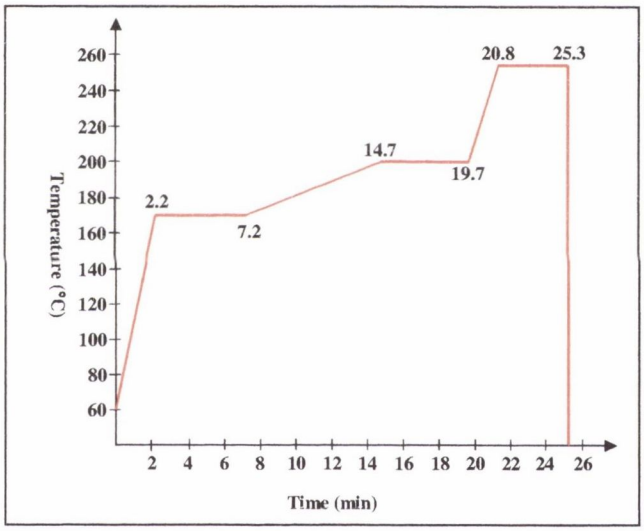


Figure 7.8.3.1 Temperature program for GC

7.8.3.3 Validation of GC method

As this study was a comparative study of the fatty acid profiles of *Taxus baccata* 'Fastigiata' needles, seeds and cell cultures only limited validation in order to ensure accuracy and precision was necessary. For a qualitative procedure of this type, precision is the parameter of greatest importance both in terms of peak area and retention time. Huber (Huber 1999) recommends in his book, 'Validation and

qualification in analytical laboratories', that these parameters should be evaluated as follows;

1. Precision of peak area

The peak area of five 10µl replicate injections of a known concentration (1mg/ml) of a fatty acid methyl ester (methyl stearate (C_{18})) was recorded. The percent relative standard deviation (% RSD) between the five peak areas was found to be 1.25%, below the 2 % recommended limit. This is sufficient to demonstrate that the injection volume of 10µl was consistently reproducible.

2. Precision of retention time

The retention time of the same series of five replicate injections of a 1mg/ml solution was recorded. The % RSD between the five peak areas was 0.63%, les than the 1% recommended limit. This was therefore sufficient to show that the method was reliable.

7.8.4 Identification of the FAMEs

The methylated fatty acids were identified using the linear relationship between the logarithm of retention time (Log Rt) and the carbon number (EP 1997). Nine different fatty acid methyl esters were used as standards for the qualitative analysis (see **Table 7.8.4.1**). These are the most common FAMEs in *Taxus* leaves (Mongrand et al. 2001).

Table 7.8.4.1 FAMEs used in qualitative GC analysis

FAME	Rt	Log Rt	Carbon number
Capriate	5.87	0.77	10
Laurate	8.21	0.91	12
Myristate	10.00	1.00	14
Palmitate	13.24	1.12	16
Stearate	15.74	1.20	18
Oleate	16.36	1.21	18.3*
Linoleate	17.55	1.24	18.9*
γ-Linolenate	18.86	1.28	19.7*
Linolenate	19.22	1.28	19.7*

*equaivalent chain length on polyetyleneglycol adipate (EP 1997)

8 Results and Discussion

8.1 Plant Material

8.1.1 Origin and maintenance of cell culture

Two cell cultures of *Taxus baccata* 'Fastigiata' were used in the present study. The first was initiated from embryos by researchers from Coillte Research Laboratories, Newtownmountkennedy, Co. Wicklow, Ireland in 1997 and the second from needles (Hook 1999). Details and development of suspension cultures can be found in Materials and Methods (section 7.1).

8.1.2 Growth characteristics of cell lines

Some evaluations of growth kinetics, i.e. biomass and taxane production, as well as effect of culture medium composition were carried out previously (Dempsey 2000, Hook 1999). All cultures grew very slowly. Comparison of the Growth Indices (fresh wt of cells at harvest/fresh wt of inoculum at subculture) of Cell Lines 19, 8 and 3 showed that Line 19 had superior growth characteristics. It was therefore selected for further study. Maximum biomass was found to be produced on day 24 (5.5g dry cell wt/L), with maximum paclitaxel accumulation being reached on day 30 (132 mg/kg) (Dempsey 2000, Hook 1999). These findings coincides with those reported by Schuler and his research group for *T. baccata* suspension cultures, where they found that maximum growth was reached at day 25 and maximum paclitaxel accumulation was recorded on day 30 (Srinivasan et al. 1995). When *Taxus baccata* suspension cultures were grown in an airlift bioreactor the highest productive state (taxane production) was reported to be from day 24 to day 28 (Navia-Osorio et al. 2002).

Variations in culture medium composition were also investigated (Dempsey 2000, Hook 1999). Cell Lines 19, 8 and 3 were grown under the same conditions in two media - 1902A and 1902B (see formulation in **Table 7.2.1** in Materials and Methods, section 7.2). They differed only in their glutamine content. No statistically significant difference in biomass production or taxane content between the two media was found (Dempsey 2000, Hook 1999). Further studies including three different media were

undertaken (Hook 1999). Cell line 3 was grown in Rugini's Olive (RO), Westvako 3 (WV3) and Westvako 5 (WV5) media (see formulation in Appendix I) in addition to 1902A and 1902B. Since the biomass production in these media was significantly lower, it was concluded that medium 1902B should be used for future studies.

Evaluation of results from all preliminary experiments led to the decision that Cell Line 19 growing in 1902B medium, with a growth cycle of 36 days would be used for further investigations.

8.1.3 Description of cell lines

Cell line 19

Cells of Line 19, grown in liquid medium, grew as soft, friable, relatively small aggregates, with some amount of dispersed single cells. Cells were light brown in colour. The culture medium turned slightly brown during the growth period, presumably due to the excretion of phenolic metabolites. Since this cell line had the most stable growth and gave the highest taxane production, it was used in elicitation experiments.

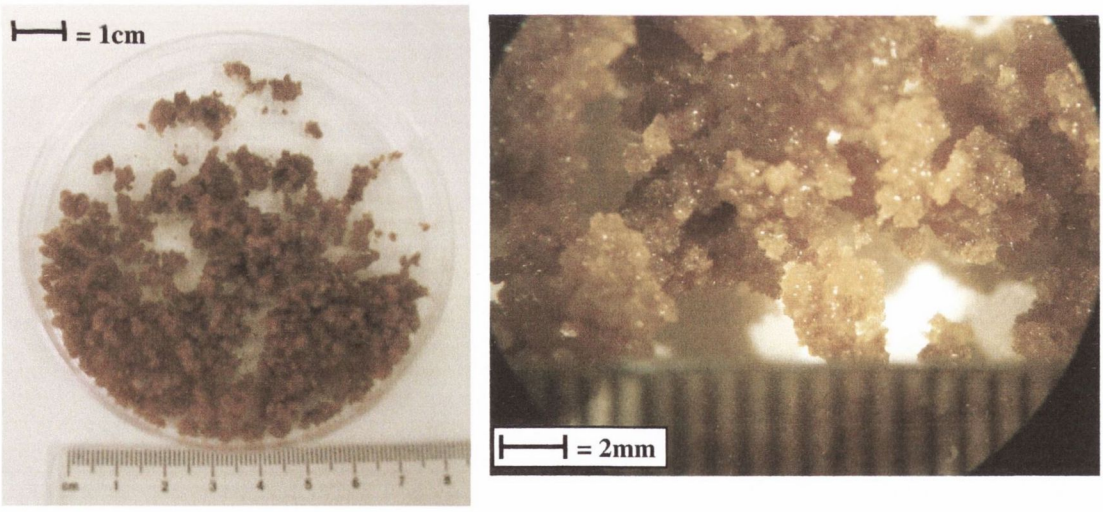


Figure 8.1.3.1 Cell line 19, freshly harvested suspension culture

Cell line 8

Cells of Line 8, grown in liquid medium, grew as soft friable aggregates, slightly larger than those of Line 19. Cells were pale yellow to beige in colour. The culture medium stayed relatively colourless during the growth period. This cell line had a

high growth rate, but because it produced metabolites that interfered with the taxane analysis, this cell line was not used in the elicitation studies performed.

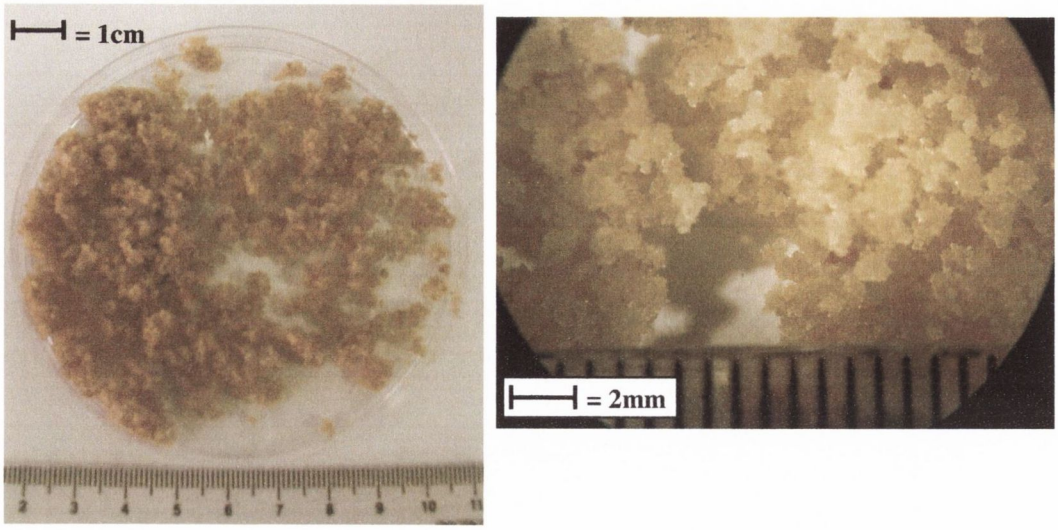


Figure 8.1.3.2 Cell line 8, freshly harvested suspension culture

Cell line 3

Cells of Line 3, grown in liquid medium, grew as soft, friable, aggregates, slightly larger than those of Cell Line 19. Cells were beige in colour, but during the course of the present study suddenly became very brown in colour and eventually died. The culture medium did not turn brown during the growth period, but stayed relatively colourless until the sudden change. This was presumably due to a large production and excretion of toxic plant phenolics.

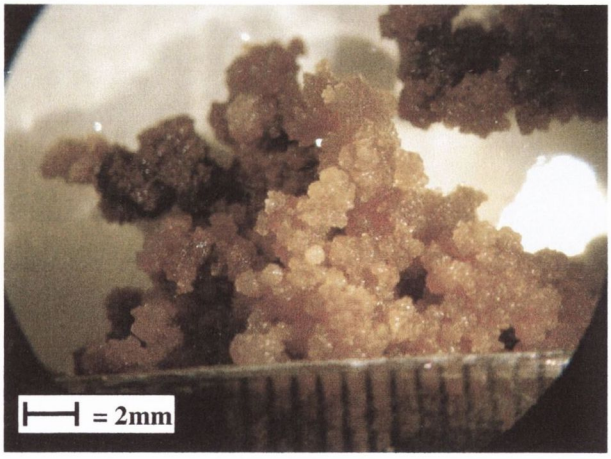


Figure 8.1.3.3 Cell line 3

Tissue darkening of both *Taxus* callus and suspension cultures has been reported by various research groups working with *Taxus* in vitro. After having added vanadium

sulphate (VSO_4) to a callus line of *T. baccata* Cusidó et al (Cusidó et al. 1999) reported that an increase in phenylalanine ammonia lyase (PAL), an enzyme that catalyses the first step in the phenylpropanoid biosynthetic pathway (Jones 1984), was followed by tissue browning of the cells. This finding indicates that tissue browning is a result of an increase in the formation of phenolics. Fett-Neto et al (Fett-Neto et al. 1992) also reported a frequent occurrence of tissue darkening in callus cultures of *T. cuspidata* and *T. canadensis*. They suggested that the darkening was a result of the production and oxidation of phenolics by the explants. By adding the phenolics-binding compound polyvinylpolypyrrolidone (PVP), to the culture medium, less tissue browning resulted. Addition of the antioxidants cysteine and ascorbic acid were found to be detrimental to the cell growth.

Cell line H* Cells of Line H*, originally established from *T. baccata* needles, grown in liquid medium, grew as soft, friable, aggregates, similar in size to those of Cell Line 19. The cells were light brown in colour, but as described for Cell Line 3, during the present study the cells turned a very dark brown/reddish colour and finally died. This was again presumably due to a large production and excretion of toxic phenolics.

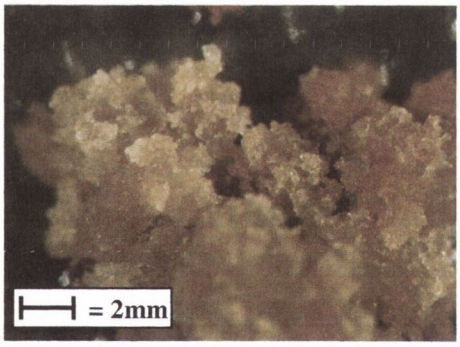
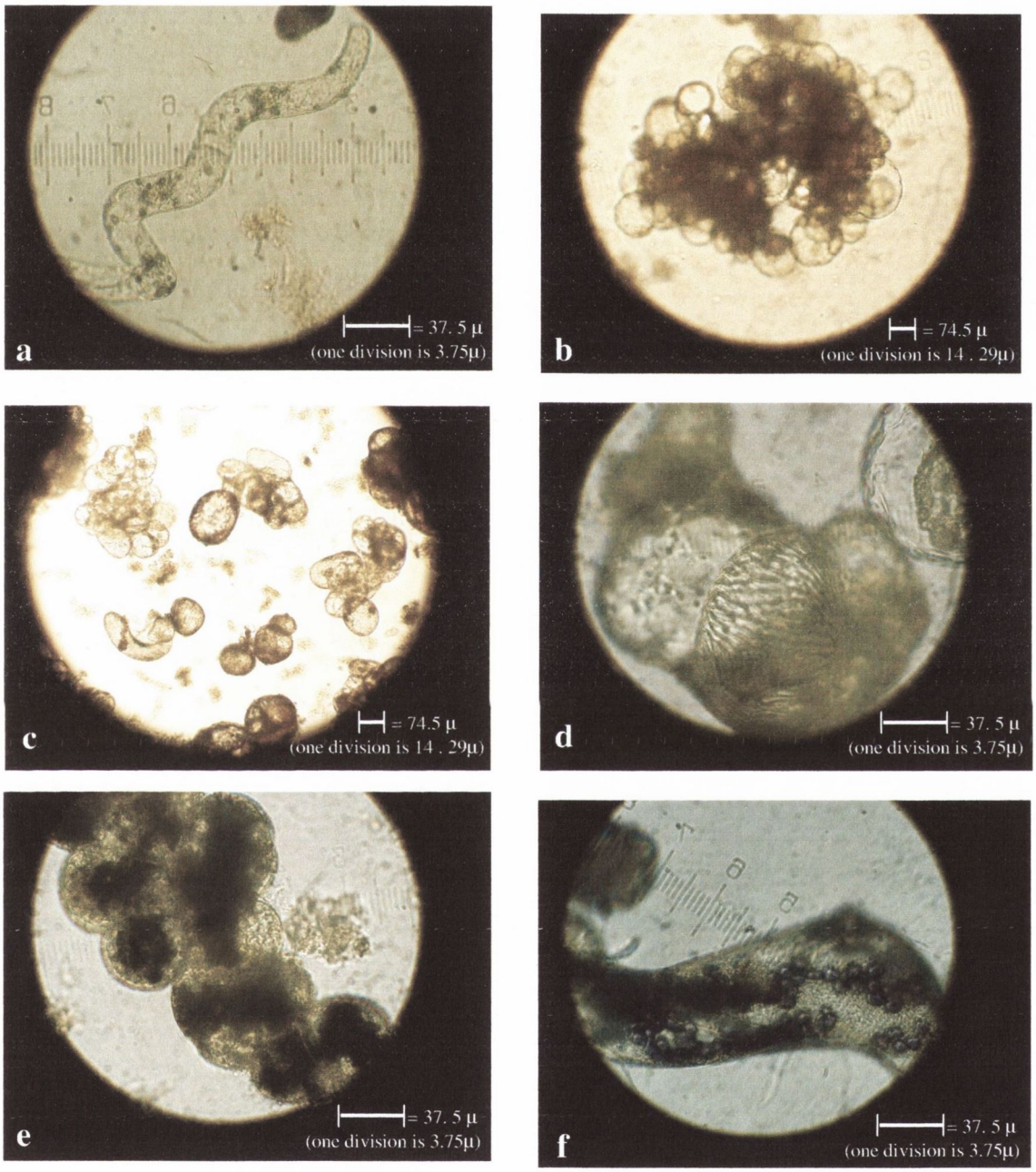


Figure 8.1.3.4 Cell line H*

Taxus cells as seen with a light microscope are shown in **Figure 8.1.3.5**. It is apparent that the cells are variable both as regards shape, size, surface markings and degree of aggregation. Many were typically parenchymatous and rich in starch grains (see **e** and **f**). The starch grains in **f** were found to be 7.8 ± 0.95 μ in size (n=10). Although few references could be found for conifer starch grains these *Taxus* grains are significantly larger than those measured by Kainulainen et al (Kainulainen et al. 2000) in Scots pine

(2.26μ ± 0.22) and Norway spruce (2.04μ ± 0.22). However, it has to be taken into account that those observations were from seedlings of Scots pine and Norway spruce grown in an open-air fumigation system and not from the corresponding cells grown as tissue cultures.



(**a**) undifferentiated elongated cell from cell line H*, (**b**) cell aggregate from cell line 8 (stained with I_2), (**c**) an overview of the variation in cell types/shapes in cell line 19 (stained with I_2), (**d**) cell showing surface sculpturing (xylem) from cell line 19, (**e**) starch accumulation in cell line 19(stained with I_2), (**f**) starch accumulation in H* cells (stained with I_2)

Figure 8.1.3.5 Microscopy of Cell lines 8, 19 and H*

8.2 Constituents of *Taxus baccata* 'Fastigiata' Suspension Cultures

8.2.1 Taxanes

Plant tissue culture of *Taxus* species is considered as one of the most promising approaches to obtain paclitaxel and related taxanes, because it is a renewable and environmentally accepted source. In this study the taxanes that were identified as present and quantifiable in the *Taxus baccata* 'Fastigiata' needles (paclitaxel, 10-DAB III, cephalomannine and baccatin III) were also present in the suspension cultures (**Figure 8.2.1.1**), however, the content was generally lower than in the needles: An example of the concentration of 10-DAB III, cephalomannine and paclitaxel in *T. baccata* 'Fastigiata' needles where, 307.53 ± 27.46 mg/kg, 124.77 ± 16.47 mg/kg and 283.26 ± 58.19mg/kg, respectively (section 4.2). The total production of 10-DAB III, baccatin III and cephalomannine in the cell cultures (section 8.4.3) where 62.40 ± 30.08 mg/kg, 136.91 ± 37.78 mg/kg and 57.13 ± 13.61 mg/kg, respectively.

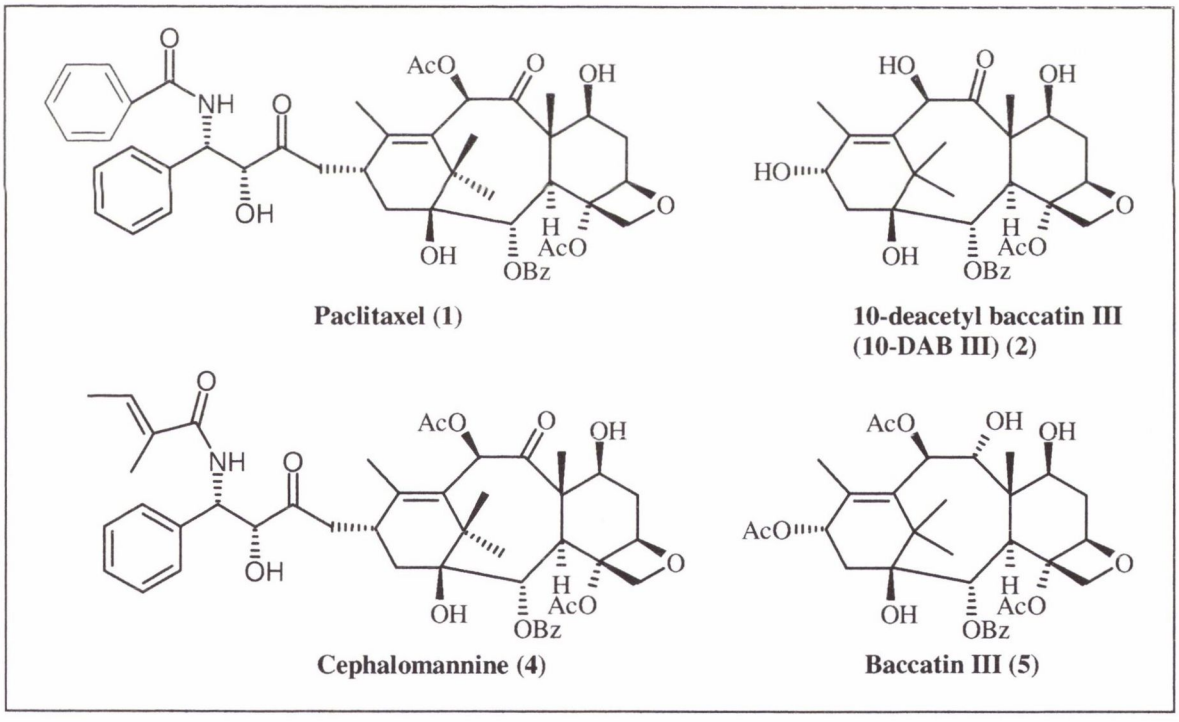


Figure 8.2.1.1 Taxanes present in *Taxus baccata* 'Fastigiata' suspension cultures

Although our experiments concentrated on the four taxanes in **Figure 8.2.1.1**, a range of other taxanes have been isolated from the cell cultures of *Taxus* species. Some examples are: 7-xylosyl-10-deacetyl taxol was isolated from *T. brevifolia* cultures

(Gibson et al. 1993). 9-dihydro-13-acetyl-baccatin III (9-DHAB III) and 9-dihydrobaccatin III was isolated from *T. canadensis* cultures (Ketchum et al. 1999b). In cultures of *T. chinensis* 16 taxanes including five novel compounds not present in the plant was isolated (Menhard et al. 1998). These were: paclitaxel, 10-DAB III, baccatin III, baccatin VI, 9-dihydrobaccatin III, 13-dehydroxy-10-DAB III (new), 9-dihydro-13-acetoxybaccatin III, 9-dihydro-13-dehydroxybaccatin III (new), 13-dehydroxybaccatin III, 13-deacetoxybaccatin I (new), yunnanxane, taxuyunnanine C, 2α,4α,7β,9α, 10β-pentaacetoxytax-11-ene (new), 2α-benzoxy-4α,7β, 9α, 10β-tetraacetoxy-1β-hydroxytax-11-ene (new), 2α,5α,10β-triacetoxy-14β-propionyloxy-4(20),11-taxadiene and 2α,5α,10β-triacetoxy-14β-(2'-methyl)butyryloxy-4(20),11-taxadiene.

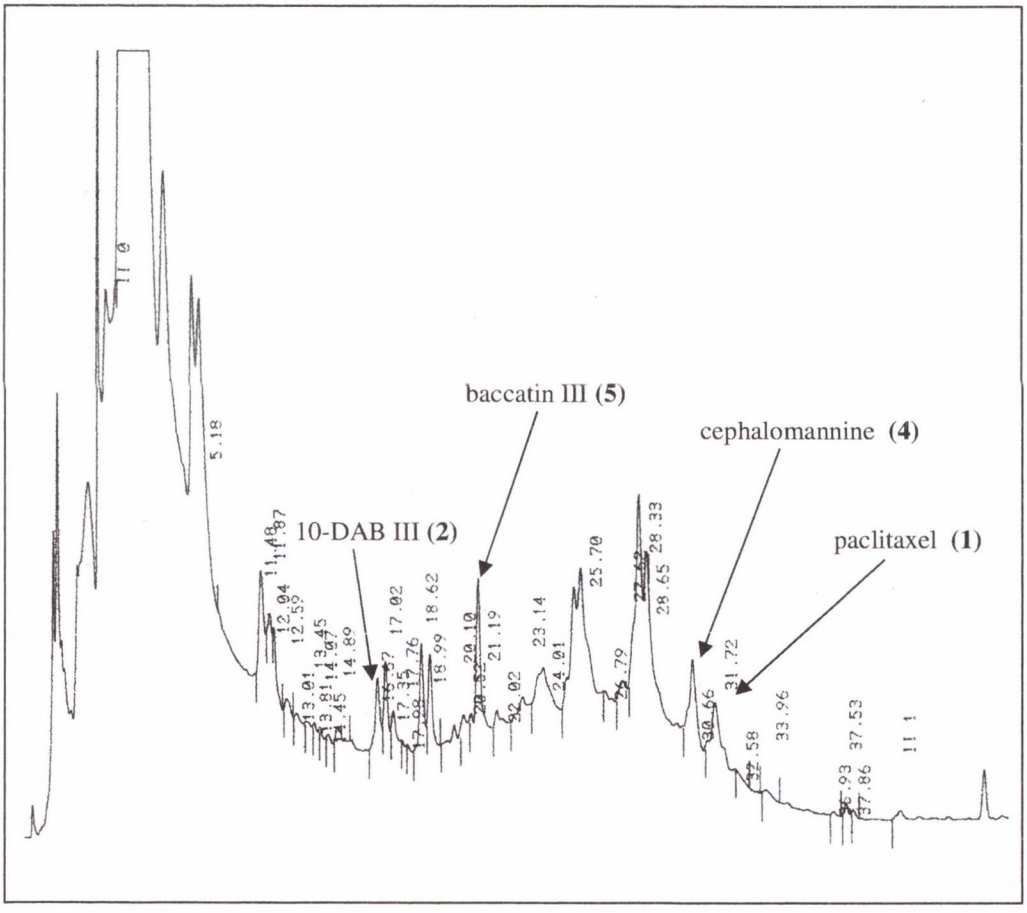


Figure 8.2.1.2 HPLC profile of taxane constituents in *T. b.* 'Fastigiata' cell cultures

8.2.2 Flavonoids

Taxanes are known to be increased by elicitation. During the elicitation experiments using *T. baccata* 'Fastigiata' cells tissue browning was observed in some cell lines (see

description of cell lines in section 8.1.3). Phenolic compounds produced as a response to the oxidative burst created in the event of an elicitation could be the reason for this tissue colouring. As mentioned in section 8.1.3, Cusidó et al. reported that this tissue-browning indicated an increase in phenolic compounds in the cells (Cusidó et al. 1999). Cells were therefore examined for phenolics, especially the phenylpropanoids – flavonoids. Flavonoids are part of the plant defence mechanism, and play an important role as scavengers of active oxygen species (AOS) formed during an oxidative burst resulting from elicitation of or pathogen attack on the plant (see Introduction, section 6.3.1).

HPLC analysis of a crude methanol extract prepared from *T. baccata* 'Fastigiata' cell extract (Cell Line 19) (see HPLC trace in **Figure 8.2.2.2**) showed that the major flavonoids present were catechin (**6**) and *epi*-catechin (**7**). Other minor flavonoids such as taxifolin (**10**), myricetin (**11**), quercetin (**8**), kaempferol (**9**) and amentoflavone (**12**) were also present. The minor flavonoids have all been isolated from the *Taxus* needles (Baczek et al. 2001, Parmar et al. 1999), however, to our knowledge, there is no report of isolation of these flavonoids from cells cultures of *Taxus* species.

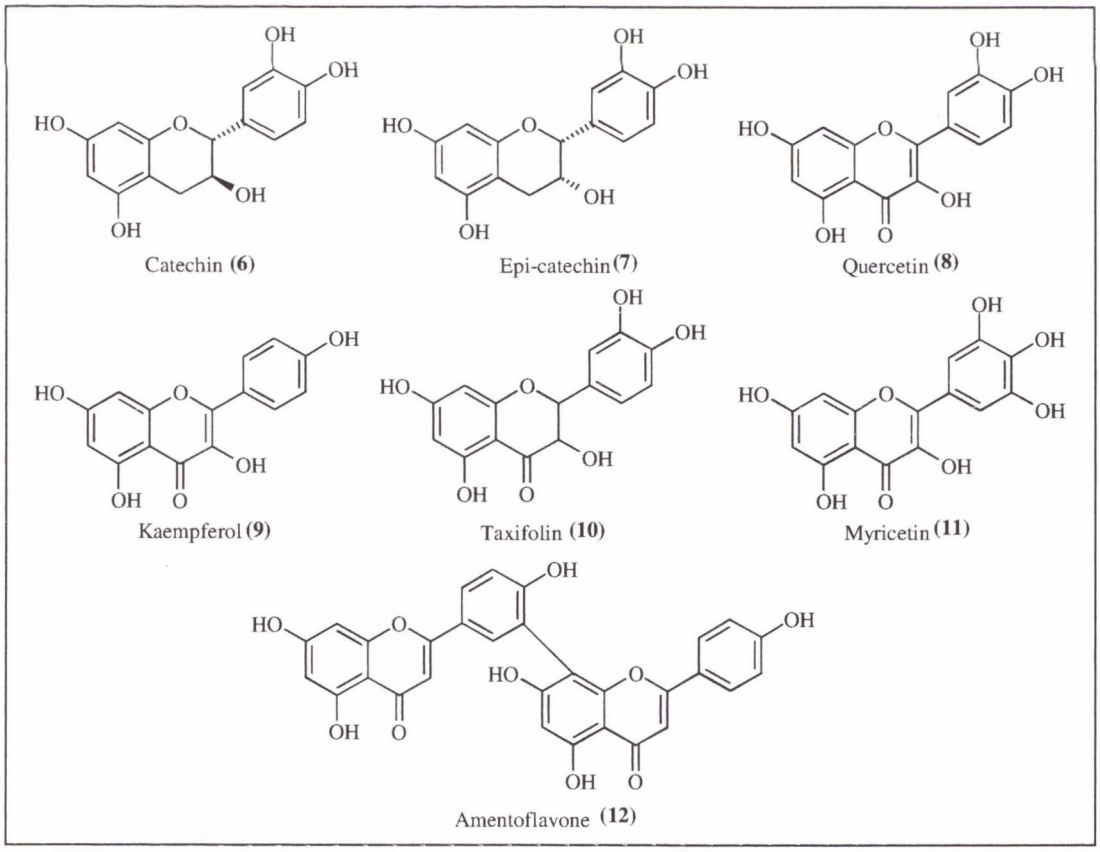


Figure 8.2.2.1 Structures of flavonoids analysed by HPLC

Catechin (**6**) was first isolated from the needles of *T. baccata* 'Fastigiata' by Dempsey (Dempsey 2000). Catechin has also been isolated from the stem wood and needles of other *Taxus* species (Chattopadhyay et al. 1999, Chen and Chen 1996, Kim et al. 1999). It was found to be the major phenolic constituent present in plant cell cultures of *T. chinensis* (Kim et al. 2002, Pyo et al. 2004) and (+)-catechin was isolated in the form of its penta-acetate from the callus cultures of *T. wallichiana* (Agrawal et al. 2003). To our knowledge there is no report of isolation of catechin and epi-catechin from cell cultures of *T. b.* 'Fastigiata'.

Flavonoids were identified by comparison to standards, both by HPLC and TLC. In order to confirm that catechin and *epi*-catechin were the major flavonoids in the extract, both were phytochemically isolated and the structures elucidated by nuclear magnetic resonance spectroscopy (NMR) and mass spectroscopy (MS) (see section 8.6.1).

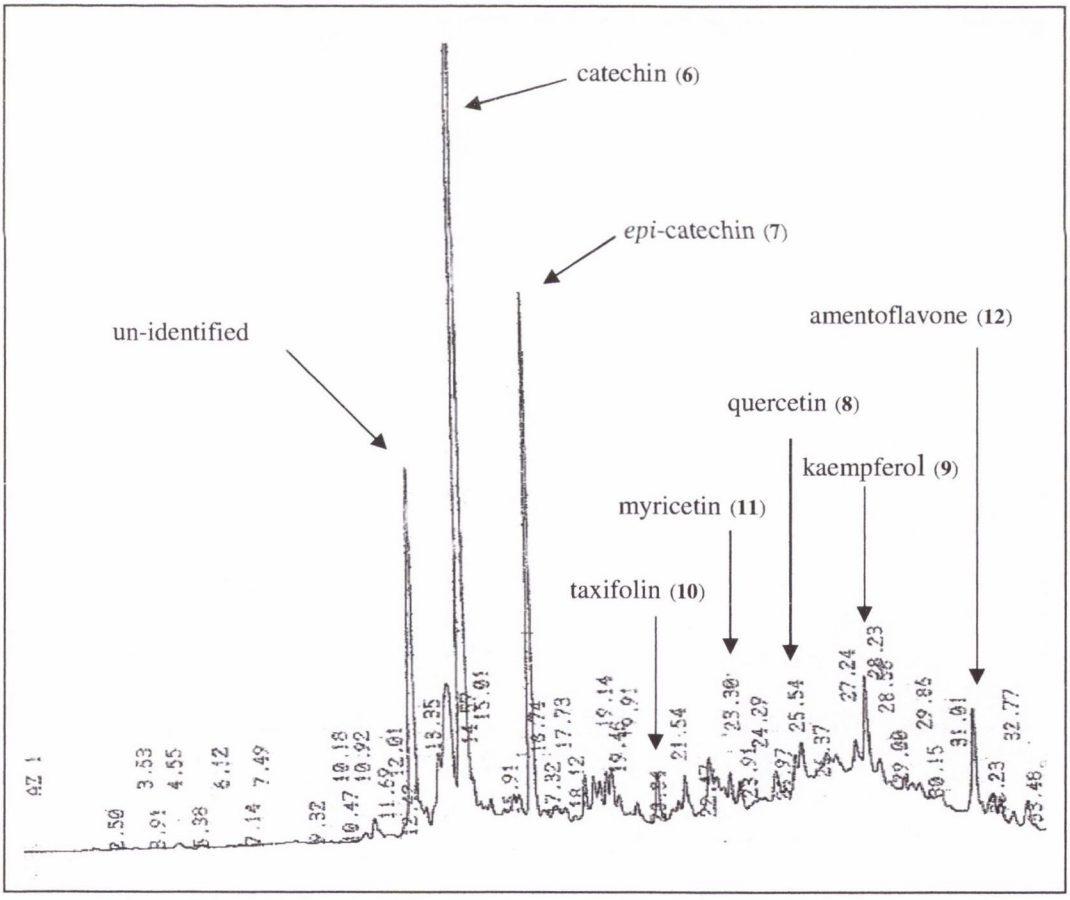


Figure 8.2.2.2 HPLC profile of flavonoid constituents from ethyl acetate extract of *Taxus baccata* 'Fastigiata' cell culture

8.3 Influence of Carbon Sources in Culture Medium on Taxane Yields

8.3.1 Taxanes

Plant cell cultures are usually grown heterotrophically using simple sugars as carbon sources. The effect of various sugars and their concentrations have been investigated by many researchers, hoping to optimise the production of secondary metabolites, including taxanes, in cell cultures (de Piava Neto and Otoni 2003, Kim et al. 1995, Kim et al. 2001, Wickremesinhe and Arteca 1994). The preferred carbon source for growth in plant cell cultures is sucrose. Increasing the sucrose concentration has been found to suppress cell growth but enhance the accumulation of secondary metabolites (Panda et al. 1992). This effect was also reported in cell cultures of *Taxus chinensis*, where a high initial sucrose concentration resulted in smaller cell aggregates, reduction in ratio of dry cell weight to fresh cell weight and high paclitaxel production (Kim et al. 2001).

Wickremesinhe and Arteca (Wickremesinhe and Arteca 1994) tested eleven different sugar regimes (consisting of sucrose, glucose and fructose) in order to optimise the growth index for *T.* X *media* 'Hicksii' cell suspension cultures. They found that three regimes (w/v): 8% sucrose; 4% fructose and 4%fructose + 4%glucose gave the highest growth index. Kim and co-workers (Kim et al. 1995) looked at the effect different sugars (sucrose, lactose, galactose, glucose, fructose, mannitol and sorbitol (all 8% w/v)) had on growth and paclitaxel production in *T. brevifolia* cell suspension cultures. They reported that contrary to expectation, the best growth was achieved with galactose. Sucrose gave the second best growth, but there was relatively little difference between the five sugars. The sugar alcohols mannitol and sorbitol are non-metabolic sugars, and these were not able to support cell growth. The highest level of paclitaxel was obtained with fructose (0.172 mg/l). This was considerably higher than the level obtained by the conventional sugar sucrose (0.012 mg/l). Even if mannitol and sorbitol gave poor biomass, paclitaxel was produced using them as the carbohydrate source (0.049 and 0.135 mg/l respectively).

Since fructose was found to give the highest paclitaxel production one objective in the present study was to examine what effect changing the sugar used in the growth medium from sucrose to inulin would have. Inulin is an uncharged polyfructose molecule with an average molecular weight of ~5000. The inulin used was in powdered form, and dissolved in water when heated. Two concentrations of inulin were used; Test 1 used 3% (w/v) inulin, and Test 2 used 1% (w/v) inulin. These were compared to a control medium with 3% sucrose as the carbohydrate source. The effects on the production of the taxanes 10-DAB III, cephalomannine and paclitaxel were analysed. The results are shown in **Figure 8.3.1.1** and in the corresponding **Table 8.3.1.1**.

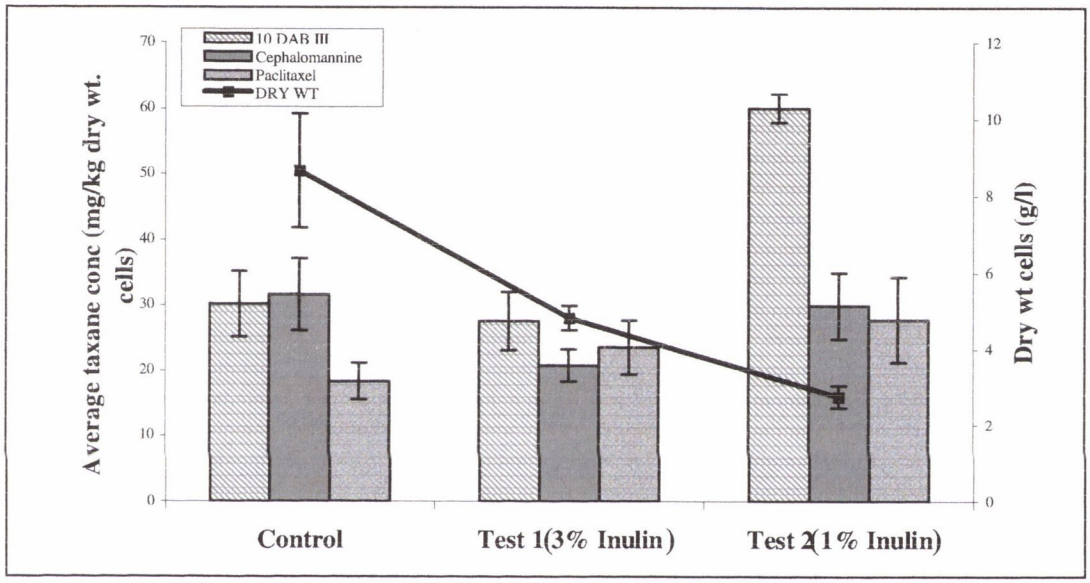


Figure 8.3.1.1 Effect of change of carbohydrate source on taxane production

Table 8.3.1.1 Effect of change of carbohydrate source on taxane production

Sample	Dry weight (g/l)	Growth Index	Taxanes (mg/kg dry wt. cells)		
			10-DAB III	Cephalomannine	Paclitaxel
Control	8.70 ± 1.50	3.43 ± 0.15	30.20 ± 5.00	31.64 ± 5.47	18.39 ± 2.81
Test 1: 3% inulin	4.80 ± 0.30	3.20 ± 0.18	27.61 ± 4.47	20.82 ± 2.46	23.61 ± 4.14
Test 2: 1% inulin	2.70 ± 0.30	1.73 ± 0.08	60.08 ± 2.15	29.99 ± 5.07	27.86 ± 6.61

From the growth index it is obvious that changing the carbohydrate source and concentration had a dramatic effect on the cell growth. The decrease in biomass was

nearly 70% lower in the Test 2 (1% inulin) formulation than the control. This strongly suggests that this low concentration of inulin will not support cell growth, and may lead to severe cell damage and ultimately death. However, the production of 10-DAB III was significantly enhanced ($P>0.001$) compared to that of the control. The effect on cephalomannine and paclitaxel was not significant due to high standard deviation, but the trend shows that there was an increase in paclitaxel accumulation. The effect on cell growth was not as severe using 3% inulin, where the reduction in biomass was only 40%. Inulin was therefore considered not to be a carbon source suitable for sustaining cell growth, although it had a positive effect on secondary metabolite production.

The change of sugars and sugar concentrations in the medium can have two fundamentally different effects on the physiology of the plant cells. The physical change in the cellular environment by changing the osmotic pressure could affect the cells. The other effect is the change in available carbohydrate sources as biochemical substrates. The osmotic contribution of the different carbon sources used should be taken into consideration when the effect the carbon sources have on cell cultures is analysed. The osmotic contribution values for the various carbon sources can be calculated using Van't Hoff's equation:

$$\psi_s = -CiRT$$

where **C** corresponds to the solute concentration (mol l^{-1}), **i** (Van't Hoff's constant) corresponds to the solute's ionisation constant, **R** is the gas constant (0.00831 kg $MPamol^{-1}$ K^{-1}) and **T** is the temperature (K).

Table 8.3.1.2 The osmotic potentials of the various carbon sources

Carbon source	Molecular weight	Concentration	Osmotic Potential (MPa)
Glucose	180.16	$30gl^{-1}$	-0.4120
Fructose	180.16	$30gl^{-1}$	-0.4120
Galactose	180.16	$30gl^{-1}$	-0.4120
Sucrose	342.00	$30gl^{-1}$	-0.2172
Lactose	360.31	$30gl^{-1}$	-0.2062
Mannitol	182.17	$30gl^{-1}$	-0.4078
Sorbitol	191.18	$30gl^{-1}$	-0.3884
Inulin	~5000	$30gl^{-1}$	-0.01486
Inulin	~5000	$10gl^{-1}$	-0.00495

It has been reported that a higher osmotic potential gives an enhancement in paclitaxel production (Kim et al. 2001). In that study the sucrose level was kept constant, so that the available carbohydrate sources used by the cells for biochemical substrates were constant. They then increased the osmotic pressure by addition of the non-metabolic sugar alcohol mannitol. The increase in osmotic pressure resulted in an increase in paclitaxel production.

In our experiment the osmotic pressure decreased significantly (hypoosmotic). The expected result of this would be a decrease in taxane accumulation, although an enhanced 10-DAB III production was found. The experiment was repeated using 1% inulin instead of 3% sucrose to see if the effect was similar, and the same result was obtained. This suggests that even if the medium is hypoosmotic, inulin, because it is a fructose polymer, can actually be metabolised by the cells, and therefore can promote taxane production. It has been suggested that taxanes are produced as defence metabolites by the plant (Ciddi et al. 1995, Yukimune et al. 1996). A possibility would therefore be that the cells produce 10-DAB III in response to the stress which the low osmotic pressure is putting the cells under.

8.3.2 Flavonoids

Oxidative burst is an early response in plant defence towards pathogens, but active oxygen species can also be induced by other stimuli, such as physical stress in the form of physical pressure, vigorous stirring and osmotic shock (Yahraus et al. 1995). When the cells of *Nicotiana tabacum* cv Xanthi were transferred to a hypoosmotic culture medium, significant production of H_2O_2 was observed (Cazalé et al. 1998). The AOS produced in hypoosmotic conditions accumulated in the extracellular medium. In contrast the cells transferred to isoosmotic (same osmotic pressure as original culture medium) and hyperosmotic (higher osmotic pressure than original culture medium) showed only weak H_2O_2 production.

This induction of an oxidative burst as a result of lowering the osmotic pressure in the cell culture medium could in turn induce the production of the protecting flavonoids. The flavonoid concentration was therefore analysed. The results are shown in **Table 8.3.2.1** and corresponding **Figure 8.3.2.1** below. Only catechin and epi-catechin were

present in quantifiable amounts in the Test 2 cells. As there were no quantifiable amounts of flavonoids present in the Test 1 cells or in the medium of the any of the test cells, no analyses are shown.

Table 8.3.2.1 Intracellular flavonoid production

Sample	Average Intracellular Flavonoid Content (mg/kg dry wt. cells)	
	Catechin	Epi-catechin
Control	4641.75 ± 328.92	3119.75 ± 460.93
Inulin (3%)	1984.63 ± 185.83	1187.20 ± 223.41

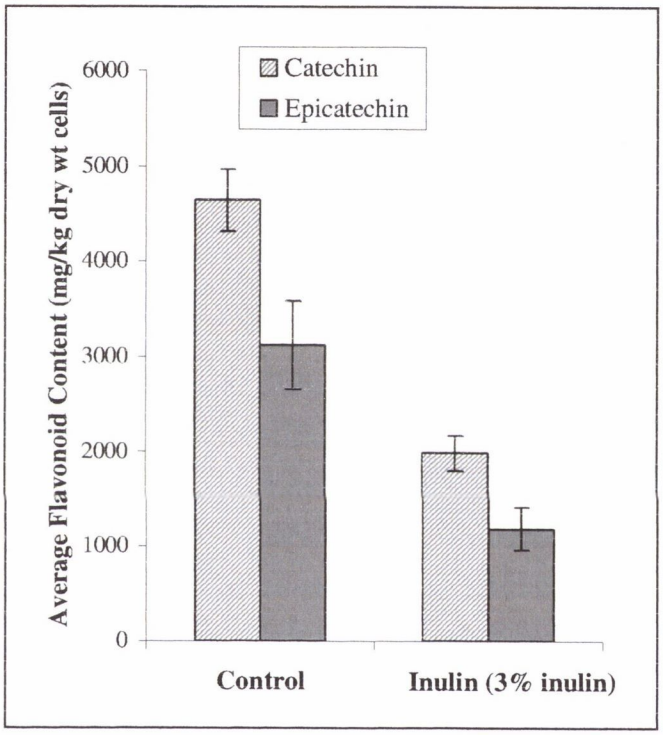


Figure 8.3.2.1 Flavonoid production, the effect of change of growth medium

The results show that changing the carbon source in the growth medium did not induce the cells to produce more flavonoids. The concentration of both catechin and epi-catechin was significantly lower ($P<0.001$) in the test cells than in the control cells. The possibility of an initial increase in the production of flavonoids should be taken into account, but this increase, however, was not reflected in the final results when cells were harvested after 36 days of growth.

8.4 Elicitation of Constituents by Induction of Oxidative Stress

8.4.1 Effect of molybdenum (Mo) on taxanes

Molybdenum (Mo) plays an essential role as the active centre in Mo-containing enzymes catalysing redox reactions in the metabolism of carbon, nitrogen and sulphur cycles (Hille 1996). Enzymes containing Mo are essential for diverse metabolic processes such as nitrate assimilation, phytohormone synthesis (Mendel and Schwarz 1999), sulphur detoxification and purine catabolism in mammals (Kisker et al. 1997). With the exception of nitrogenases that contain an iron-molybdenum-sulphur cluster, molybdenum is incorporated into proteins as the molybdenum-cofactor (Mo-co). Mo-co contains a mononuclear Mo atom coordinated to an organic cofactor named molybdopterin (Kisker et al. 1997).

An example of where molybdenum plays an important role in plant metabolism is in the reduction of nitrate (a major component in most plant tissue culture media) to ammonium. This reduction takes place in two main steps. Initially, nitrate is reduced to nitrite (NO_2^-). The reducing agent, which is gained during glycolysis, is NADH + H^+. The respective enzyme for this conversion is nitrate reductase. Secondly, nitrite is reduced into ammonia by the enzyme nitrite reductase (Bergfeld et al. 2003). The conversion of nitrate into nitrite occurs in the cytoplasm according to:

$$\mathbf{2e^- \rightarrow 2Cyt\ Fe(II/III) \rightarrow 2e^- \rightarrow 2Mo(V/VI) \rightarrow NO3^-/NO2^-}$$

Ammonium and ammonia ($NH_3 \rightarrow NH_4^+ + OH^-$) are toxic to plant cells and are rapidly incorporated into organic compounds like glutamine, asparagine, arginine, allantoin and betain. The production of ammonium compounds and nitrates is a limiting factor of plant growth (Duchefa 2003-2005).

The activity of the Mo-containing enzymes is strongly reduced during molybdenum deficiency, but can be quickly restored by adding molybdenum. It was decided to try different concentrations of molybdenum in the culture medium to see if there was any effect on growth and if this would affect the production of secondary metabolites such as the taxanes.

The 1902B growth medium used for the plant tissue suspension cultures originally contained 1.03μM molybdenum in the form of sodium molybdate (Na_2MoO_4). The Test 1 growth medium in this experiment contained an overall concentration of 42.4μM molybdenum (1mg extra molybdenum per 100ml medium), and the Test 2 growth medium contained an overall concentration of 208μM (5mg extra molybdenum per 100ml medium). These tests were compared to a control with the original molybdenum concentration. The results on growth and intracellular taxane accumulation can be seen in **Figure 8.4.1.1** and the corresponding **Table 8.4.1.1**.

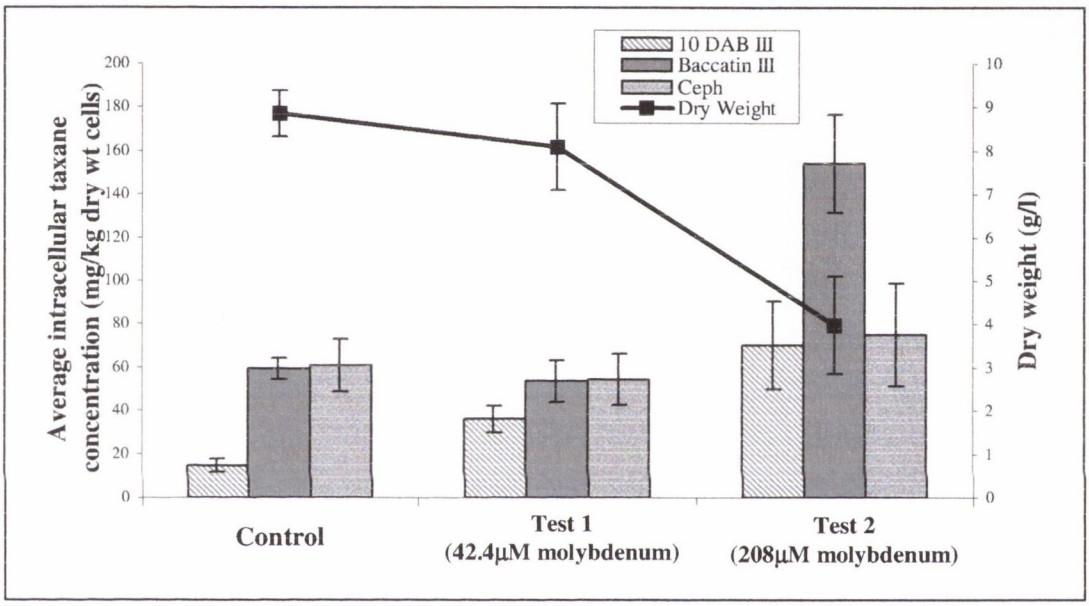


Figure 8.4.1.1 Effect of molybdenum on growth and taxane accumulation

As can be seen from the graph (**Figure 8.4.1.1**) and the corresponding **Table 8.4.1.1** it is evident that the concentration of molybdenum had an effect on the growth of the cells, reducing the biomass in Test 2 by more than half ($P<0.001$). No difference between the control and Test 1 was found. Cell growth cannot therefore be sustained in the highest concentration of molybdenum. However, there was an obvious increase in taxane accumulation in the Test 2 cells, where the production of 10-DAB III and baccatin III increased significantly ($P<0.001$). No effect was found on cephalomannine and paclitaxel production, of which only trace amounts were detected.

Table 8.4.1.1 Effect of molybdenum on growth and taxane accumulation

Sample	Dry weight (g/l)	Intracellular taxanes (mg/kg dry wt. cells)		
		10-DAB III	Baccatin III	Cephalomannine
Control (1.03 μM molybdenum)	8.90 ± 0.50	14.48 ± 4.87	59.29 ± 4.87	61.06 ± 12.23
Test 1 (42.4μM molybdenum)	8.10 ± 1.00	36.01 ± 6.27	53.88 ± 9.70	54.64 ± 11.69
Test 2 (208μM molybdenum)	4.00 ± 1.10	70.57 ± 20.19	154.22 ± 22.38	75.32 ± 23.71

The effect on extracellular taxane content in the medium is shown in **Figure 8.4.1.2** and the corresponding **Table 8.4.1.2**.

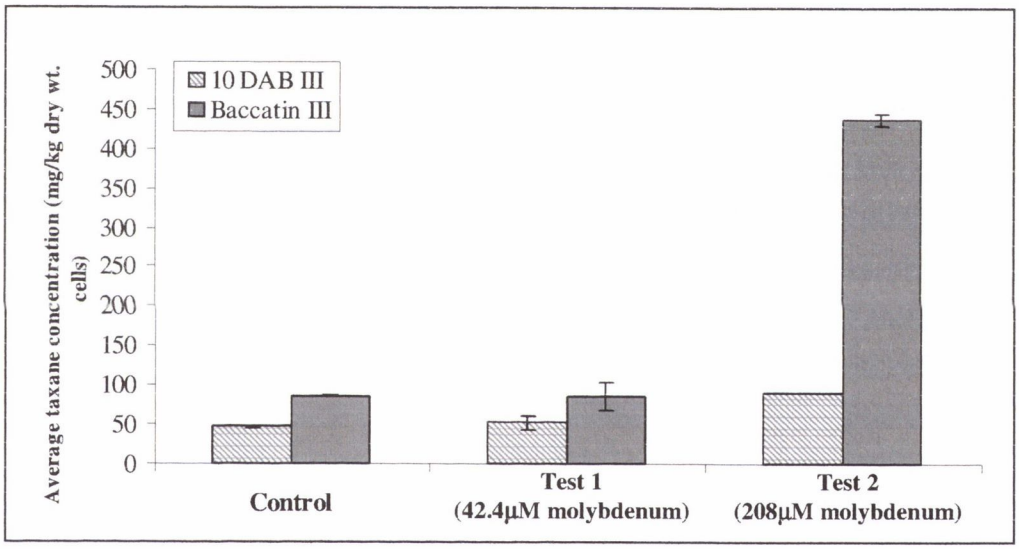


Figure 8.4.1.2 Effect of molybdenum on extracellular taxane production

Taxanes were found in significant amount only with the Test 2 concentration ($P<0.001$). The enhancement in baccatin III concentration was particularly high; where a five-fold greater concentration was found when compared to the control. This was probably due to cell lysis, since the high concentration of molybdenum was found to have a very negative effect on the cells physiology. Only trace amounts of cephalomannine and paclitaxel could be extracted from the medium.

Table 8.4.1.2 Effect of molybdenum on extracellular taxane production

Sample	Extracellular taxanes (mg/kg dry wt. cells)	
	10 DAB III	Baccatin III
Control (1.03μM molybdenum)	47.19 ± 1.28	86.33 ± 0.44
Test 1 (42.4μM molybdenum)	52.40 ± 8.92	86.45 ± 17.45
Test 2 (208μM molybdenum)	90.33 ± 0.80	437.17 ± 6.38

From the results we can conclude that the highest concentration of molybdenum (208μM) is not a suitable concentration for sustaining cell growth. The increase in taxane accumulation is probably a response due to the high stress level the cells were put under, and the increase in excretion could be due either to cell lysis or physiological changes in the cell structure. The concentration used in the Test 1 growth medium (42.4μM) is a more suitable concentration, allowing growth to occur but not significantly affecting taxane accumulation and excretion.

Molybdenum is a transition metal. Transition metals are known to participate in the production of reactive oxygen species. A typical example is the formation of $^{\bullet}OH$ from H_2O_2 in the presence of the transition metals iron and copper in a reaction called the Fenton reaction (Halliwell and Gutteridge 1999). The rare earth ions lanthanum (La^{3+}) and cerium (Ce^{4+}), which possess many of the same properties as transition metals, have been used as elicitors for enhancement of paclitaxel (Wu et al. 2001, Yuan et al. 2002d). It was found that Ce^{4+} could induce apoptosis in suspension cultures of *T. cuspidata* (Yuan et al. 2002a). However, they also found that by adding Ce^{4+} an oxidative burst was induced, followed by enhancement of paclitaxel production (Yuan et al. 2002d). Molybdenum added to the *T. b.* 'Fastigiata' cell cultures in the present study could likewise have induced an oxidative burst leading to the enhanced production of both 10-DAB III and baccatin III. Wu and co-workers (Wu et al. 2001) found that La^{3+} had a dose-dependent effect on the growth of *Taxus* cell cultures. The lower concentrations promoted cell proliferation, whereas the higher concentrations had a toxic effect on the cell growth. This effect is similar to the effect heavy metal ions, such copper (Cu), cadmium (Cd), cobalt (Co), zinc (Zn) and nickel (Ni), have on plant cells (Vangronsveld and Clijsters 1994, Winge et al. 1998). It is also similar to the effect observed after addition of molybdenum in the present study.

However, no promotion of cell growth was observed as a result of the low concentration of molybdenum in Test 1. From a commercial viewpoint the high taxane content would not transfer into an increased yield due to the poor biomass production.

8.4.2 Effect of *L*-arginine on taxanes

In mammalian cells nitric oxide (NO) is formed directly from the guanidine nitrogen of the *L*-arginine by nitric oxide synthase (NOS) (Rockel et al. 2002). Nitric oxide (NO) acts as a key signalling molecule in vertebrates, where it has numerous biological functions. Despite conflicting data for the existence of NOS in plants there is growing evidence that NO also plays an important role as a signalling molecule in higher plants (Neill et al. 2003). A pathogen-inducible nitric oxide synthase (iNOS) was isolated from the tobacco plant and characterised (Chandok et al. 2003). Guo and co-workers then isolated a hormone-activated NOS (AtNOS1) (Guo et al. 2003). However, nitrate reductase also has been found to have the capacity to generate nitrous oxide (NO). This activity in plants has been demonstrated *in vitro* (Yamasaki and Sakihama 2000) and *in vivo* (Rockel et al. 2002).

It was decided to see if the addition of *L*-arginine to the growth medium of *Taxus baccata* 'Fastigiata' cell suspension cultures could influence cell growth and taxane accumulation. Three concentrations (0.25mM, 1mM and 2.5mM) of *L*-arginine were added to the growth medium prior to autoclaving, and these three tests were compared to a control lacking *L*-arginine. The cells were grown for 35 days before harvesting. The results are shown in **Figure 8.4.2.1** and the corresponding **Table 8.4.2.1**.

The highest concentration (2.5mM) of *L*-arginine added to the growth medium had an obvious effect on the growth of the cells resulting in a statistically significant decrease in biomass production ($P<0.001$). The two other concentrations (0.25mM and 1mM) did not affect the growth significantly.

The taxane accumulation was negatively affected by the addition of *L*-arginine with a significant decrease for 10-DAB III ($P<0.001$), cephalomannine ($P<0.001$) and paclitaxel ($P<0.001$).

Table 8.4.2.1 Effect of *L*-arginine on taxane accumulation and growth

Sample	Dry weight (g/l)	Taxane production (mg/kg dry weight)		
		10 DAB III	Cephalomannine	Paclitaxel
Control	4.60 ± 0.20	60.93 ± 8.04	14.89 ± 2.59	5.79 ± 0.08
T1 0.25mM	4.60 ± 0.10	39.54 ± 4.47	7.16 ± 0.27	2.27 ± 0.00
T2 1.00mM	4.20 ± 0.30	45.24 ± 4.43	4.88 ± 0.77	1.18 ± 0.38
T3 2.50mM	2.90 ± 0.80	27.50 ± 3.91	5.48 ± 0.31	1.01 ± 0.43

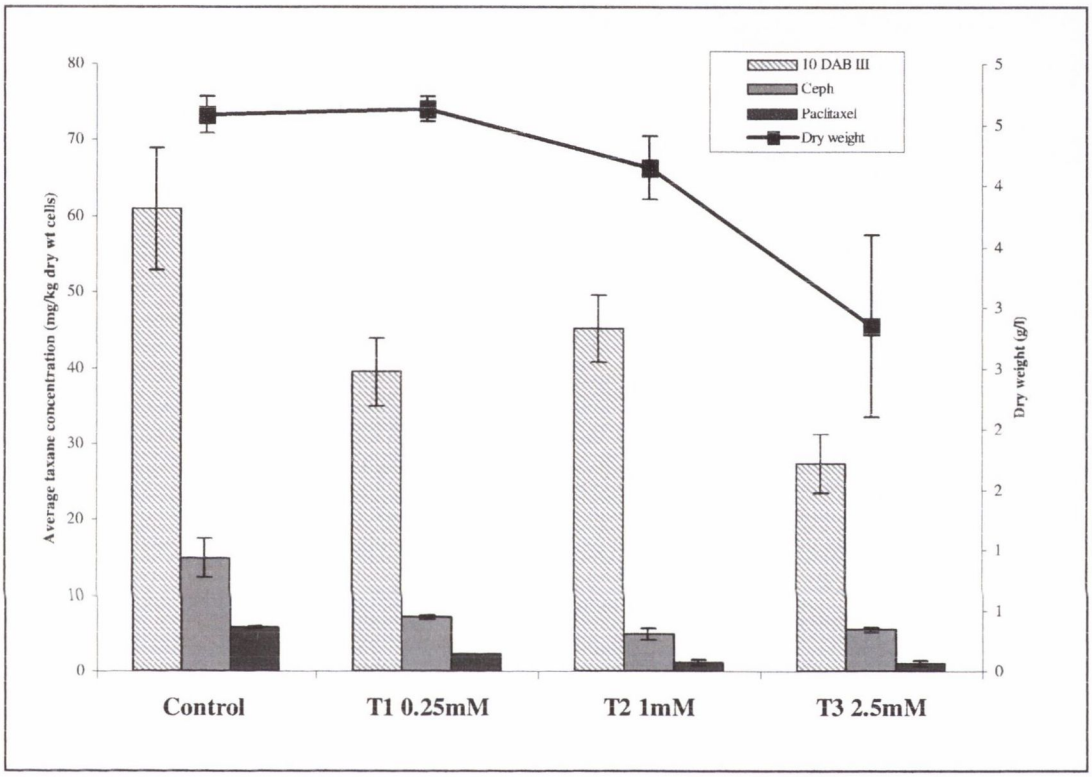


Figure 8.4.2.1 Effect of *L*-arginine on taxane accumulation and growth

Conclusion

At the concentrations used, no enhancement of growth or taxane yields was found. The high concentration of *L*-arginine is probably toxic to the cells, inducing the hypersensitive response, which in turn will lead to cell death. Another possible explanation could be that the high concentration of *L*-arginine indeed resulted in an enhanced NO production, but that the higher concentration of NO induced cell death.

Pedroso and co-workers (Pedroso et al. 2000) concluded that the hypothesis, that *Taxus* cell death under stressful conditions (mechanical stress) is preceded by an

increase of NO, was true. They did not supply *L*-arginine to the cells; instead they added the NO-donor sodium nitroprusside (SNP) to determine if NO artificially induces cell death. They found that the cell death was prevented by addition of NOS inhibitors. This suggests that there are NOS-like enzymes present in *Taxus brevifolia* cells. Wang and Wu (Wang and Wu 2004) observed that elicitor-induced H_2O_2 was suppressed by NO inhibitors suggesting that NO plays a regulatory role in activating the oxidative burst in cells.

If the high concentration of *L*-arginine resulted in enhanced NO production this could in turn lead to an overproduction of H_2O_2 causing detrimental effects to the cells. An oxidative burst is necessary but not sufficient to trigger a hypersensitive response (HR) leading to host cell death in plants. It has been proved that nitric oxide (NO) cooperates with the active oxygen species (AOS) in the activation of HR (Clarke et al. 2000, Delledonne et al. 2002, Durner et al. 1998). NO appears to activate this defence response through a salicylic acid (SA)-dependent pathway. It has also been suggested that NO plays a role in the wounding/jasmonic acid (JA) signalling pathway (Huang et al. 2004, Jih et al. 2003, Orszco-Cárdenas and Ryan 2002) (see Introduction, section 6.3.5).

8.4.3 Effect of methyl jasmonate on taxanes

Jasmonic acid and its derivatives have played a fundamental role in the intracellular signal cascade involved in the accumulation of secondary metabolites in plant cell cultures as a result of exposure of the cells to an elicitor or a pathogen (Gundlach et al. 1992). Yukimune and co-workers (Yukimune et al. 1996) reported the effects of methyl jasmonate and its analogues on the accumulation of paclitaxel and related taxanes in cell suspension cultures of *Taxus* x *media*, *T. baccata*, and *T. brevifolia*. It was found that methyl jasmonate in all the three cases strongly promoted taxane biosynthesis. Since then other researchers have reported similar effects in cell cultures of *T. cuspidata* (Mirjalili and Linden 1996), *T. canadensis* (Ketchum et al. 1999a), and *T. chinensis* (Dong and Zhong 2001, Menhard et al. 1998, Wang et al. 2004).

According to Ketchum and co-workers (Ketchum et al. 1999a) the best concentration of methyl jasmonate, for elicitation of paclitaxel in suspension cultures of *T.*

canadensis was 200μM. This gave a three-fold increase in paclitaxel production compared to the production by non-elicited cultures. In their experiment the methyl jasmonate was added to the cells halfway through the growth cycle. Earlier Yukimune and co-workers and Mirjalili and Linden (Mirjalili and Linden 1996, Yukimune et al. 1996) added the methyl jasmonate at the time of inoculation. However, Ketchum found that addition of methyl jasmonate at the time of inoculation gave a lower yield of paclitaxel than when added halfway through the cycle. The reasons for this could be (i) use of a different *Taxus* species, (ii) the addition of the elicitor at a later stage would allow the cells to recover from the stress of inoculation, (iii) the cells would be at a stage in their growth cycle when they had a larger biomass, greater taxadiene synthase activity (Hezari and Croteau 1997), and probably a greater pool of taxoid precursors.

In this elicitation experiment the final concentration of methyl jasmonate in each of the flasks was 200μM. The methyl jasmonate was added on day 21 in the growth cycle, and the cells were harvested on day 30. The parameters recorded such as subculture weight, fresh weight, dry weight and pH measurements can be found in **Appendix CD**.

In our experiment the addition was not halfway through the growth cycle, but it was at the time found to give maximum biomass production, and also in the phase just before the highest accumulation of taxanes. This would according to Hezari and co-workers (Hezari et al. 1997) be the time when there is a rise in taxadiene synthase activity, which would be a critical enzyme in the biosynthesis of taxanes.

8.4.3.1 Taxanes

The cells used in our experiment were found to produce only traces of paclitaxel. They did, however, produce structurally related taxanes such as 10-DAB III, baccatin III, and cephalomannine. These taxanes were therefore quantified, and the increase in taxane production after elicitation with methyl jasmonate was measured. Since MJ was dissolved in ethanol, a control for this solvent was included in the experimental design. As can be seen from the graph (**Figure 8.4.3.1**) and the corresponding table (**Table 8.4.3.1**) there was a significant decrease in taxane production as a result of the addition of ethanol. Since it was reported by Wang and co-workers (Wang et al. 2002)

that a concentration higher than 1% ethanol would cause biological changes in *T. cuspidata* cells, and ultimately cell death, the concentration of ethanol in each of the flasks was not more than 1%. Even with a concentration less than 1%, the ethanol appeared to have a detrimental effect on the cells, although they still produced taxanes.

Table 8.4.3.1 Average intracellular taxane production

Sample	Growth Index	Intracellular taxanes (mg/kg dry wt. cells)		
		10-DAB III	Baccatin III	Cephalomannine
Control	2.06 ± 0.06	7.73 ± 1.94	23.14 ± 7.71	51.18 ± 13.50
Control + EtOH	1.93 ± 0.04	1.60 ± 0.85	7.37 ± 4.27	12.81 ± 2.69
Methyl jasmonate	1.48 ± 0.28	41.96 ± 8.28	55.15 ± 5.87	115.74 ± 33.83

There was a statistically significant ($P<0.01$) increase in the content of the three taxanes in the elicited cells compared to that of the controls. The elicited cells had a 5.43 times higher content of 10-DAB III ($P<0.001$). There was a two-fold increase in both baccatin III and cephalomannine content ($P<0.001$ and $P<0.01$ respectively).

These results only show the intracellular taxanes produced by the cells. Wang and co-workers (Wang et al. 2004) showed that the extent of taxane excretion was enhanced with methyl jasmonate elicitation. The same effect was also reported by Wu and co-workers when *T. yunnanensis* cells were elicited by lanthanum nitrate at different concentrations (Wu et al. 2001). This increase in extracellular taxane concentration was originally thought to be attributed in part to cell lysis caused by addition of elicitors. However, Fornalé and co-workers (Fornalè et al. 2002) suggested that *Taxus* cells have an ATP-dependent "taxane transport system" that excretes 55-60% of taxanes to the medium.

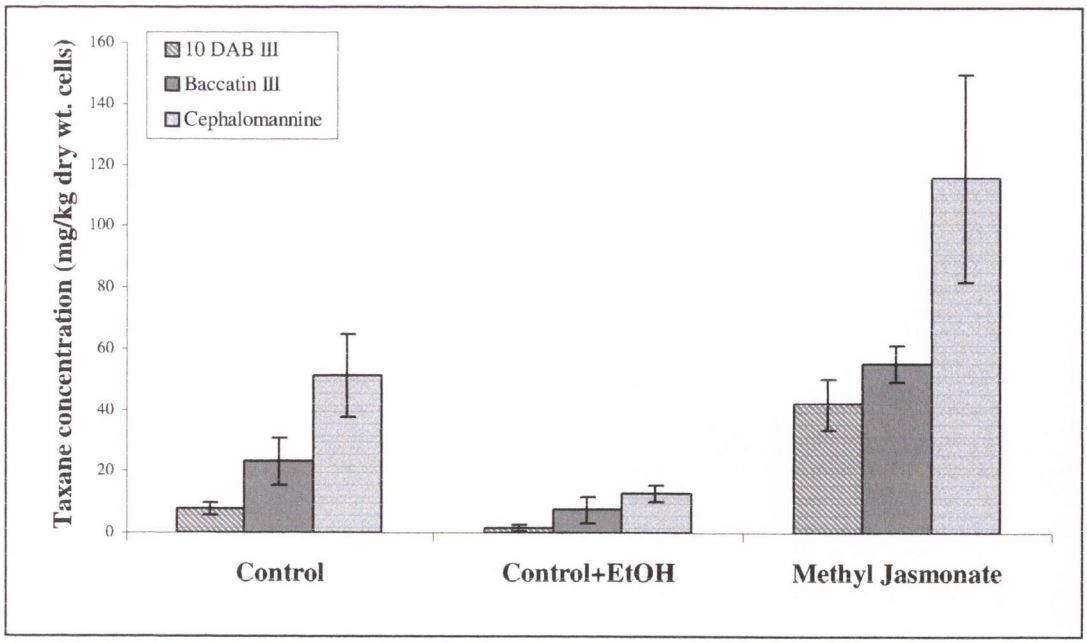


Figure 8.4.3.1 Effect of methyl jasmonate on intracellular taxane accumulation

Figure 8.4.3.2 and **Table 8.4.3.2** show the excretion of taxanes into the growth medium. The results show that there was a very statistically significant increase in 10-DAB III and baccatin III excretion ($P<0.01$ in both cases) in the elicited cultures compared to that of the control cultures. The increase in cephalomannine was less significant ($P<0.05$).

From the growth index it is apparent that methyl jasmonate did have a slight effect on cell growth in the cultures, where there was a decrease in cell growth in the flasks elicited compared to the controls. However, the effect was not statistically significant due to the low number of replicates (n=4) giving a high standard deviation. It has been reported that an increase in paclitaxel or taxanes resulted in a decrease in biomass (Laskaris et al. 1999, Yukimune et al. 1996).

Table 8.4.3.2 Extracellular taxane production

Sample	Growth Index	Extracellular taxanes (mg/kg dry wt. cells)		
		10-DAB III	Baccatin III	Cephalomannine
Control	2.06 ± 0.06	54.67 ± 30.44	113.77 ±32.97	7.93 ± 4.51
Control + EtOH	1.93 ± 0.04	25.78 ± 7.55	30.85 ± 6.01	11.83 ± 7.85
Methyl jasmonate	1.48 ± 0.28	172.47 ± 53.63	245.01 ± 58.30	40.09 ± 15.42

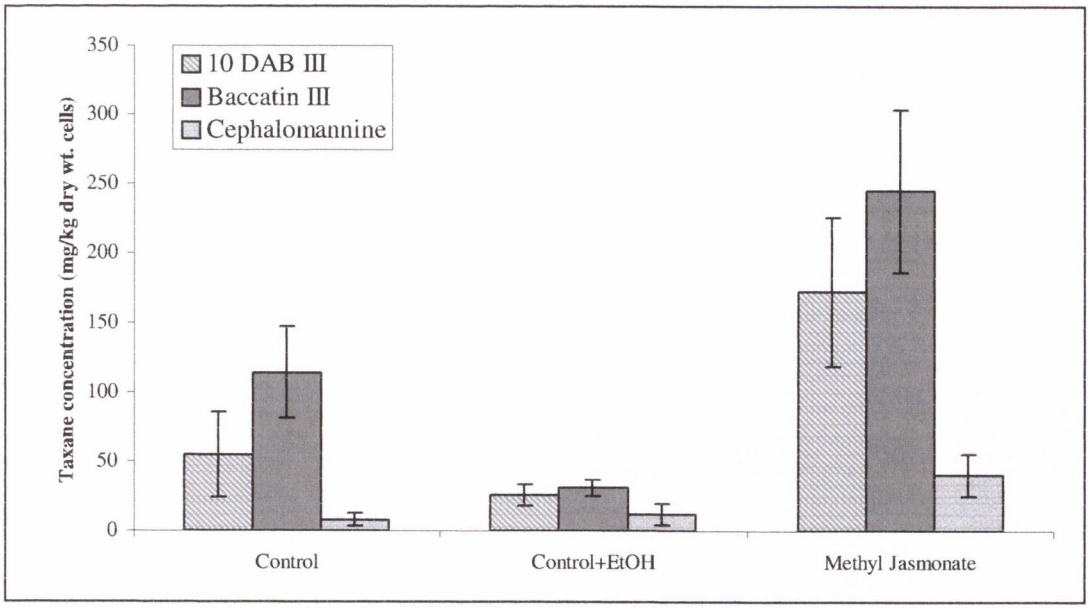


Figure 8.4.3.2 Effect of methyl jasmonate on extracellular taxane accumulation

When the intracellular and the extracellular taxane production were combined to give a total value, one can see a significant increase ($P<0.0001$) in total taxane production in the elicited cultures, where 10-DAB III had a three-fold increase, baccatin III had a two-fold increase and cephalomannine had a three fold increase (**Figure 8.4.3.3** and corresponding **Table 8.4.3.3**).

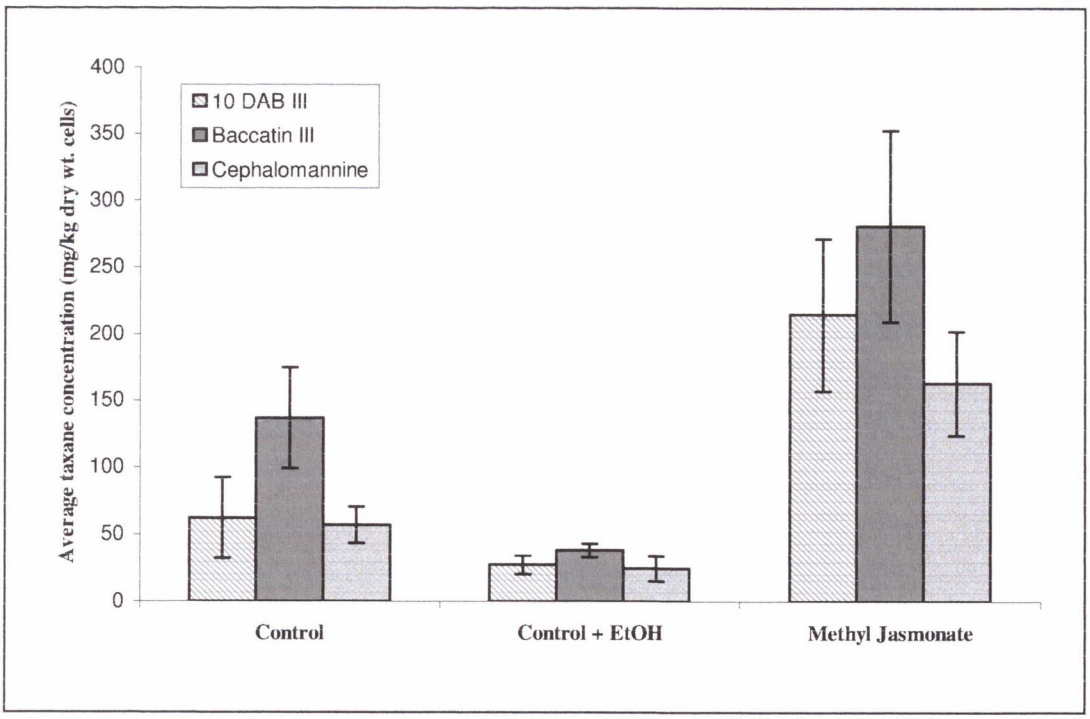


Figure 8.4.3.3 Effect of methyl jasmonate on total taxane production

Table 8.4.3.3 Effect of methyl jasmonate on total taxane production

Sample	Growth Index	Total taxanes (mg/kg dry wt. cells)		
		10-DAB III	Baccatin III	Cephalomannine
Control	2.06 ± 0.06	62.40 ± 30.08	136.91 ± 37.78	57.13 ± 13.61
Control + EtOH	1.93 ± 0.04	27.38 ± 6.84	38.25 ± 4.96	24.64 ± 9.33
Methyl jasmonate	1.48 ± 0.28	214.43 ± 57.13	280.90 ± 72.06	163.23 ± 39.05

Conclusion

These results show that methyl jasmonate can stimulate the synthesis and excretion of taxanes in our suspension cultures of *Taxus baccata* 'Fastigiata'. Wang and co-workers found that methyl jasmonate induced biosynthesis of paclitaxel, but had no effect on cephalomannine production in cell cultures of *T. chinensis*. In the same study they reported that salicylic acid promoted the biosynthesis of cephalomannine, whereas it had no influence on paclitaxel biosynthesis (Wang et al. 2004). In the present study methyl jasmonate did enhance the biosynthesis and excretion of cephalomannine with a three-fold increase. However, the biosynthesis of paclitaxel itself was not enhanced.

Ketchum (Ketchum et al. 1999a) reported that the elicitation of paclitaxel by methyl jasmonate is cell line specific, i.e. not all cell lines responded to elicitation by increasing paclitaxel production. They also found that the month-to-month production of paclitaxel by their cell lines fluctuated even after elicitation with methyl jasmonate. Bonfill et al. (Bonfill et al. 2003) examined the effect of three different elicitors on the taxane production in *Taxus media* cells. They found that the maximum contents of the various taxanes analysed was not on the same day in the growth cycle. Baccatin III reached a maximum yield on day 16 after elicitation with methyl jasmonate, while paclitaxel had a maximum yield on day 24. 10-DAB III reached a maximum yield on day 24 when the cells were elicited using vanadium sulphate, and cephalomannine had a maximum on day 24 when arachidonic acid was used as the elicitor. It is obvious therefore that different *Taxus* cultures respond to elicitors in different ways, i.e. cell line specific. It is also important to keep in mind the biosynthetic pathway of the taxanes (see General Introduction, section 1.2.2), where 10-DAB III and baccatin III

are non-side chain taxanes synthesised before the side chain addition. Cephalomannine and paclitaxel, which both contain the phenylalanine-derived side chain, are synthesised downstream of 10-DAB III and baccatin III (Walker and Croteau 2001). After elicitation with methyl jasmonate, 10-DAB III and baccatin III were found to peak around day 12, whereas cephalomannine and paclitaxel peaked around day 20-25 in cultures of *T. cuspidata* (Naill and Roberts 2005).

8.4.3.2 Flavonoids

The effect on flavonoid production by addition of methyl jasmonate (200mM) to the cell cultures are shown in **Figure 8.4.3.4** and in the corresponding **Table 8.4.3.4.**

Table 8.4.3.4 Intracellular flavonoid production

Sample	Intracellular Flavonoid Content (mg/kg drywt. cells)				
	Catechin	Epicatechin	Taxifolin	Quercetin	Kaempferol
Control	4452.92 ± 359.41	2880.53 ± 473.32	154.17 ± 22.44	66.88 ± 10.16	48.76 ± 10.10
Control + EtOH	3407.54 ± 675.74	2067.62 ± 105.99	129.31 ± 19.27	111.47 ± 65.69	105.72 ± 71.95
Methyl Jasmonate	2667.84 ± 337.29	541.19 ± 77.88	33.14 ± 6.97	376.52 ± 71.33	64.95 ± 6.97

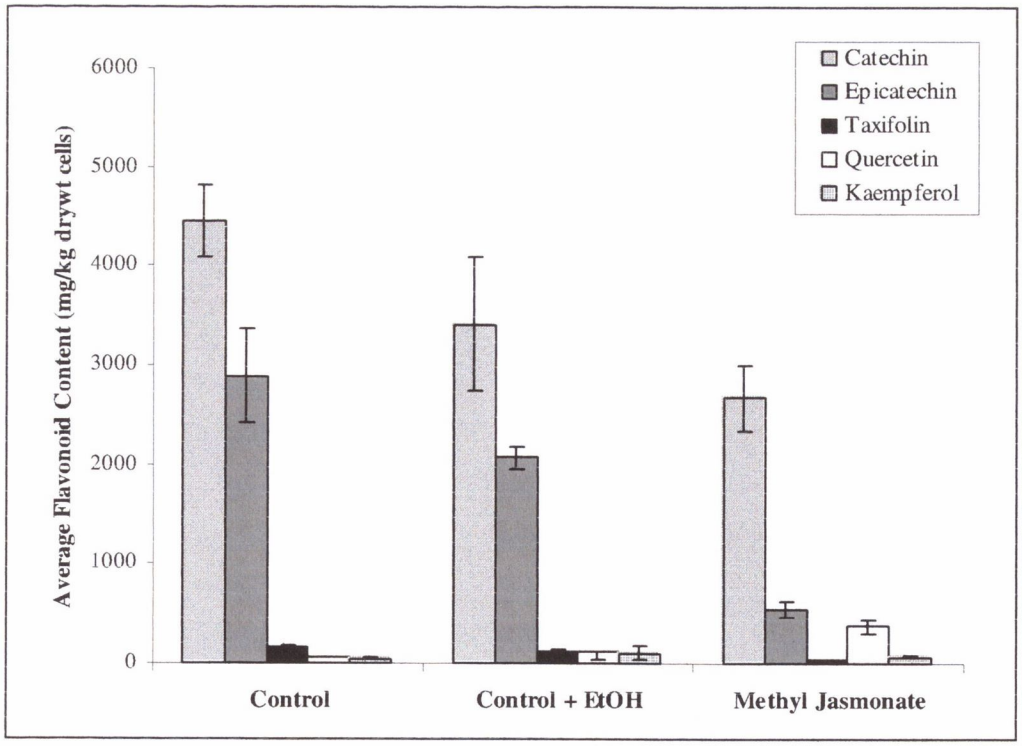


Figure 8.4.3.4 Effect of methyl jasmonate on production of intracellular flavonoids

In these cells only catechin, epi-catechin, taxifolin, quercetin and kaempferol were detected. As can be seen from the graph (**Figure 8.4.3.4**), there was a significant decrease in the catechin and epi-catechin content of the cells when treated with methyl jasmonate ($P<0.001$). Addition of ethanol also led to a significant reduction ($P<0.001$) in catechin and epi-catechin content in the cells when compared to control cells. The concentration of taxifolin was only slightly lower in the control + EtOH cells than it was in the overall control cells ($P<0.05$). Treatment of the cells with the elicitor resulted in a highly significant reduction in taxifolin ($P<0.001$). For quercetin there was no significant difference between the two types of control cells. The concentration of this flavonoid in the treated cells was considerably higher than that found in the control cells ($P<0.001$). Quercetin was the only flavonoid to have been enhanced by elicitation with methyl jasmonate. There was no significant difference, in the concentration of kaempferol.

The amounts of extracellular flavonoids in the liquid culture medium were also analysed. The results of this analysis can be seen in **Table 8.4.3.5** and in the corresponding **Figure 8.4.3.5**.

Table 8.4.3.5 Effect of methyl jasmonate on extracellular flavonoid production

Sample	Extracellular Flavonoid Content (mg/kg dry wt cells)		
	Catechin	Epi-Catechin	Taxifolin
Control	7.69 ± 1.69	12.08 ± 2.45	128.69 ± 7.64
Control + EtOH	16.56 ± 6.74	13.40 ± 3.13	111.78 ± 11.95
Methyl Jasmonate	25.68 ± 7.52	38.02 ± 3.43	172.60 ± 17.47

From the results it is clear that the amounts of flavonoids present in the medium was much lower than that found in the cells, i.e. very little flavonoids were excreted into the medium. The only quantifiable flavonoids were catechin, epi-catechin and taxifolin. The major difference between the intracellular and the extracellular profiles was that taxifolin was the major flavonoid in the extracellular analysis. There was no significant difference between the taxifolin content in the overall control and the cells treated with methyl jasmonate. The taxifolin content in the control + EtOH cells was lower than in the overall control and the MJ treated cells ($P<0.001$). The catechin and the epi-catechin levels in the medium of the elicitor treated cells were significantly

higher than in the medium of the control cells ($P<0.001$), but only in the case of epi-catechin was it statistically higher than that of the control + EtOH medium.

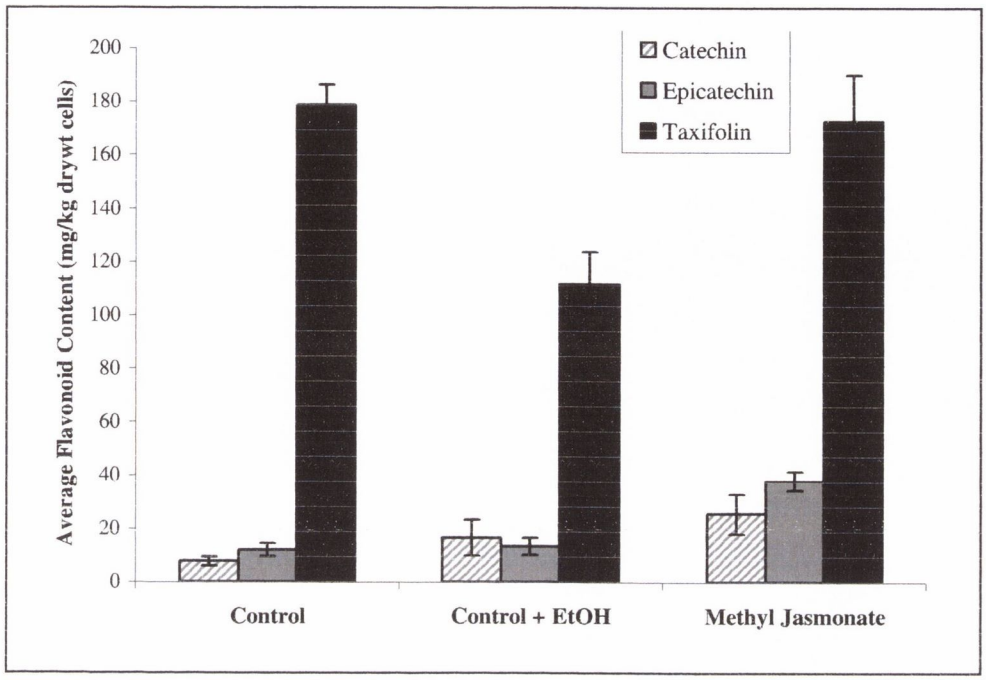


Figure 8.4.3.5 Effect of methyl jasmonate on extracellular flavonoid production

Conclusion

The results of the flavonoid analysis did not confirm the theory that there are more phenolics of the flavonoid type in the cells that have been treated with an elicitor. Both in the extracellular and the intracellular analysis the control cells had a significantly higher content of the various flavonoids. Flavonoids are synthesised by the plant as a response to pathogen attack or stress situations. It could be suggested that the maximum flavonoid content could occur earlier in the growth cycle. Our cells were harvested 9 days after the addition of the elicitor. However, Wu and Li (Wu and Lin 2003) found that almost immediately after (1-2 days) addition of methyl jasmonate to *Taxus chinensis* cells there was an increased intracellular activity of phenylalanine ammonia lyase (PAL), the key enzyme in the initial step of the phenylpropanoid pathway responsible for the synthesis of most plant phenolics (see section 6.3.2).

8.4.4 Effect of *tert*-butyl hydroperoxide (tBH) on taxanes

As was noted seen in the introduction (section 6.3.1) AOS is a rapid response by the plants to a pathogen or an elicitor. Yu et al. and Yuan et al. found that the oxidative burst in suspension cultures of *T. chinensis* induced by a fungal oligosaccharide extracted from *Fusarium oxysporum* is generally accompanied by enhanced taxane production (Yu et al. 2002, Yuan et al. 2001, Yuan et al. 2002b, Yuan et al. 2002c, Yuan et al. 2002d). The oligisaccharide was added to the cell cultures at the late exponential stage (day 18) and the cells were collected at predetermined time intervals (typically 24, 48, 72 hours after addition) for analysis. The PAL activity and phenolics production was monitored at regular time intervals from time of addition (15, 30, 45, 60 and 90 minutes). Further investigation by Yuan et al (Yuan et al. 2001) concluded that an oxidative burst is actually necessary for the taxane accumulation.

Tert-butyl hydroperoxide (tBH) is an organic peroxide. It has been used as a model compound for the study of mechanisms of oxidative cell injury in the form of lipid peroxidation, DNA damage and mutagenicity in mammalian cells (Aherne and O'Brien 2000, Coleman et al. 1989, Edenharder and Grünhage 2003, Hix et al. 1995). It has also been shown to cause lipid peroxidation of biomembranes in plant cells resulting in increased levels of lipid hydroperoxides in the cell (Matsuo et al. 1989).

Suspension cultures of soybean (*Glycine max*) treated with tBH were found to accumulate a higher concentration of the phytoalexin glyceollin than that of the un-treated cultures, due to the oxidative burst created (Degousée et al. 1994). The PAL activity was used as a marker for the induced phenypropanoid pathway leading to glyceollin production. They found that in the cultures treated with tBH there was an immediate increase in lipid peroxidation, and that the addition of a transition metal enhanced this peroxidation activity.

The decomposition of tBH is catalysed by transition metals producing various peroxyl radicals. Hematin, an iron-complex (**Figure 8.4.4.1**), has been found to catalyse the decomposition of tBH (Davis 1988, Kalyanarama et al. 1983) as outlined in **Figure 8.4.4.2.** Upon β-scission *tert*-butyl radicals, which form via the reactions of the peroxyl radical, will decompose and form methyl radicals and acetone (Hix et al.

1995). The methyl radical will then react rapidly with oxygen forming a peroxyl radical, which in turn will self-react to form a alkoxy radical (Kalyanarama et al. 1983).

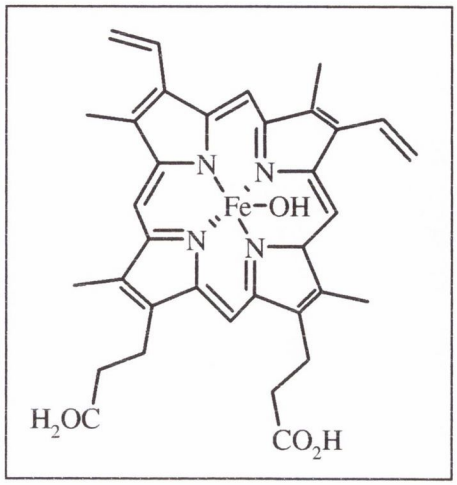


Figure 8.4.4.1 The iron-complex, hematin

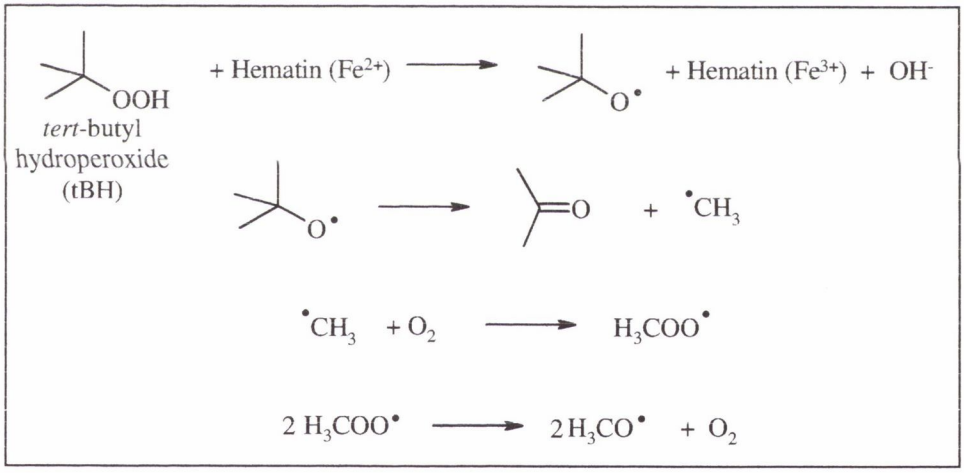


Figure 8.4.4.2 Decomposition of tBH catalysed by hematin

A number of studies have indicated that active oxygen species generated as a result of an oxidative burst not only activates plant defence mechanisms, but also enhances the biosynthesis of secondary metabolites in the plant. The close relationship between oxidative burst induced by elicitors and taxane production found by Yuan and co-workers for both *T. chinensis* and *T. cuspidata* cells (Yuan et al. 2001, Yuan et al. 2002d) is an example of this. They found that the accumulation of phenolics in treated cultures were higher than in that of controls (Yuan et al. 2002c). When Ce^{4+} was used

to induce an oxidative burst in suspension cultures of *T. cuspidata*, the taxane production was also enhanced (Yuan et al. 2002d).

It was decided to induce an oxidative burst in the *T. baccata* 'Fastigiata' suspension cultures using tBH in conjunction with hematin. In addition to analysing the effect this oxidative burst would have on the taxane production of the cells, the flavonoid content was also monitored. Flavonoids have been found to have a high scavenging activity of the activated oxygen species formed by decomposition of tBH (Afanas'ev et al. 1989, Aherne and O'Brien 2000, Akaike et al. 1992). In addition to indicating an activation of the plant defence mechanism, an increase in flavonoid production would also be indicative of an increase in secondary metabolite production.

8.4.4.1 Preliminary Experiment 1 and 2

In an experiment carried out with *E. coli* researchers decided that the optimal concentration of tBH was 20mM in conjunction with a 200µM concentration of hematin (Nakao et al. 1998). Degousée and co-workers found that a concentration of 10mM tBH was more suitable for the soybean cells (Degousée et al. 1994). In our Experiment 1 two different concentrations were used: Test 1 (T1) 10mM tBH in conjunction with 100µM hematin were added to each flask. In Test 2 (T2) 30mM tBH in conjunction with 300µM hematin were added. The addition was done on day 29 of the growth cycle, and the cells were harvested by filtration 24 hours later. In Experiment 2 the highest concentration (30mM tBH with 300µM hematin) was added at two different times. In Test 1 the elicitor was added on day 14 of the growth cycle and in Test 2 the addition was done as before on day 28. The cells were harvested 24 hours later.

The cells became very brown in colour, and the physical appearance was also different. The results of the taxane analysis in both preliminary experiments showed great variation, with the HPLC profiles of control and tests extracts being very different. This was indicating that the treatment was causing major cell injury or maybe even cell death. When the addition of tBH and hematin were done on day 14 a 54% decrease in cell growth was recorded, indicating that the treatment was detrimental to the cells.

In both the preliminary experiments the hematin solutions were made up using ethanol. Since ethanol can have a damaging effect on plant cells if the concentration is above 1% in the growth medium, or as we found, even at less than 1% (section 8.4.3) it was decided to change the solvent used to dimethyl sulphoxide (DMSO), which is less damaging on the cells (Wang et al. 2002).

8.4.4.2 Experiment 3 - "Long time course"

In order to see what effect tBH has on the cells over time, the cell were harvested at different time intervals after addition of tBH and hematin. The times of harvest were 6, 12, 24 and 72 hours after addition of tBH in conjunction with hematin. The hematin solution was made up using DMSO instead of ethanol, and the final concentration of DMSO in each flask was kept under 1%. Because of the adverse effects the treatment caused in the preliminary experiments the concentration of tBH and hematin was lowered from 30mM tBH and 300μM hematin to 20mM and 200μM respectively. Each of the tests was compared to a control where only the same amount of DMSO and sterile water was added.

The results of the effect the tBH treatment had on the flavonoid content in the cells is shown in **Figure 8.4.4.3** and corresponding **Table 8.4.4.1** below. The concentrations of catechin, epi-catechin, taxifolin, quercetin, kaempferol and amentoflavone were combined in order to see the overall effect on flavonoid production.

It is apparent that tBH and hematin had an effect both on the growth of the cells and the flavonoid content. There was a dramatic decrease of flavonoids between control and test, and the decrease was greater over time. In fact there were no quantifiable flavonoids present in the extract of the cells that were harvested 72 hours after the addition of the oxidative burst inducer. At 12 hours after treatment the cells were very dark brown. At 72 hours after addition they appeared dead. The oxidative burst caused by the tBH in conjunction with hematin obviously resulted in major cell injury, DNA damage and eventually cell death. Because of this dramatic effect a further adjustment to the treatment concentrations used was necessary. The effect on taxane production could not be analysed in this experiment due to the detrimental effect which the treatment had on the cells.

Table 8.4.4.1 Average flavonoid content in cells over time after tBH treatment

Sample	Total Flavonoid Content (mg/kg dry weight cells)	Dry Weight (g/l)	Growth Index (g)
C 6 hrs	8191.51 ± 988.09	2.98 ± 0.42	2.02 ± 0.11
T 6 hrs	1400.10 ± 369.14	1.71 ± 0.07	1.97 ± 0.35
C 12 hrs	5751.69 ± 915.51	2.35 ± 0.30	2.25 ± 0.44
T 12 hrs	289.96 ± 52.04	1.86 ± 0.10	2.35 ± 0.37
C 72 hrs	5006.66 ±1038.12	2.55 ± 0.12	2.21 ± 0.12
T 72 hrs	NQ	1.82 ± 0.21	1.47 ± 0.28

NQ = Non-quantifiable, C=control, T= test

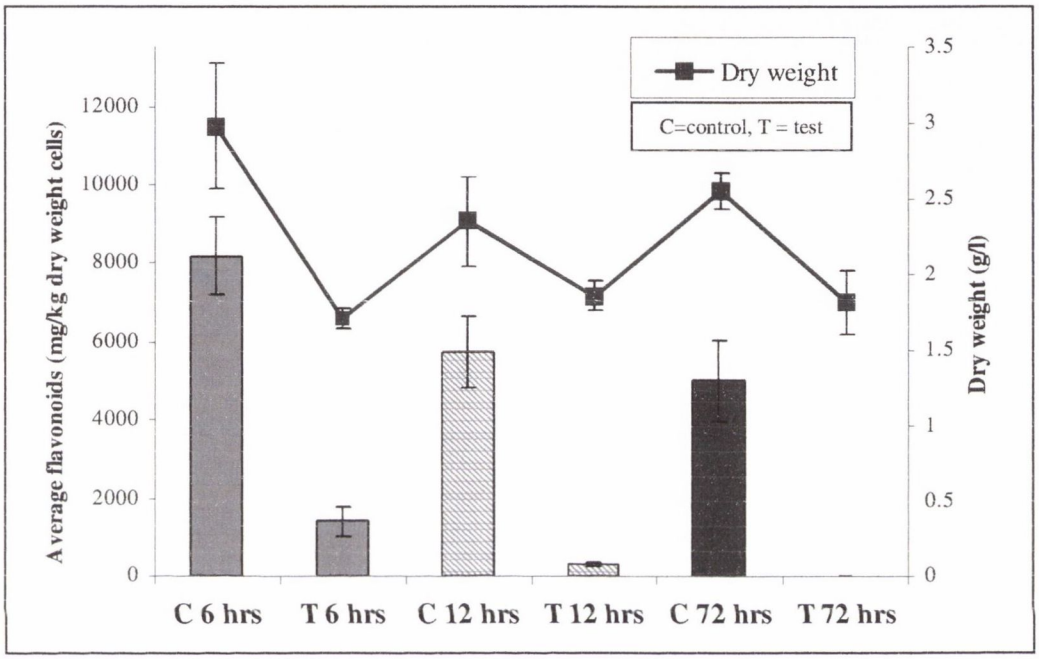


Figure 8.4.4.3 Total flavonoid content in cells over time after tBH treatment

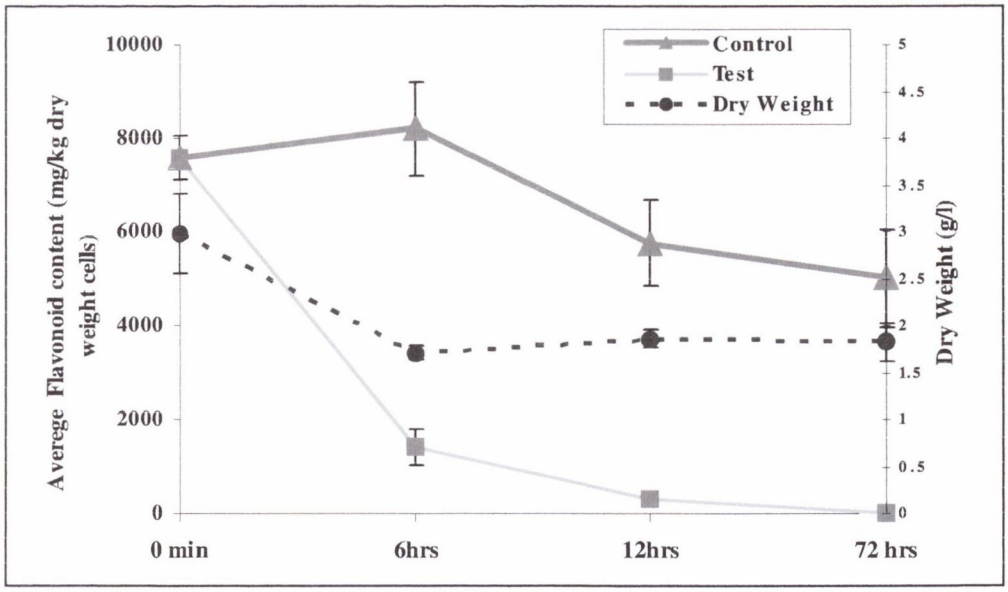


Figure 8.4.4.4 Time-course effect of tBH treatment on flavonoid content and growth

8.4.4.3 Experiment 4 - "Long time course"

The concentrations of tBH and hematin were reduced to 10mM and 100μM respectively. These elicitors were added to the cell culture on day 28 of the growth cycle, and the cells were harvested 24, 48 and 72 hours after the addition. The experiment was designed as a factorial experiment using Minitab (Minitab^{TM}). Because there is no interaction between the different concentrations of the elicitor, and because there is one factor with two levels (concentration) and one factor with four levels (time), the design was decided to be a 2x4 instead of 2^4 factorial design.

Effect on taxanes

The effects which elicitation with tBH and hematin had on the taxane production are shown **Figure 8.4.4.5** and **Table 8.4.4.2**. 10-DAB III, baccatin III and cephalomannine were analysed in this experiment. Only non-quantifiable trace amounts of paclitaxel were present in the cells used.

Table 8.4.4.2 Effect of tBH and hematin on the taxane production of the cells

Sample	Growth Index	Dry weight (g/l)	Average taxane Production (mg/kg dry weight cells)		
			10-DAB III	Baccatin III	Cephalomannine
Con	2.83 ± 0.04	4.60 ± 0.61	90.64 ± 10.04	293.93 ± 14.52	124.52 ± 12.26
C24	3.55 ± 0.33	4.16 ± 0.02	38.28 ± 5.74	243.30 ± 12.83	164.16 ± 3.57
T24	3.04 ± 0.14	4.33 ± 0.21	34.40 ± 7.43	68.52 ± 10.64	79.60 ± 13.74
C48	3.84 ± 0.38	5.52 ± 0.38	34.18 ± 0.85	137.47 ± 11.04	201.23 ± 18.53
T48	3.05 ± 0.18	4.62 ± 0.15	60.19 ± 2.33	283.33 ± 13.65	199.90 ± 25.39
C72	3.49 ± 0.23	4.83 ± 0.10	46.32 ± 6.61	175.25 ± 21.41	178.53 ± 13.14
T72	3.54 ± 0.25	4.68 ± 0.28	53.82 ± 5.88	258.29 ± 27.31	169.49 ± 17.13

Con=overall control, C24= control 24hrs, T24= test 24hrs, C48=control 48hrs, T48=test 48hrs, C72=control 72hrs, T72=test 72hrs

The overall control (Con) had a significantly higher production of 10-DAB III ($P<0.001$) than any of the other experimental units. There was an initial drop in the production of this taxane both in C24 and in T24. With ethanol it had previously been seen that addition to the cells resulted in a drop in the overall taxane yield (section 8.4.3). The addition of DMSO plus sterile water had a similar though less pronounced effect with the production of cephalomannine being hardly affected. With 10-DAB III, the induction of the oxidative burst resulted in no effect in test cells harvested after 24 hours. However, cells (T48) harvested after 48 hours had a significantly higher ($P<0.001$) concentration than the control (C48). The increase in 10-DAB III

production was in fact 76% at this time. The 10-DAB III concentration stayed relatively constant after this increase, with only a 16% increase between control and test cells at 72 hours.

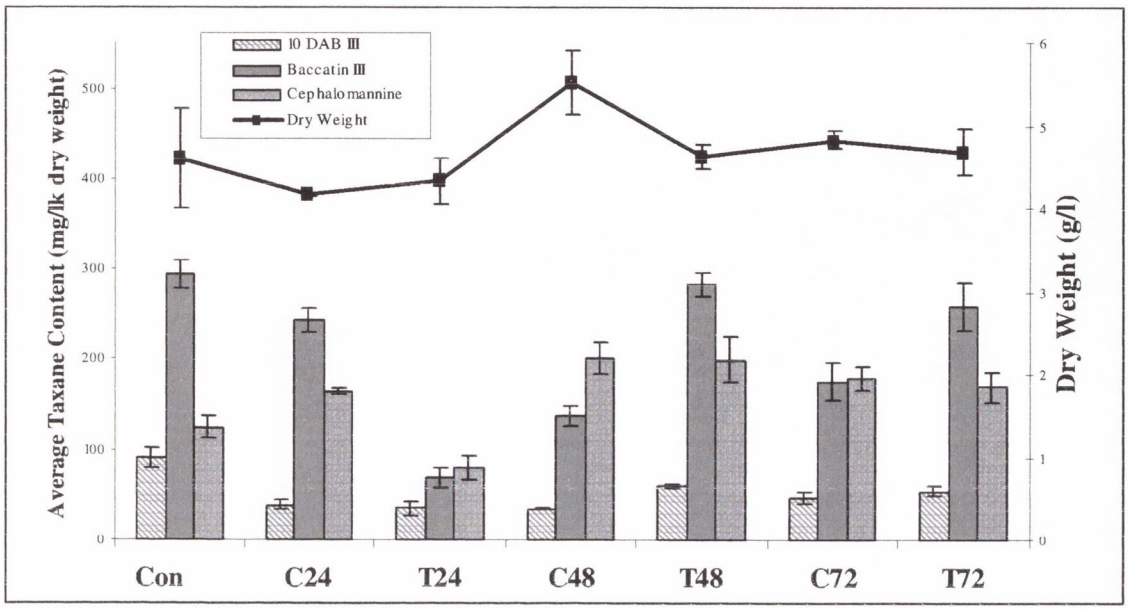


Figure 8.4.4.5 Effect of oxidative burst on the taxane production

For baccatin III the initiation of the oxidative burst had a severe effect on the production of the compound in the test cells harvested 24 hours after addition of the organic peroxide, resulting in a significant decrease in production between C24 and T24 ($P<0.001$). At 48 hours the opposite effect is apparent, with the concentration of baccatin III having increased from 68.52 (± 10.64) mg/kg to 283.33 (±13.65) mg/kg (in T24 and T48, respectively). This is a four-fold increase in production in 24 hours. The concentration of baccatin III in the test cells at this time was also significantly higher than that of the control ($P<0.001$). Finally there was no significant difference in the production of this taxane in the test cells at 48 hours and at 72 hours ($P>0.05$), but the T72 still had a higher baccatin III content than C72.

With cephalomannine, the induction of the oxidative burst by tBH and hematin caused a significant initial decrease in the biosynthesis of this taxane ($P<0.001$). This decrease was followed by a significant increase ($P<0.001$) in the cells harvested after 48 hours, but this increase was also present in the control cells at this time, i.e. the oxidative burst did probably not cause the increase. After this increase the production

of cephalomannine both by control cells and test cells was stable for the remaining period of the experiment.

In the initial long time experiment (Experiment 3) using a 20mM concentration of tBH and a 200μM concentration of hematin to induce the oxidative burst, it was obvious that the cells could not sustain the large oxidative burst created, and lost viability over time. From **Figure 8.4.4.5** we can conclude that the use of a lower concentration of both hematin and tBH did not affect the growth of the cells to the same extent indicating that the oxidative burst created by the organic peroxide did not lead to cell injury or death, but it had a significant effect on the taxane biosynthesis of the cells 48 hours after addition of the elicitor.

Figure 8.4.4.6 illustrates the overall effects of the oxidative burst over time on both the taxane generation and the growth of the cells. It is apparent from the graph that cell growth (growth index) and biomass are relatively constant over the 72 hour growth period. *Taxus* cells are slow-growing and any changes in metabolite concentrations are thought due to the treatment and not loss of cell viability.

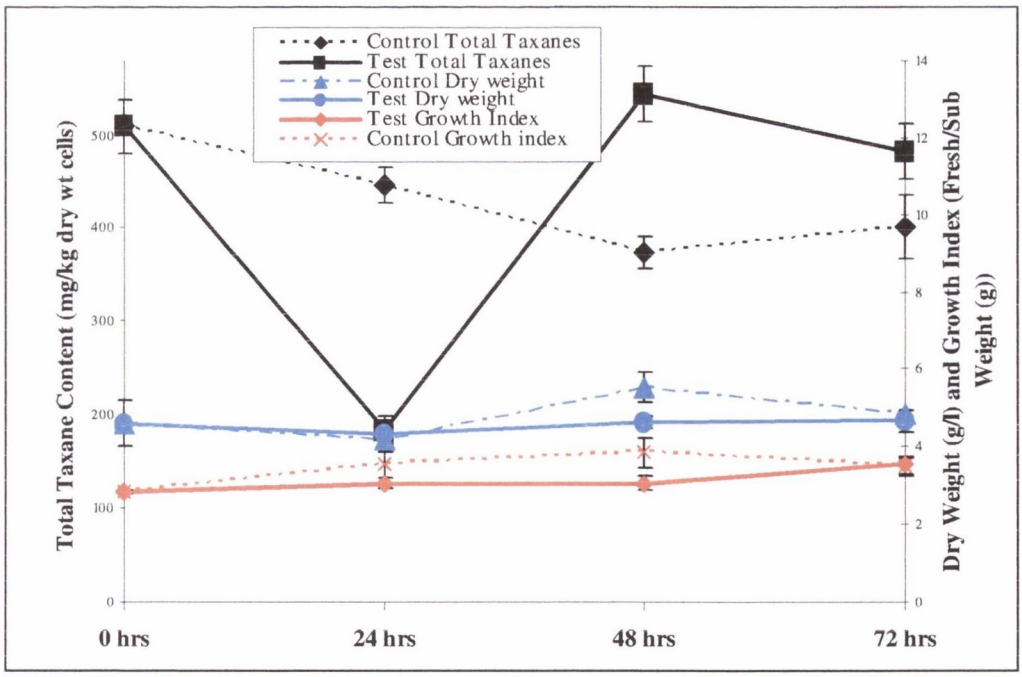


Figure 8.4.4.6 Effect of oxidative burst on total taxane production and cell growth

Effect on flavonoids

The results of the flavonoid analysis are shown below in **Table 8.4.4.3** and in the corresponding **Figure 8.4.4.7**. From these it is clear that there was a significant difference in the concentration of the various flavonoids.

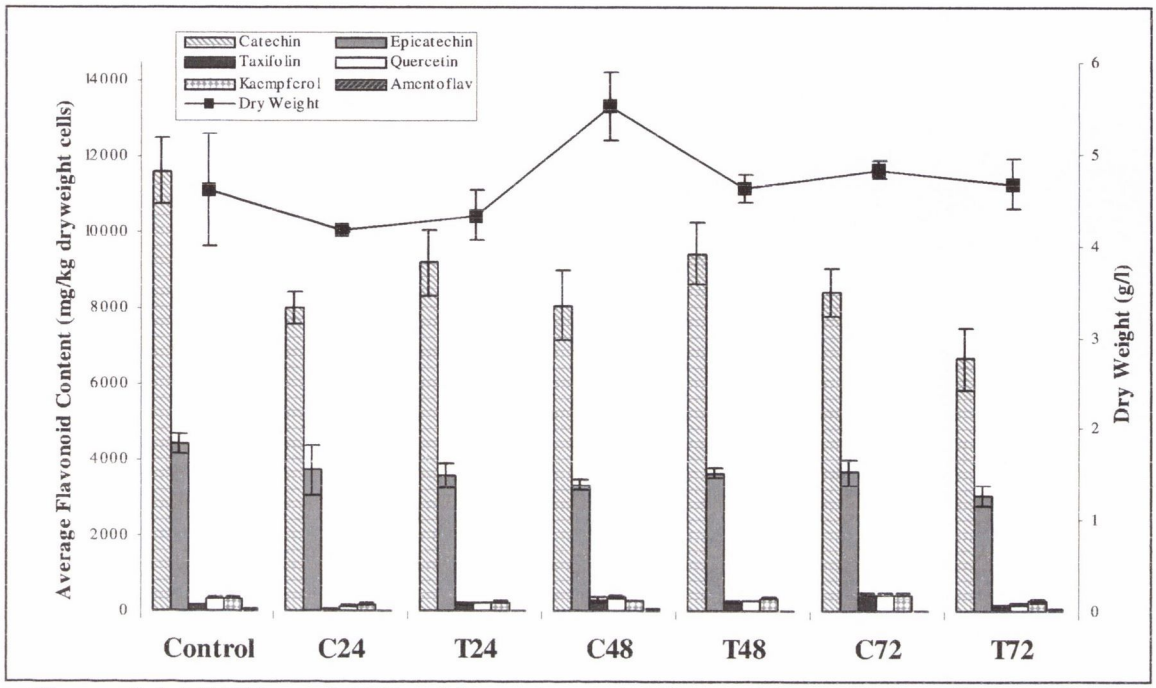


Figure 8.4.4.7 Effect of tBH and hematin on the flavonoid content in cells over time

Table 8.4.4.3 Effect of tBH and hematin on flavonoids over time

Sample	Dry Weight (g/l)	Average Flavonoid Content (mg/kg dry weight cells)		
		Catechin	Epi-catechin	Taxifolin
C	4.60 ± 0.61	11620.18 ± 888.35	4422.49 ± 246.95	159.51 ± 16.74
C24	4.16 ± 0.02	8015.76 ± 440.09	3727.99 ± 667.64	20.52 ± 12.14
T24	4.33 ± 0.21	9208.79 ± 859.23	3585.55 ± 304.68	192.53 ± 24.17
C48	5.52 ± 0.38	8091.56 ± 946.94	3343.09 ± 127.30	282.87 ± 62.59
T48	4.62 ± 0.15	9453.29 ± 819.82	3652.83 ± 143.19	227.88 ± 15.56
C72	4.83 ± 0.10	8449.16 ± 645.59	3688.89 ± 341.01	435.02 ± 15.39
T72	4.68 ± 0.28	6685.55 ± 810.26	3049.23 ± 261.49	131.84 ± 25.73
	Quercetin	Kaempferol	Amentoflavone	
C	327.49 ± 29.94	331.74 ± 27.75	21.20 ± 8.06	
C24	117.24 ± 21.39	180.27 ± 13.55	7.73 ± 1.15	
T24	217.55 ± 15.75	220.56 ± 20.37	20.26 ± 4.36	
C48	359.03 ± 65.64	248.58 ± 6.53	25.96 ± 1.67	
T48	265.75 ± 20.47	335.20 ± 11.66	15.89 ± 4.82	
C72	442.27 ± 33.58	445.09 ± 40.21	14.49 ± 7.72	
T72	194.59 ± 25.36	246.37 ± 53.97	17.73 ± 15.75	

Con=overall control, C24= control 24hrs, T24= test 24hrs, C48=control 48hrs, T48=test 48hrs, C72=control 72hrs, T72=test 72hrs

The concentrations of catechin and *epi*-catechin were considerably greater than those of taxifolin, quercetin, kaempferol and amentoflavone. Therefore the results of the effect tBH and hematin had on the minor flavonoids were presented in a separate graph in **Figure 8.4.4.8**. The concentration of myricetin was so variable and non-significant that it was omitted from the analysis

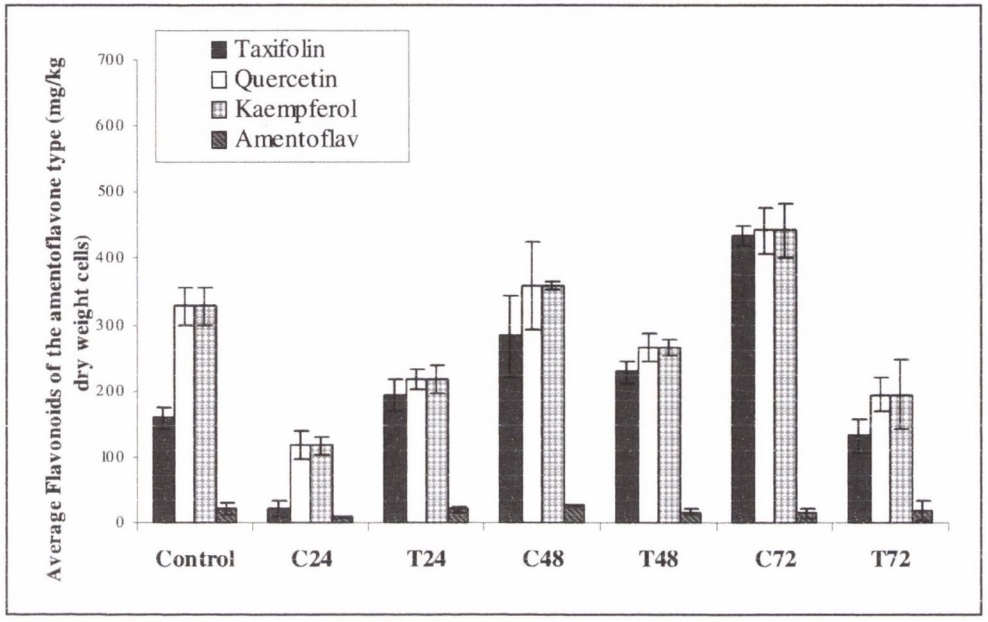


Figure 8.4.4.8 Effect of tBH on the minor flavonoid constituents in the cells

Because of this large difference the results were also presented as a time-course in **Figure 8.4.4.9** and the corresponding **Table 8.4.4.4**, where catechin and *epi*-catechin was grouped together and called "The catechin group" on one axis and the remaining flavonoids were grouped together and called "The quercetin group" on the other axis.

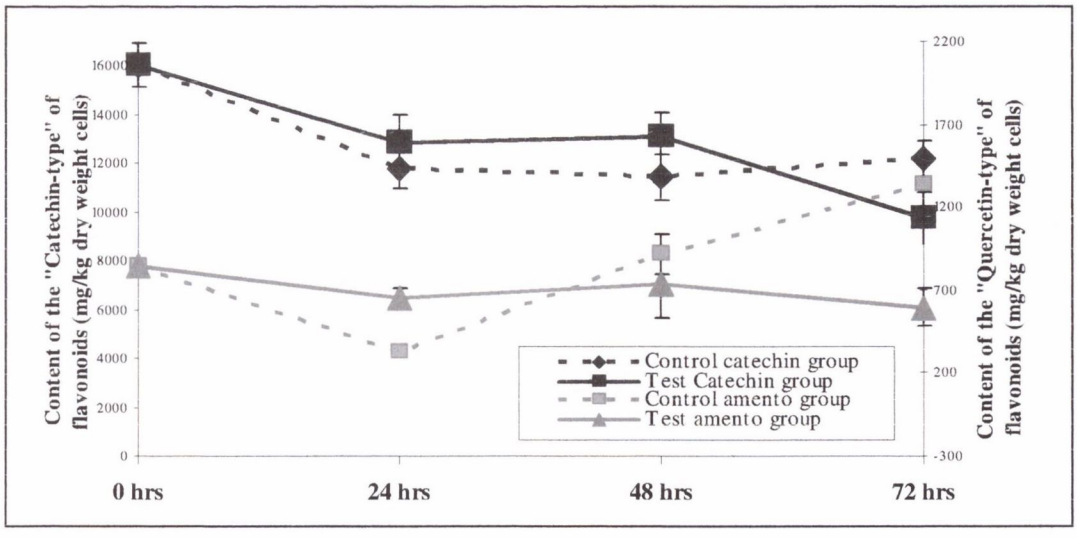


Figure 8.4.4.9 Time-course effect of tBH on the two groupings of flavonoids

Table 8.4.4.4 Effect of tBH on the two groupings of flavonoids

Time	Sample	
	Control Catechin Group	**Test Catechin Group**
0 hrs	16042.66 ± 909.51	16042.66 ± 909.51
24 hrs	11743.75 ± 829.92	12794.34 ± 1149.45
48 hrs	11434.64 ± 941.87	13106.12 ± 957.39
72 hrs	12138.04 ± 734.87	9734.78 ± 1060.02
	Control Quercetin Group	**Test Quercetin Group**
0 hrs	839.95 ± 32.43	839.95 ± 32.43
24 hrs	325.76 ± 21.13	650.90 ± 58.82
48 hrs	916.44 ± 123.17	732.98 ± 208.38
72 hrs	1236.88 ± 29.13	590.53 ± 114.65

Catechin and *epi*-catechin are closely related being diastereomers, whereas the other flavonoids are structurally different (see **Figure 8.2.2.1**). So the grouping done can actually be structurally supported.

For the catechin the overall control (sample with no addition) had a significantly higher ($P<0.001$) concentration than any of the other controls or test samples. The concentration of catechin decreased from the overall control to the control at 24 hours after addition of oxidative burst inducer. There was no significant difference between C24 and T24 ($P>0.05$). There was a slight significant difference ($P<0.05$) between C48 and T48 where there was a higher concentration of catechin in the test sample than in the control. But T48 and T24 were not significantly different to each other. There was a significant decrease ($P<0.001$) in the catechin content at T72 compared to the test samples at 24 hours and 48 hours after addition.

Examination of the *epi*-catechin concentration the trend is quite similar, but the variation is not as apparent as in the catechin concentration. The overall control was also here significantly higher than the rest of the samples (ranging from $P<0.01$ to $P<0.001$). The control samples at the various harvest times did not vary significantly. There was however, as for the catechin concentration, a significant decrease ($P<0.001$) in *epi*-catechin content in the test sample at 72 hours after induction. It can therefore be concluded that in "the catechin group" catechin itself created the greatest variation. The variation in the *epi*-catechin followed a similar trend to that of the catechin, but

the variation was less extensive and did not influence the overall result to the same degree.

For the minor flavonoids the degree of variation was not as great as it was for the major flavonoids. For amentoflavone there was almost no variation only a slight decrease from the overall control to the control at 24 hours after addition ($P>0.05$). This would suggest that the concentration of amentoflavone was not affected by the induction of an oxidative burst by tBH and hematin. For taxifolin, quercetin and kaempferol the trends were quite similar. For taxifolin and quercetin the overall control was significantly lower than that of C48 and C72 ($P<0.001$). For kaempferol the overall control was higher than that of C48 ($P<0.001$). The controls at 24 hours in the case of taxifolin and quercetin are significantly lower than the rest of the samples ($P<0.001$). The same holds for kaempferol, the only difference being that there was no statistical difference between T24 and C24. There was also no significant difference in the quercetin and taxifolin concentration between T24 and T48, but there was a slight increase in kaempferol between these two times. There was a significant drop ($P<0.001$) in the concentration of all the three flavonoids in the test samples harvested 72 hours after induction. The control, however, was significantly higher than that of the control at 48 hours.

The overall trend in the test results of both "the catechin group" and "the quercetin group" is that there is an initial drop in flavonoid concentration after addition of the organic peroxide (at 24 hours). This drop is followed by a slow increase with a maximum at 48 hours after addition. That the test sample is significantly higher than that of the control in "the quercetin group" at 24 hours after oxidative burst induction, and that the same is seen for the test sample in "the catechin group" at 48 hours after induction strongly suggests that the oxidative burst created has induced the cells to produce protective antioxidants. Finally there is a considerable decrease in the overall flavonoid content at 72 hours after oxidative burst induction. In the control results the trend is similar, but there is no drop in flavonoid concentration at 72 hours.

Figure 8.4.2.10 shows the effect the treatment had on the total flavonoid generation and the total yield of taxanes in the *T. b.* 'Fastigiata' cell cultures. From this one can see that at 48 hours after oxidative burst total taxanes were nearly 50% higher than

control (C48) amounts. This increase was due mainly to doubling of the baccatin III content (283 mg/kg (T48) vs. 137mg/kg (C48)). Flavonoid increases were not as dramatic, but 72 hours after oxidative burst significant reductions in flavonoids were noted (20% reduction in "catechin-group" and 48% reduction in "quercetin-group").

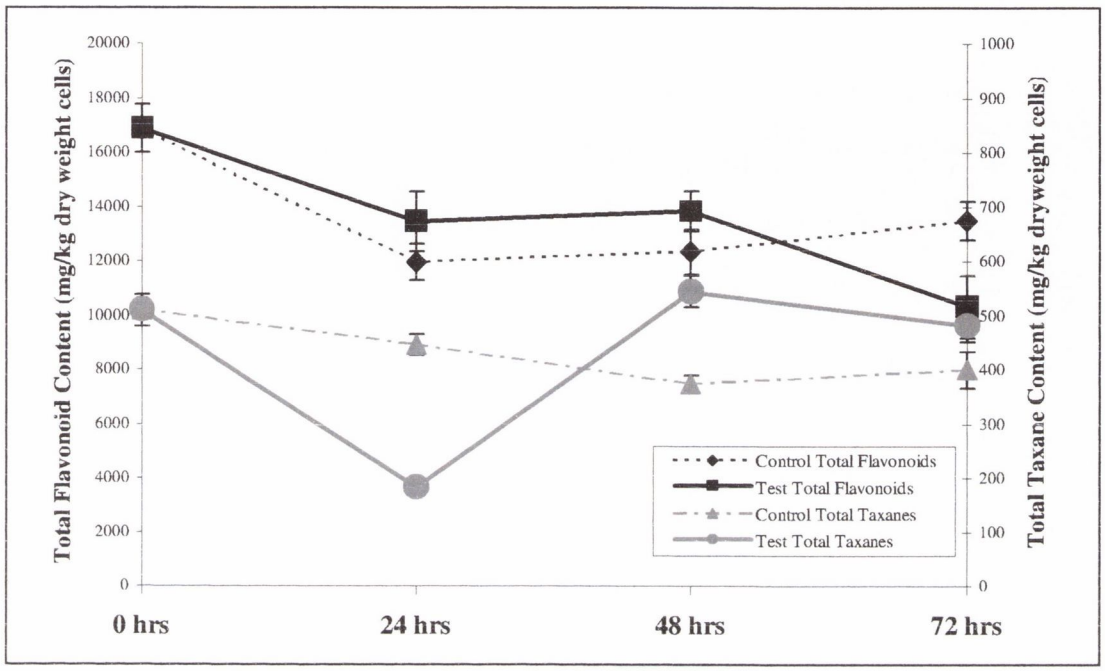


Figure 8.4.4.10 Total taxane and total flavonoid production by the cells over time

8.4.4.4 Experiment 5 - "Short time course"

The plants defence system to an oxidative burst is complex and varies depending on the pathogen, elicitor, and a whole range of other circumstances. As mentioned earlier, flavonoids have several protective functions in the plants cells, and one of these is their importance as antioxidants. The initiation of the phenylpropanoid pathway is a rapid response of the plant cells to oxidative stress. In order to see if there was an initial burst of the protective flavonoid production after the induction of an oxidative burst in the *Taxus baccata* 'Fastigiata' cell culture, the times intervals of cell harvest after tBH and hematin addition were shortened to 10 minutes, 30 minutes and 1 hour. The same concentrations of tBH and hematin as in the above experiment (10mM and 100μM respectively) were added on day 28. As in *Experiment 4 -"Long time course"* the effects on taxane and flavonoid production were investigated.

Effect on taxanes

The effects which tBH and hematin had on the taxane production was analysed, and the results of this analysis can be seen in **Table 8.4.4.5** and the corresponding **Figure 8.4.4.11**. While in the long-time experiment only 10-DAB III, baccatin III and cephalomannine were analysed (as only trace amounts of paclitaxel were present in the cells), in this short-time experiment quantifiable amounts of paclitaxel were present and results are shown below.

Table 8.4.4.5 Effect of oxidative stress on the taxane production of the cells

Sample	Dry weight (g/l)	Growth Index (g)	Average Taxane Production (mg/kg dry weight cells)			
			10-DAB III	Baccatin III	Cephalo-mannine	Paclitaxel
Con	2.34 ± 0.13	2.39 ± 0.27	14.85 ± 1.68	66.03 ± 9.57	162.78 ± 5.94	21.66 ± 2.91
C10	2.40 ± 0.31	2.47 ± 0.20	22.42 ± 1.93	51.42 ± 4.88	154.67 ± 1.59	29.81 ± 1.91
T10	2.60 ± 0.20	2.18 ± 0.19	12.22 ± 1.61	67.96 ± 6.20	175.79± 6.29	13.60 ± 1.92
C30	3.05 ± 0.51	2.06 ± 0.11	20.05 ± 0.22	82.98 ± 5.13	143.21 ± 0.79	20.79 ± 0.75
T30	3.03 ± 0.28	2.05 ± 0.18	14.89 ± 1.46	70.32 ± 2.16	206.74 ± 5.73	12.63 ± 1.62
C60	2.61 ± 0.36	2.28 ± 0.18	16.12 ± 2.17	114.80 ± 7.66	147.51 ± 10.60	11.97 ± 1.77
T60	2.80 ± 0.43	2.21 ± 0.29	12.94 ± 0.35	108.94 ± 7.96	158.38 ± 5.36	36.54 ± 1.25

Con=overall control, C10= control 10min, T10= test 10min, C30=control 30min, T30=test 30min, C60=control 60min, T60=test 60min

No decrease in 10-DAB III concentration was observed between the overall control (Con) and the controls containing DMSO + sterile water (C10, C30 and C60) as was observed in the long time course (*Experiment 4*). In fact, in C10 and C30, where the concentration of 10-DAB III was higher than in the Con cells. There was no significant difference between the Con cell and the control cells at 1 hour. This would suggest that the addition of DMSO and sterile water did not have an immediate effect on the cells. In the test cells at both 10 minutes and 30 minutes there was a significant reduction ($P<0.001$) in production compared to their respective control. At 1 hour the difference between control cells and the test cells was only slightly significant ($P<0.05$).

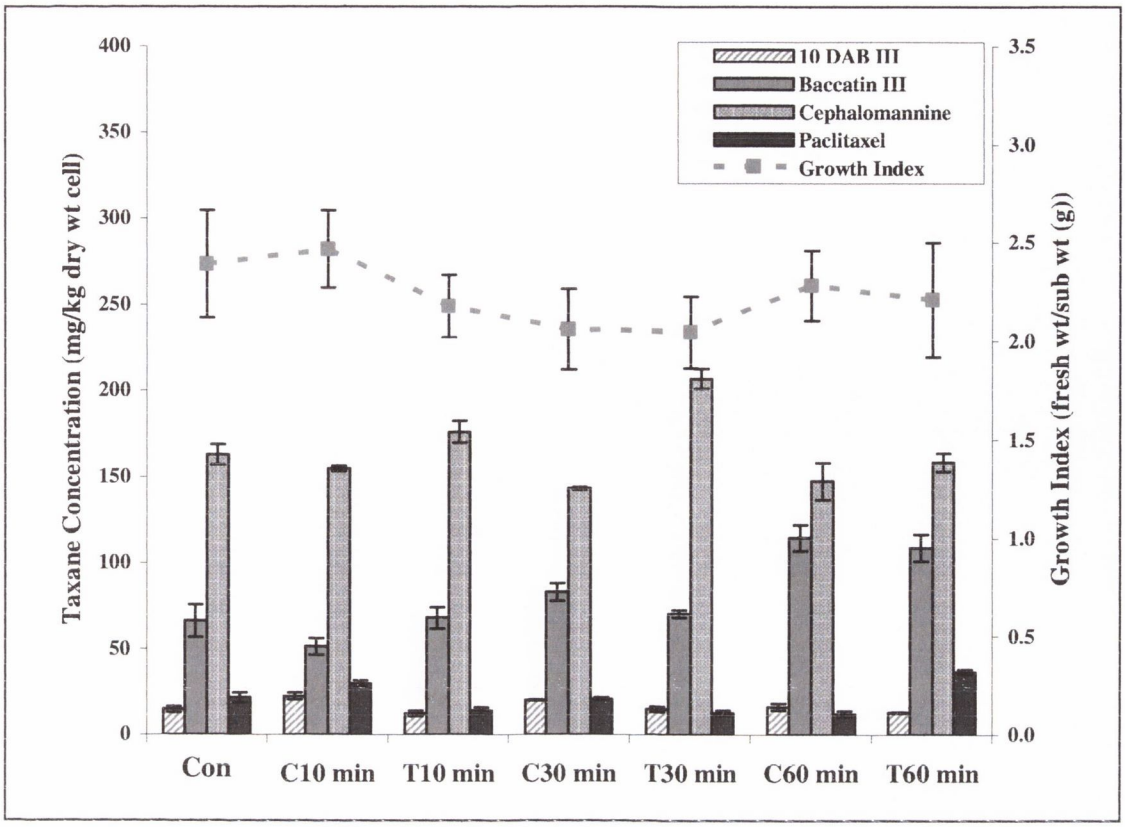


Figure 8.4.4.11 Effect of oxidative stress on growth index and taxane production

For baccatin III there was a slight decrease ($P<0.01$) in production after addition of the sterile water and DMSO in the control cells (C10) compared to the overall control (Con). The initiation of the oxidative burst caused a slight increase in the production of baccatin III in the test cells after 10 minutes ($P<0.01$) compared to the control cells (C10). The concentration of baccatin III was higher in the control cells at C30 and C60 than that found in the test cells at this time, but the concentration was nearly doubled after 60 minutes (both in T60 and C60) compared to the concentration in the cells at time 0 (Con). Because the concentration was high also in the control cells the reason for the increase in baccatin III concentration can not be exclusively linked to the oxidative burst induced.

In the production of cephalomannine it is quite apparent that there was very little variation in the production over time in the control samples. The production of cephalomannine in the overall control was higher than that of the control at 30 minutes and at 60 minutes ($P<0.001$ and $P<0.01$ respectively). However, between the control at 10, 30 and 60 minutes there was very little difference, only the control at 30 minutes

was slightly statistically lower than the other two ($P<0.05$). The induction of the oxidative burst by tBH and hematin caused significant variation over time in the biosynthesis of this taxane. Even after just 10 minutes of the induction there was a significant increase in the production between the test cells and the control cells ($P<0.001$). However, the concentration found in the T10 cells was only slightly higher than that of the overall control ($P<0.05$). The production of cephalomannine in the test cells harvested after 30 minutes after induction of oxidative burst was significantly higher than that of any of the other samples ($P<0.001$), producing a 44% increase compared to the control cells, and a 27% increase compared to the overall control. One hour after inducing the oxidative burst cephalomannine production decreased to a normal level.

With regard to paclitaxel, the addition of tBH in conjunction with hematin, initially decreased production, with concentration in T10 and T30 being significantly lower than that in the controls at these times ($P<0.001$). After 1 hour there was an apparent change in the paclitaxel production, with significant increase in production in the test cells (T60). This was a 3.1-fold increase compared to the control cells (C60), and a 1.7-fold increase compared to the overall control (Con).

In the long-time experiment it was concluded that the lower concentration of tBH and hematin (10mM and 100μM, respectively) was less toxic, allowing cell growth over time. From the graph in **Figure 8.4.4.11** it is obvious that no significant effects were produced by treatments on cell growth, with no statistically significant differences in Growth Index figures (ANOVA). Any effects on metabolite production were therefore due to oxidative burst caused by the treatment.

The overall effects of the oxidative burst over time on both the taxane generation and the growth of the cells are better illustrated in the time-course graph in **Figure 8.4.4.12**. A sustained increase in total taxane content is obvious up to 1 hour after the oxidative burst. However, the increase is not as significant as the increase in total taxane content in the long time course experiment (*Experiment 4*).

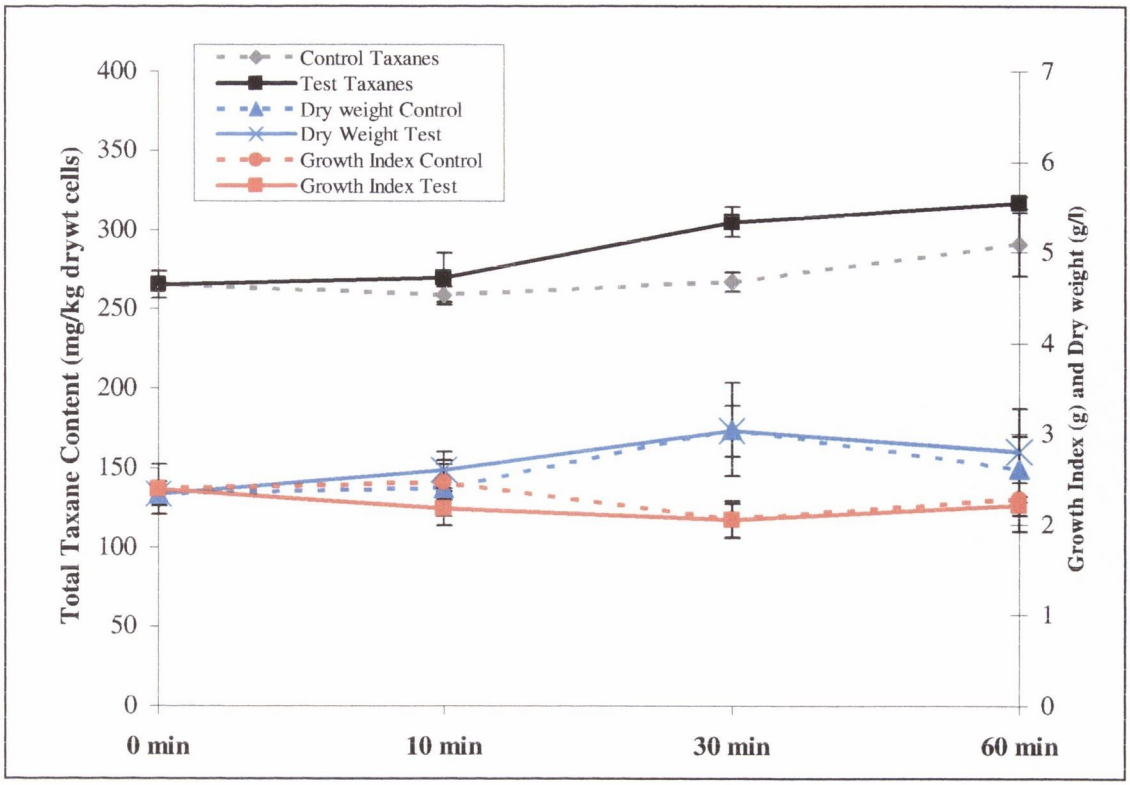


Figure 8.4.4.12 Effect of oxidative burst on total taxane production and cell growth

Effect on flavonoids

The results of the flavonoid analysis are shown in **Figure 8.4.4.13** and in the corresponding **Table 8.4.4.6**. The same significant difference in the concentration of the various flavonoids as in the long time course experiment was also noted in the short time course experiment. The concentration of catechin and *epi*-catechin was considerably greater than that of the quercetin, kaempferol and amentoflavone. Therefore the results of the effects which tBH and hematin had on the minor flavonoids were presented in a separate graph in **Figure 8.4.4.14**. The concentrations of myricetin and taxifolin were so variable and so insignificant that they were left out of the analysis.

Table 8.4.4.6 The short-time effect of tBH on the overall flavonoid production

Sample	Growth Index (g/l)	Average Flavonoid Content (mg/kg dry weight cells)		
		Catechin	Epi-catechin	Quercetin
Con	2.39 ± 0.27	2867.08 ± 106.10	739.54 ± 37.39	120.80 ± 43.73
C10	2.47 ± 0.20	1643.43 ± 311.80	428.31 ± 20.54	252.07± 94.69
T10	2.18 ± 0.19	2676.14 ± 189.49	1236.30 ± 132.91	271.36 ± 104.30
C30	2.06 ± 0.11	2133.06 ± 445.88	806.15 ± 152.12	185.66 ± 81.34
T30	2.05 ± 0.18	2034.61 ± 279.68	832.62 ± 126.96	175.56 ± 78.14
C60	2.28 ± 0.18	2395.17 ± 290.28	823.08 ± 86.63	201.14 ± 102.33
T60	2.21 ± 0.29	2094.73 ± 155.06	626.98 ± 40.47	187.51 ± 80.84
	Kaempferol	Amentoflavone		
Con	236.86 ± 81.92	67.38 ± 54.18		
C10	225.14 ± 54.47	40.91 ± 16.63		
T10	275.00 ± 59.27	84.50 ± 29.33		
C30	268.97 ± 58.18	68.00 ± 17.00		
T30	224.87 ± 29.16	68.45 ± 12.31		
C60	267.50 ± 38.30	77.01 ± 16.22		
T60	234.55 ± 42.68	54.54 ± 20.32		

Con=overall control, C10= control 10min, T10= test 10min, C30=control 30min, T30=test 30min, C60=control 60min, T60=test 60min

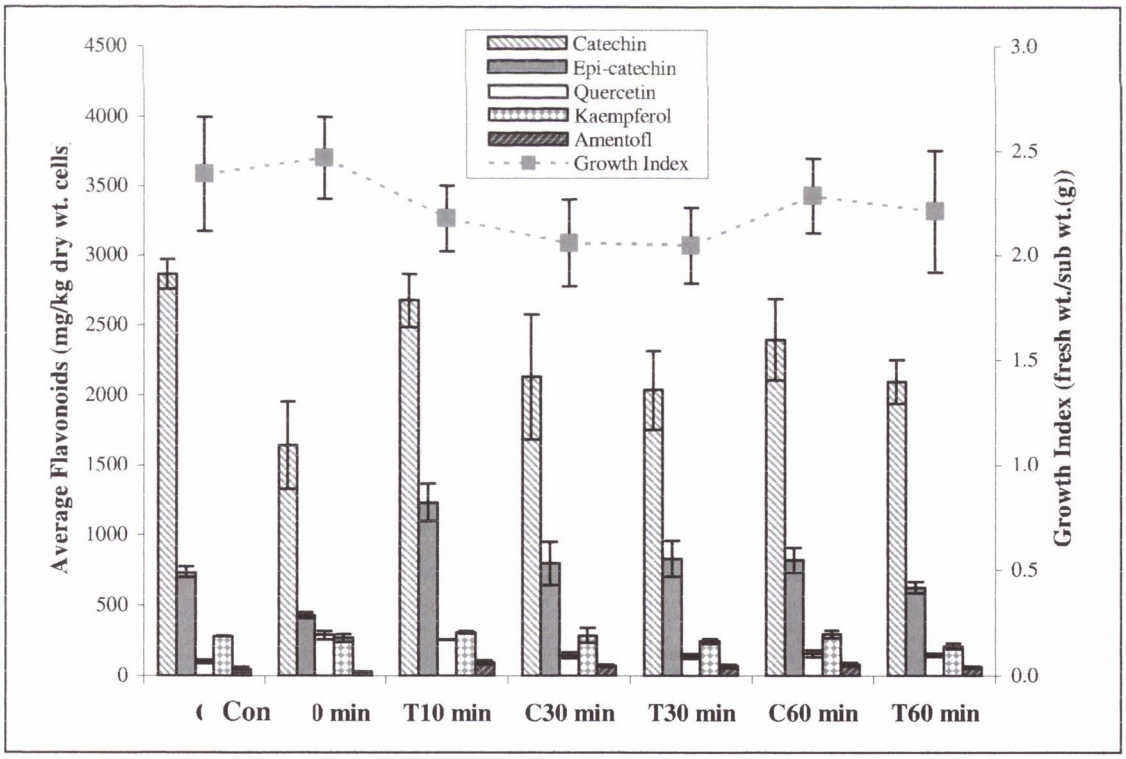


Figure 8.4.4.13 The short-time effect of oxidative stress on the overall flavonoid production

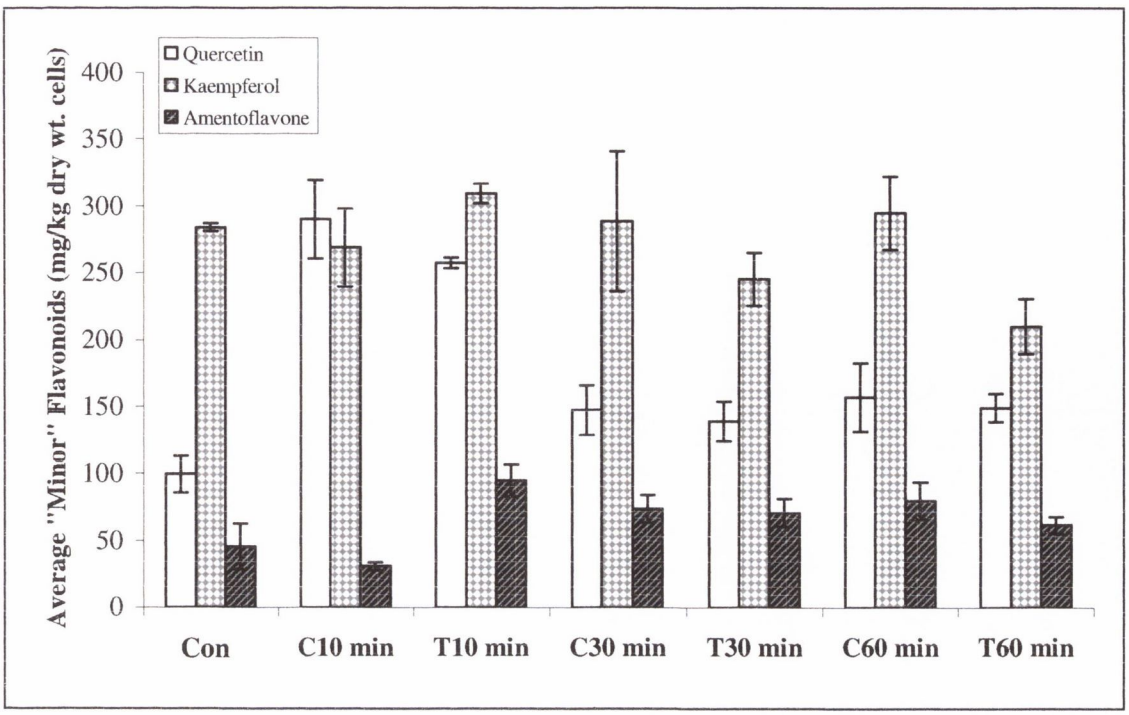


Figure 8.4.4.14 Short-time effect of oxidative stress on the generation of minor flavonoids

The effect which the oxidative burst had on the test cells compared to the response given by the control cells are shown in **Figure 8.4.4.15** and the corresponding **Table 8.4.4.7**. Because of the large difference between the two groups of flavonoids the results are here presented as "The catechin group" (including catechin and *epi*-catechin) on one axis and the remaining flavonoids "The quercetin group" (including quercetin, kaempferol and amentoflavone) on the other axis, as before.

Table 8.4.4.7 Effect of oxidative stress on the two groupings of flavonoids

Time	Sample	
	Control Catechin Group	**Test Catechin Group**
0 min	3606.67 ± 70.64	3606.67 ± 70.64
10 min	2071.74 ± 322.91	3912.43 ± 312.60
30 min	2933.20 ± 567.38	2867.23 ± 394.42
60 min	3218.25 ± 371.11	2721.71 ± 135.01
	Control Quercetin Group	**Test Quercetin Group**
0 min	429.70 ± 20.67	429.70 ± 20.67
10 min	590.29 ± 20.91	662.27 ± 16.75
30 min	510.26 ± 66.82	455.80 ± 42.62
60 min	532.57 ± 53.58	421.34 ± 23.76

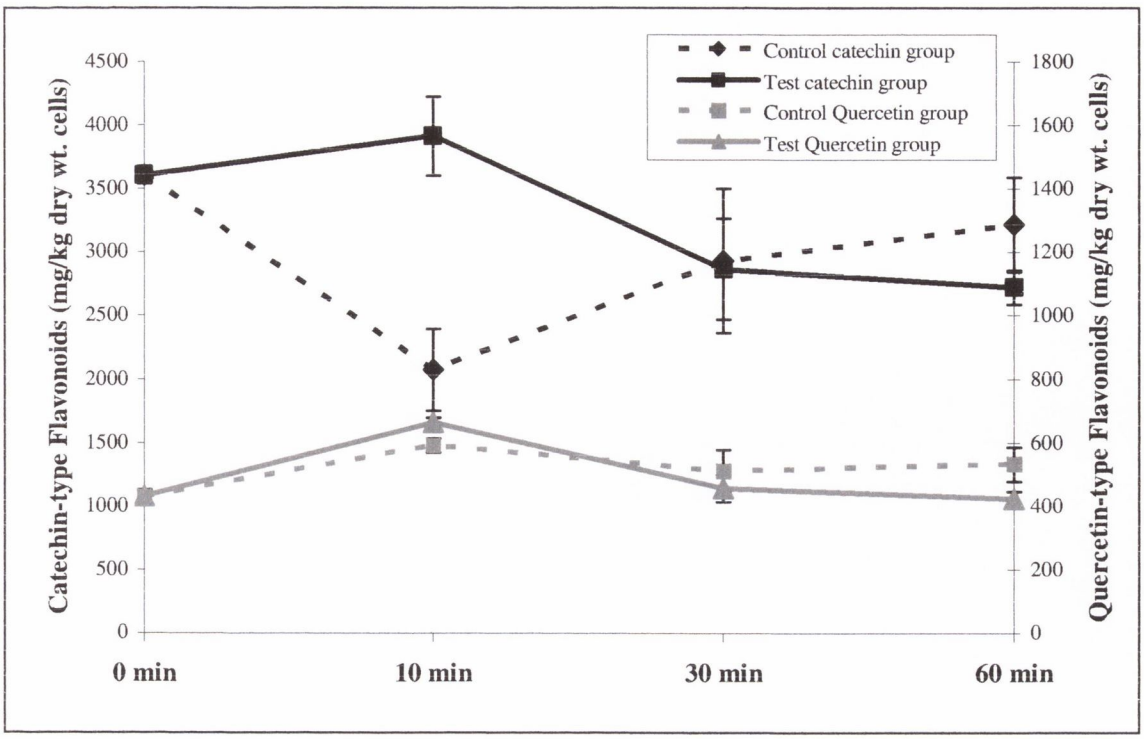


Figure 8.4.4.15 Time-course effect of tBH on the two groupings of flavonoids

From the graph in **Figure 8.4.4.15** it can clearly be seen that there was an initial burst of flavonoids of both "the catechin group", showing an 89% increase, and "the quercetin group", showing an 12% increase, after the addition of the organic peroxide in conjunction with the catalyst hematin (at 10 minutes). After this the concentration slowly decreased, and at the final harvest time the concentration of flavonoids in the test cells was lower than that in the control cells. The difference between the two groupings of the flavonoids was that in "the catechin group" the concentration of flavonoids in the control cells actually decreased after the addition of water and DMSO, whereas in "the quercetin group" the concentration slightly increased.

In the short-term, as well as in the long-term experiments, catechin is the major flavonoid present. The overall control (sample with no addition of solvent) had a significantly higher ($P<0.001$) concentration than any of the other controls (with solvent) with the exception of the control harvested after 1 hour. The concentration of catechin decreased from the overall control to the controls at 10 minutes and 30 minutes after addition of oxidative burst inducer. There was a very significant difference between C10 and T10 ($P>0.001$) indicating a burst of catechin synthesis in T10. In the same manner as for the catechin concentration there was a burst of *epi-*

catechin after 10 minutes of oxidative stress induction. This concentration was significantly higher than any of the concentrations recorded in the run of the experiment ($P<0.001$). There was no significant difference between C30 and T30 ($P>0.05$). There was, however, a drop in *epi*-catechin generation between C60 and T60 ($P<0.01$). From the graph (**Figure 8.4.4.13**) one can therefore conclude that catechin and epi-catechin followed similar trends during the course of this experiment. In both the components there was a burst of concentration in the cells harvested 10 minutes after addition of tBH and hematin.

For the minor flavonoids the degree of variation was not as large as that of the major flavonoids. The flavonoid with the least variation during the course of the experiment was kaempferol, although there was a slight difference between the control and the test cells 30 minutes after induction ($P<0.05$). The greatest variation was observed 1 hour after induction where there was a significant drop in kaempferol production between the control and the test cells ($P<0.001$). For quercetin there was a significantly greater concentration in all the treated cells (C and T) than in the control (Con) cells ($P<0.001$). However, there was no statistical difference between any of the treated (C and T) cells. The trend of the amentoflavone production followed that of the catechin and epi-catechin generation above. There was a burst of production in the test cells harvested at 10 minutes after induction. The concentration analysed at this time was significantly higher than any of the other concentrations found (P ranging from <0.01 to <0.001).

The time course effects on the production of the two secondary metabolite groups (taxanes and flavonoids) were combined in one graph in **Figure 8.4.4.16**. From this it is apparent that the increase in taxane production began after the initial burst in flavonoid synthesis in cells harvested 10 minutes after oxidative stress induction. The immediate effects of the organic peroxide and its catalyst were not reflected in the production of taxanes, but in the biosynthesis of the protecting flavonoids.

The results from this short-term experiment confirm those found in the long-term experiment, i.e. that the tBH and hematin have an effect on secondary metabolite production. The initial burst of flavonoids can be a result of the rapid response of the

plants defence system to oxidative stress. This could also result in the enhanced production of taxanes.

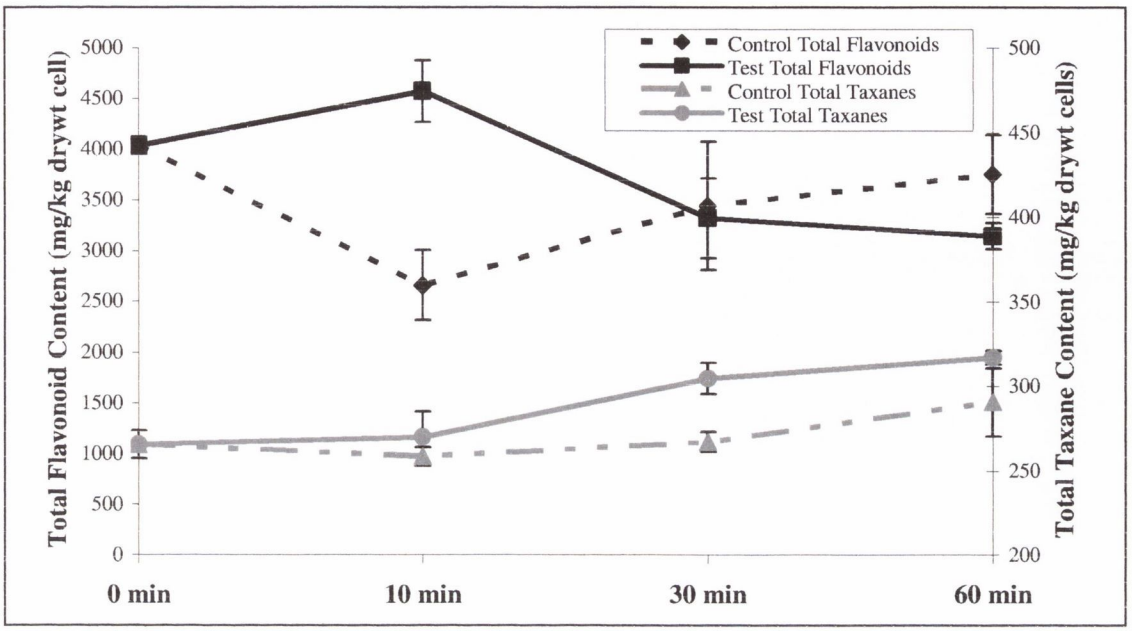


Figure 8.4.4.16 Total taxane and flavonoid production by cells over time

8.4.4.5 Conclusion

Both in *Experiment 4 – 'Long-time'* and *Experiment 5 – 'Short-time'* the addition of the organic peroxide tBH in conjunction with the catalyst hematin was found to have an effect on the secondary metabolism of the *T. b.* 'Fastigiata' cells.

In the *Experiment 5 – 'Short-time'* experiment the burst of flavonoids, especially catechin and epi-catechin, produced by the cells harvested 10 minutes after treatment clearly shows that the tBH stimulates the cells defence system. The physical appearance of the cells treated with the oxidants also changed, with treated cells being darker in colour presumably due to the presence of greater amount of phenolics. In *Experiment 4 – 'Long-time'* there was no sign of a further burst of flavonoids, and the concentration decrease compared to the overall control after 72 hrs, showing that the effect of an oxidative burst on phenolics production is immediate, again suggesting that the flavonoids are produced in order to defend the cells against the detrimental effect of overproduction of AOS. Catechin and *epi*-catechin were found to have a higher scavenging activity than that of the potent scavengers β-carotene, *L*-ascorbate

and superoxide dismutase (SOD) when added as an antioxidant after *E. coli* cells were treated with tBH and hematin (Nakao et al. 1998). The reason for its effectiveness was found to be because the AOS formed in the decomposition of tBH are lipophilic $ROO^{\bullet}$ radicals (Akaike et al. 1992). Catechins have the property of both hydrophilicity and lipophilicity, which enhances their capability to access the radicals formed. SOD, β-carotene and *L*-ascorbate are all potent scavengers of $O_2^{\cdot-}$, $OH^{\bullet}$ and $^1O_2$ (singlet oxygen), but being more hydrophilic they have reduced accessibility, hence they are less effective. Other flavonoids such as rutin and quercetin have also been found to be good scavengers of the AOS produced in the decomposition of tBH (Afanas'ev et al. 1989, Aherne and O'Brien 2000).

The induction of an oxidative burst leading to oxidative stress in the plant cells also affected the taxane production in the cells. In the *Experiment 4 –'Long-time'* the effect of the oxidative burst was clearly seen in the cells harvested after 48 hours. The concentration of both 10-DAB III and baccatin III increased compared to the controls at this harvest time (1.76-fold and 2-fold, respectively). It has to be taken into account that there was a considerable drop in taxane production in the test cells at 24 hours. This probably came as a result of the addition of a "foreign agent" to the cell system. But the increase in total taxane biosynthesis in the test cells was 45% greater than that in the control at 48 hours. Cephalomannine and paclitaxel production did not get affected by the oxidative burst induced in *Experiment 4 – 'Long-time'*. Yuan et al. found that the maximum accumulation of paclitaxel was at 48 hours after addition of the oligosaccharide from *Fusarium oxysporum*, the oxidative burst inducer they used (Yuan et al. 2001). They used electron spin resonance (ESR) to measure the radicals formed in the cells after treatment with the oligosaccharide and found that the difference in ESR signal intensity between oligosaccharide-treated and control cells was apparent at 15 minutes and that the difference in their oxidative burst reached a maximum at 4 hours after the elicitor treatment.

The immediate effect of the oxidative burst was not reflected in any great extent in the taxane production. In *Experiment 5 – 'Short-time'* the concentration of cephalomannine was increased after 30 minutes, but decreased to the normal level after 1 hr, begging the question as to the actual reason for the elevated level after 30

minutes. This observation could be due to the oxidative burst, but could also be due to experimental variation during the analysis. The increased paclitaxel concentration after 1 hour (three-fold increase), however, is much more significant, and would probably be a result of the oxidative burst induced. When *T. chinensis* cultures were treated with a fungal elicitor, Yuan et al (Yuan et al. 2002c) reported that neomycin, an inhibitor of phospholipase C, not only blocked the induction of the oxidative burst it also blocked the biosynthesis of taxanes. In a similar study with *T. chinensis* cultures (Yu et al. 2002) it was reported that when a low concentration (F1), middle-range concentration (F2) and a high concentration (F3) of the same fungal elicitor was used F3 gave a lower paclitaxel production than F2. The oxidative stress was higher in F3 treated cells than in F2 treated cells. But when ascorbic acid was added with F2 to regulate the oxidative burst the paclitaxel production was also lowered. F3 + ascorbic acid had a higher paclitaxel production than F3 on its own. This finding and the finding of Yuan et al (Yuan et al. 2002c) indicates that an oxidative burst is necessary for paclitaxel (and other taxane) production, but if the oxidative burst is too great the effect is negative probably due to membrane damage.

The results of the short-time and the long-time experiment show that accumulation of taxanes is a downstream event of signal transduction resulting from the oxidative burst. The activation of defence genes is a more upstream and immediate response to the treatment.

8.5 Influence of a Liquid-Solid Culture System on Constituent Yields

A poor accumulation of secondary metabolites in cell cultures may not always be due to lack of biosynthetic enzymes, biosynthetic precursors or the right signalling cascade. In many cases it is due to feedback inhibition, degradation of the metabolites produced by enzymes, or it could be due to the actual toxicity of the substances produced. The traditional methods for productivity enhancement, such as cell line selection, medium optimisation, elicitation and metabolic engineering may still be partially limited because of unfavourable conditions for biosynthesis and product metabolism.

One method of overcoming this is to introduce an extracting phase into the cell culture system, a so called secondary metabolite sink. This leads to *in situ* product removal, which diverts the flux away from product metabolism and enhances the metabolic flux towards biosynthesis (Wong et al. 2004).

The most commonly used adsorbents for *in situ* product removal in plant cell culture are the non-ionic Amberlite® XAD resin series. Since the resin will mix completely with the cells, and therefore make it difficult to separate for extraction, two approaches are usually employed. The resin can be immobilised or encapsulated by sodium alginate (Choi et al. 1996), resulting in some loss of the adsorption ability, but simplifying separation of the resin from the cells. The other method is to enclose the resin in a cloth (either nylon, Miracloth® or any other porous material), where it will have almost the same adsorption ability as free resin but allowing easy separation and further processing (Wong et al. 2004).

Choosing an optimum adsorbent depends on trial and error. The XAD-2, -4 and -16 types of Amberlite resin are based on the cross-linked polystyrene structure, and have a hydrophobic character. The XAD-7 type is based on the cross-linked polymethacrylate structure, and is more hydrophilic. It has been suggested that since paclitaxel is highly hydrophobic the XAD-2, -4 and -16 type of Amberlite resin could easily adsorb the taxanes, whereas XAD-7 would not adsorb these compounds as effectively. Kwon et al used the XAD-4 type of the resin for their studies (Kwon et al. 1998). Because of the higher specific surface area of XAD-4 ($725m^2/g$) compared to that of XAD-2 ($300m^2/g$) they chose XAD-4.

For our experiments bags with the XAD-2 type of Amberlite resin were added on day 9/7 and the cells were cultivated for 30/36 days before harvesting.

8.5.1 Preliminary experiment

In a preliminary experiment Miracloth® was used as the resin enclosing material. However, problems with the preparation of those bags occurred, leading to leakage of resin making separation of resin from the cells difficult. This made it impossible to quantify the effect which the resin had on secondary metabolite accumulation in the cells. The preliminary experiment was therefore used to find optimal resin-enclosing materials and optimal conditions for the further experiment. The only effect analysed was that of flavonoid production as a result of stress.

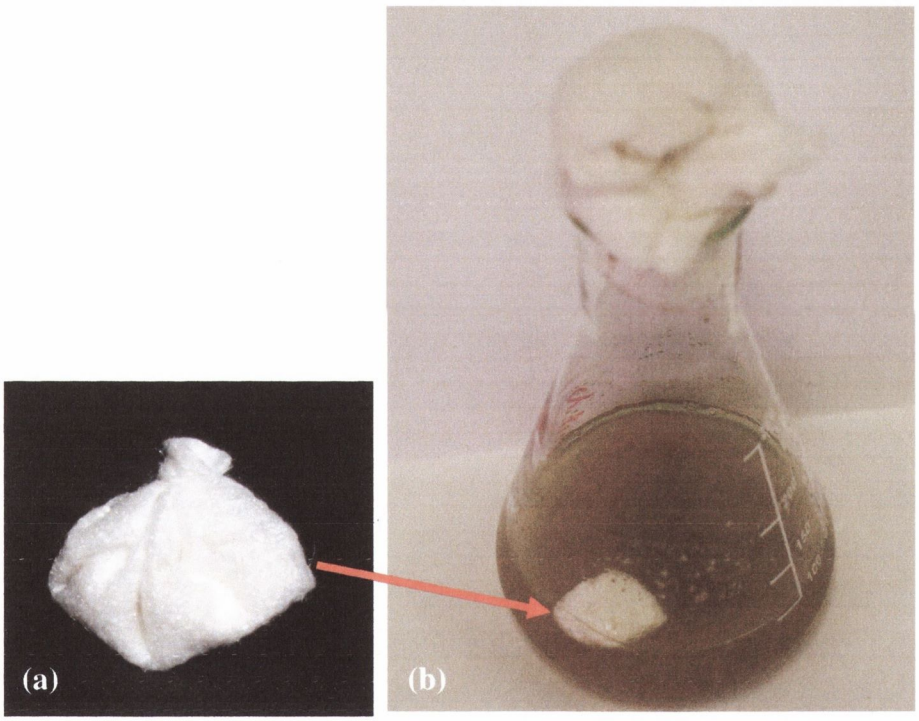


Figure 8.5.1.1 (a) Amberlite reisn bag, (b) Flask with cells and resin bag added

The results are presented in **Table 8.5.1.1** and **Figure 8.5.1.2**. As an additional condition to the preliminary experiment, free resin was added to one flask in order to compare this with the flasks containing enclosed resin. As controls to the cells that contained resin-bags, an empty Miracloth® bag was added.

The following samples were extracted and analysed:

- **Control cells**: the cells growing with no Miracloth ® or resin present
- **Cells with loose resin**: the cells growing with loose resin mixed with the cells (the cells and resin was extracted together as the resin could not be separated from the cells)
- **Cells with control bag**: the cells growing with empty Miracloth® bag present as a control. The cells themselves were extracted.
- **Control bag**: the empty Miracloth® bag material itself (control bag) was extracted in order to see if the material had adsorbed secondary metabolites.
- **Cells with resin bag**: the cells growing with a resin-filled Miracloth® bag present. The cells themselves were separated from the bag and extracted.
- **Resin bag**: the resin-filled Miracloth® bag itself was extracted in order to see if the amberlite adsorbed secondary metabolites.

The resin-filled bag was extracted on its own in order to determine the amount of secondary metabolites absorbed by the resin. The empty bag was also extracted in order to find out if the material itself absorbed secondary metabolites.

The addition of loose resin to the cells resulted in an increased concentration of epi-catechin, taxifolin, quercetin and kaempferol ($P<0.001$) compared to the control. Catechin was the only flavonoid that decreased significantly ($P<0.001$) after addition of loose resin. Since the resin was loose with the cells it is not possible to decide if the flavonoids were absorbed by the resin or if they were extracted from the cells themselves. The results show that the empty Miracloth®-bag absorbed a very small amount of flavonoids. But because of the added shear-stress the bag has on the cells a control containing an empty bag must be included in the experimental design. The cells that had a resin-bag present had a significantly lower concentration of catechin and epi-catechin than the two types of control cells (control cells and cells with control bag). Only trace amounts of the minor flavonoids such as taxifolin, quercetin, kaempferol and amentoflavone were present in the cells with the resin bag present. The analysis of the resin bag and control bag showed that catechin and epi-catechin and the minor flavonoids were also absorbed by the resin and the bag-material.

Table 8.5.1.1 Intracellular flavonoid production in preliminary experiment

Sample	Intracellular Flavonoid Content (mg/kg dry wt. cells)		
	Catechin	**Epi-catechin**	**Taxifolin**
Control	4516.34 ± 367.62	2897.50 ± 194.84	154.15 ± 22.42
Cells with loose resin	1368.87 ± 288.36	4594.89 ± 305.11	531.92 ± 55.97
Cells with control bag	3537.96 ± 28.55	1463.33 ± 101.61	
Control bag	95.56 ± 9.78	93.33 ± 0.89	14.57 ± 2.22
Cells with resin bag	2543.62 ± 55.95	863.33 ± 26.85	
Resin bag	1369.67 ± 17.85	559.84 ± 4.40	790.49 ± 229.52
	Quercetin	**Kaempferol**	
Control	66.87 ± 10.15	48.76 ± 10.09	
Cells with loose resin	416.17 ± 40.32	365.32 ± 32.20	
Cells with control bag	109.44 ± 3.40	117.78 ± 0.00	
Control bag	90.28 ± 10.23		
Cells with resin bag			
Resin bag	277.71 ± 4.17	238.85 ± 9.50	

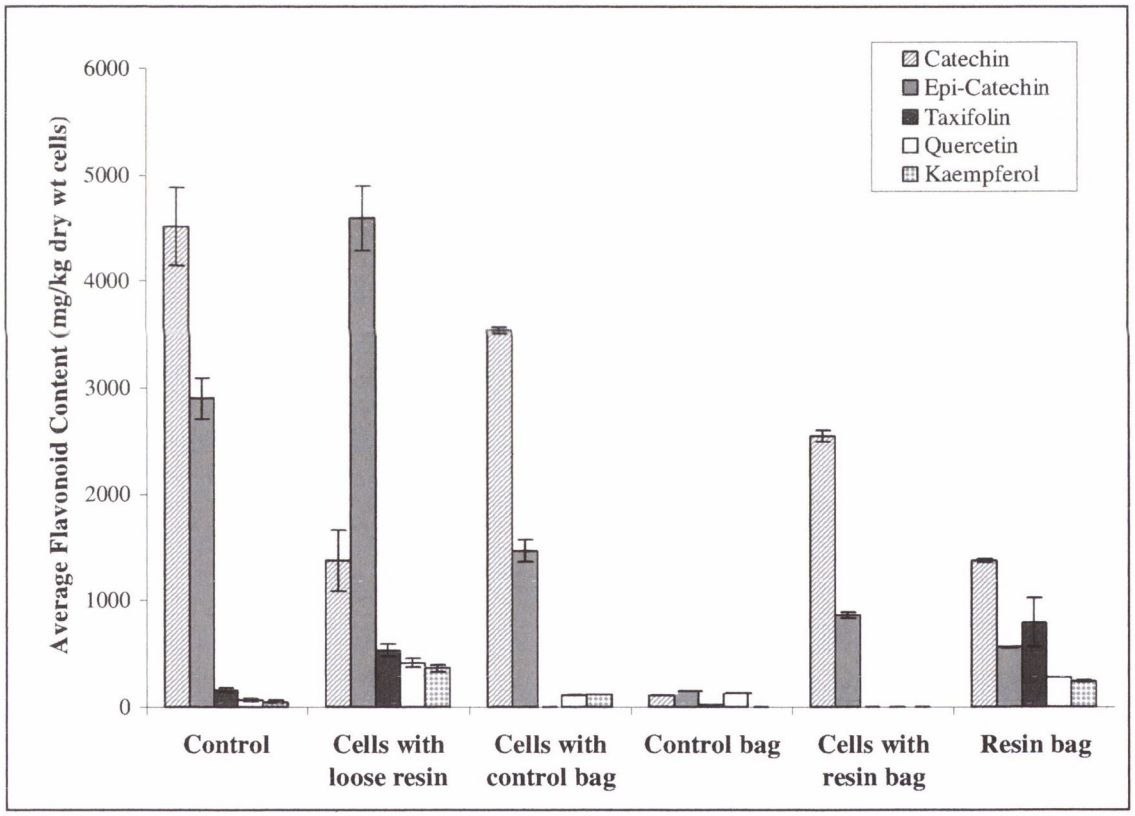


Figure 8.5.1.2 Intracellular flavonoid production in preliminary experiment

For further studies a non-woven fabric consisting of a synthetic non-cellulosic material was used instead of the Miracloth®. Although the substitute material had the same pore-size (typically 22–25 μm) as Miracloth® it was found to give a better closure around the resin and no leaks occurred.

8.5.2 Effect of *in situ* absorption on taxane production

The results of the intracellular taxane production in the final Amberlite experiment are shown in **Figure 8.5.2.1** and in the corresponding **Table 8.5.2.1**. The following samples were extracted and analysed:

- **Control cells**: the cells growing with no Miracloth ® or resin present cells with control bag (empty bag present)
- **Cells with resin bag**: the cells growing with a resin-filled Miracloth® bag present. The cells themselves were separated from the bag and extracted.
- **Resin bag**: the resin-filled Miracloth® bag itself was extracted in order to see if the amberlite adsorbed secondary metabolites.

The taxane concentration from the cells with resin bag, and the taxane concentration from the resin bag were combined to yield the total taxane production by the cells. As can be see from **Table 8.5.2.1** the cell growth is clearly affected by the addition of resin bags or control bags ($P<0.001$). Kwon and co-workers (Kwon et al. 1998) did not comment on any change in the growth of the *T. cuspidata* cells using resin bags. However, Wong and co-workers (Wong et al. 2004) reported that the resin bags did not appear to have any detrimental effect to the growth of the *Catharanthus roseus* cells that they used. The effect the bags had on our cells was probably due to shear stress. The bags could also have altered the hydrodynamics of the cultures. Even though the growth was negatively affected, the cells continued to produce secondary metabolites.

The non-woven material itself (the empty bags) had only a slight effect on the secondary metabolite production of the cells. But due to variation this effect was not statistically significant ($P>0.05$). The same was reported by Wong et al (Wong et al. 2004). However, the results of the experiment will be compared to the control cells with the empty bag present because of the slight effect the empty bags caused. The resin bag addition increased the production of 10-DAB III, cephalomannine and paclitaxel significantly ($P<0.001$) compared to the control cells (both the control without resin present and the control with empty bag present). As the graph in **Figure 8.5.2.1** shows, most of the taxanes were adsorbed onto the resin, only a small amount of cephalomannine and paclitaxel was extracted from the cells themselves. This

indicated that there might be an increase in excretion of taxanes from the cells, but the overall increase would also indicate that there was an enhanced stimulation of the biosynthesis. The total production of cephalomannine had the highest increase, where the cultures with adsorbent produced 4- to 5-fold more than the cultures without adsorbent. The increase in paclitaxel was 4-fold, and the production of 10-DAB III was increased 3.84 times.

Table 8.5.2.1 Intracellular taxane production using XAD-2 Amberlite

Sample	Dry weight (g/l)	Intracellular Taxanes (mg/kg dry wt.)			
		10 DAB III	Baccatin III	Cephalo..	Paclitaxel
Control	5.90 ± 1.30	23.95 ± 2.86	67.96 ± 14.07	88.41 ± 35.18	34.30 ± 3.75
Cells with control bag	3.40 ± 0.80	37.63 ± 11.08	91.19 ± 30.40	187.95 ± 93.46	82.28 ± 17.42
Cells with resin bag	2.60 ± 0.50	-	-	98.39 ± 83.72	146.28 ± 47.73
Resin bag		144.60 ± 25.20	-	751.32 ± 170.51	191.73 ± 21.00
Concentration from cells + resin bag combined		144.60 ± 25.20	-	849.71 ± 165.83	338.01 ± 135.71

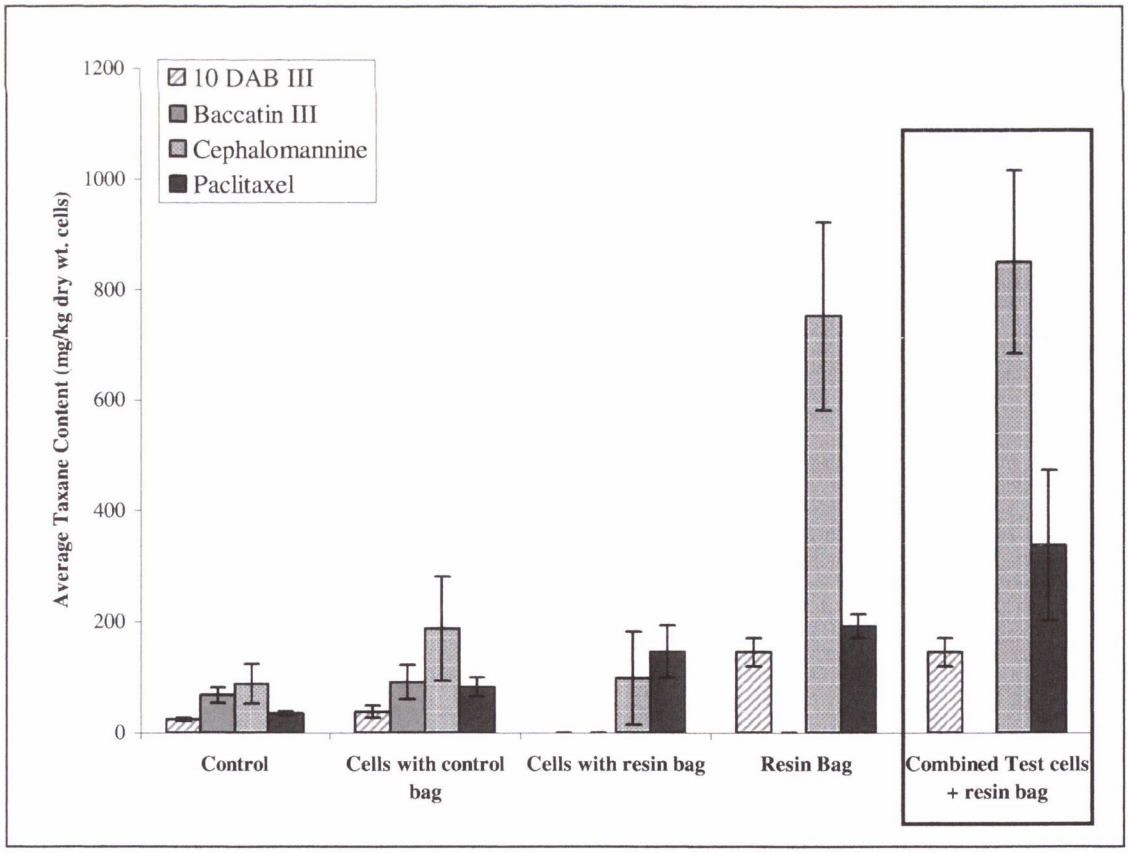


Figure 8.5.2.1 Intracellular taxane production using the adsorbent Amberlite XAD-2

A very apparent change with the use of an adsorbent was that the cells produced a non-identified group of metabolites that masked the baccatin III peak in our HPLC chromatogram. It was at first thought that the interfering compounds were extracted from the bag-material or resin, but a blank extraction ruled out this possibility. We were therefore unable to measure the change in baccatin III production by the cells. Kwon reported that the production of baccatin III by the *T. cuspidata* cells was significantly enhanced by the presence of XAD-4 (Kwon et al. 1998).

In order to see if most of the taxanes were adsorbed onto the resin the medium was extracted. The results are shown in **Figure 8.5.2.2** and the corresponding **Table 8.5.2.2**.

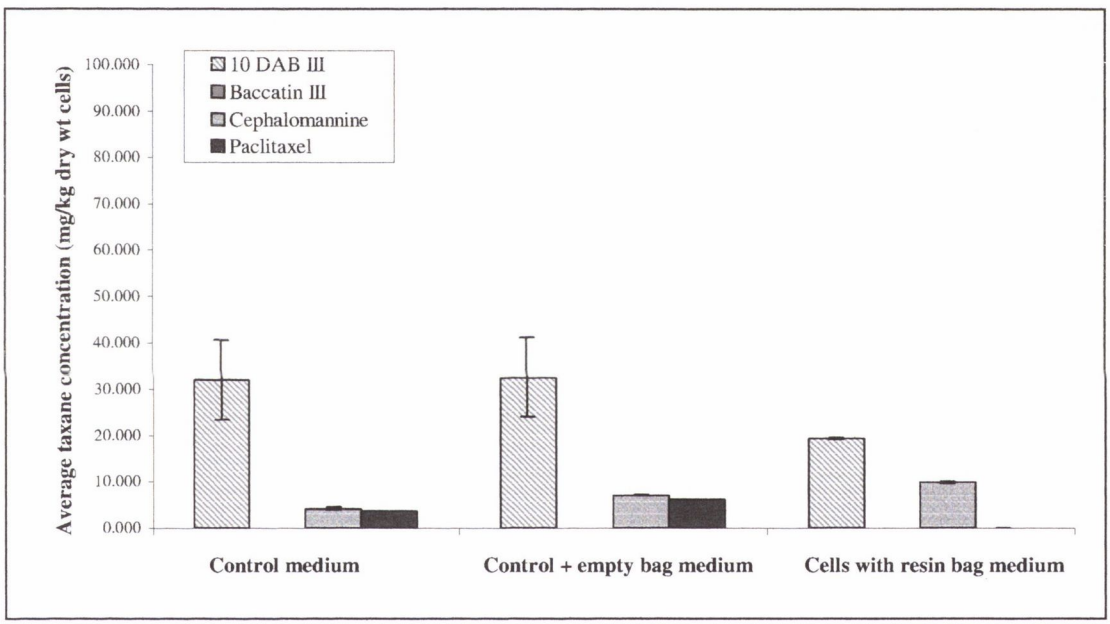


Figure 8.5.2.2 Extracellular taxanes using XAD-2 as an adsorbent

As can be seen from **Figure 8.5.2.2** there was no excretion of baccatin III at all in either of the control cultures or the test cultures. There was only as small amount of excretion of taxanes into the medium even from the control cells. The extracellular total taxane concentration in the test medium (containing the resin bags) was significantly lower ($P<0.01$) than that of the control medium, although the concentration of cephalomannine was slightly higher. This shows that the excretion of secondary metabolites was enhanced using an adsorbent. The resin is in fact acting as a taxane sink.

Table 8.5.2.2 Extracellular taxane production using an adsorbent

Sample	Dry weight (g/l)	Extracellular Taxanes (mg/kg dry wt.)			
		10 DAB III	Baccatin III	Ceph..	Paclitaxel
Control medium	5.90 ± 1.30	32.03 ± 8.57	-	4.19 ± 0.38	3.69 ± 0.02
Control + empty bag medium	3.40 ± 0.80	32.59 ± 8.51	-	7.03 ± 0.12	6.21 ± 0.04
Cells with resin bag medium	2.60 ± 0.50	19.29 0.22	-	9.90 ± 0.13	-

8.5.3 Effect of *in situ* absorption on flavonoid production

The flavonoid production was analysed for the same experiment. The results can be seen in **Table 8.5.3.1** and in the corresponding **Figure 8.5.3.1**.

Table 8.5.3.1 Intracellular flavonoid production, the effect of Amberlite

Sample	Intracellular Flavonoid Content (mg/kg dry wt. cells)		
	Catechin	Epi-catechin	Taxifolin
Control	3084.65 ± 80.76	4563.58 ± 519.38	90.95 ± 17.87
Cells with control bag	9114.08 ± 393.47	6719.90 ± 677.97	221.92 ± 36.15
Control bag	2577.53 ± 242.37	793.19 ± 52.38	
Cells with resin bag	4982.38 ± 604.78	5262.65 ± 745.06	247.47 ± 42.31
Resin bag	785.18 ± 101.08	358.50 ± 124.37	243.17 ± 32.25
	Quercetin	**Kaempferol**	**Amentoflavone**
Control	303.93 ± 52.26	239.36 ± 36.51	
Cells with control bag	622.53 ± 53.70	437.97 ± 67.68	2.59 ± 5.19
Control bag			
Cells with resin bag	346.70 ± 48.60	451.51 ± 53.31	5.67 ± 11.34
Resin bag	353.06 ± 156.3	530.412 ± 86.67	25.40 ± 21.65

It is apparent from these results that the addition of the control bags to the cells resulted in an increase ($P<0.001$) in the production of all of the flavonoids analysed, where the increase in catechin and epi-catechin was 3.79-fold and 1.6-fold, respectively. Taxifolin, quercetin and kaempferol all had a 2-fold increase as a result of the addition of the control bag. Only amentoflavone remained unaffected with only non-quantifiable amounts present in both samples. This increase was probably a result of the extra shear-stress and the alternation of the hydrodynamic of the cultures caused by the bags. As noted in the preliminary experiment it was found that the bag material

itself adsorbed some flavonoids, mainly catechin and epi-catechin. In the cells that were growing with a resin bag present the concentration of catechin and epi-catechin decreased compared to the cell growing with a control bag (2.0 and 1.6 times lower concentrations, respectively ($P<0.001$). The rest of the minor flavonoids did not differ significantly between the two. All of the flavonoids were present in quantifiable amounts in the resin bag itself, showing that the flavonoids were excreted into the medium for then to be adsorbed onto the Amberlite resin.

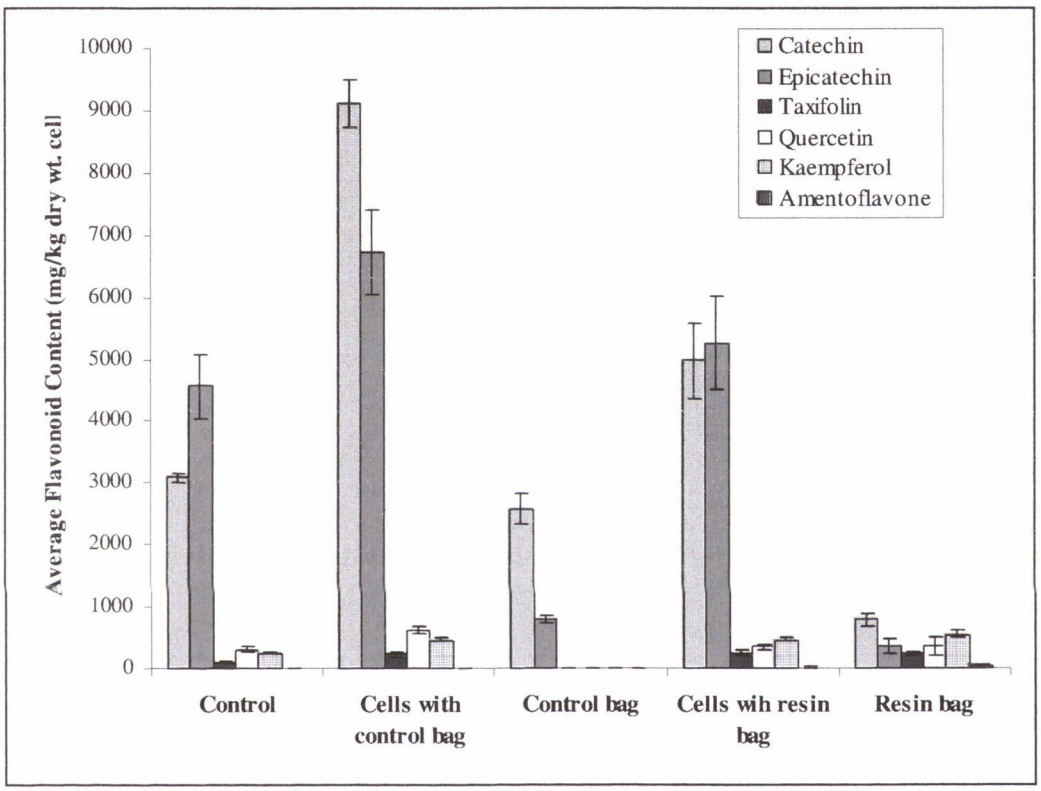


Figure 8.5.3.1 Intracellular flavonoid production, effect of Amberlite

8.5.4 Conclusion

For *in situ* product removal in plant cell culture it has been found that a liquid-solid culture system, consisting of the aqueous growth medium and a solid polar absorbent, is superior to a liquid-liquid system. The reason being that many products of plant cells are polar in nature and bind weakly in the lipophilic phase of liquid-liquid systems (Choi et al. 1996). Robins and Rhodes (Robbins and Rhodes 1986) reported that addition of Amberlite XAD-7 resin stimulated the production of anthraquinones in *Cinchona ledgeriana* 15-fold in comparison to the medium without adsorbent. An improved alkaloid production by *Catharanthus roseus* by in situ recovery has also

been reported (Payne et al. 1988, Wong et al. 2004). Similarly production of sanguinarine by *Papaver somniferum* cell cultures was enhanced using XAD-4 or XAD-7 resins (Archambault et al. 1996a, Archambault et al. 1996b, Williams et al. 1992).

A 4-fold increase in overall taxane production was observed as a result of the addition of Amberlite filled bags to the *T. b.* 'Fastigiata' cultures. It could be concluded that the Amberlite in fact acted like a secondary metabolite sink, adsorbing the taxanes that were excreted into the medium from the cells. Kwon and co-workers (Kwon et al. 1998) investigated the effect of XAD-2, XAD-4, XAD-7, XAD-16 and activated charcoal (Norit-PK1) on the taxane production of *T. cuspidata* cell suspension cultures, where they reported an increase of 40-70% in taxane production by the cells. However, it was also clear that the bags had a negative effect on the growth of the cells during the experiment. This was probably due to an increased level of mechanical stress in the form of shear. The bags could also have affected or altered the hydrodynamics of the cultures.

From the results we found that the bag material itself had only a slight effect on the biosynthesis of taxanes by the cells. This effect was not statistically significant. But the effect the material had on the secondary metabolism of flavonoids was quite considerable. Phenolics are produced as a response to mechanical stress, where the PAL activity increases. Wu and Lin (Wu and Lin 2003) observed an increase in PAL activity as a response to ultrasound treatment of *Taxus chinensis* cell cultures. Ultrasound is a form of mechanical stress that will alter the hydrodynamic of the cells and will lead to an increased level of shear-stress.

8.6 Phytochemical Analysis of *Taxus baccata* 'Fastigiata' Tissue Culture

Extensive analysis and research have been done in order to phytochemically screen the needles, roots and heartwood of various *Taxus* species and hybrids. Significant publications such as the review article by Parmar and co-workers (Parmar et al. 1999) include listings of all chemical constituents isolated from different varieties of yew up to December 1997. Because of the importance of the anticancer activity associated with this species, *Taxus* has become one of the most intensely investigated genus of all the plant genera. However, no further review papers have been published (1998-2005) which include novel constituents isolated. The quest for novel metabolites is ongoing and so far in 2005 11 new constituents have been isolated and characterised from various *Taxus* species (Li et al. 2005, Shen et al. 2005a, Shen et al. 2005b, Xia et al. 2005).

Plant tissue cultures of *Taxus* have not undergone the same degree of phytochemical screening as the tree itself has. Because of the commercial interest in the elicitation and improvement of taxane production, the generation of other secondary metabolites by the *Taxus* cell cultures has not been investigated to any great extent.

In our HPLC analysis of the flavonoid content in the extracts of *Taxus baccata* 'Fastigiata' cell suspension cultures, we found that catechin and epi-catechin were the major flavonoids present. The results were established by comparison with reference data of standards using HPLC. Parmar et al (Parmar et al. 1999) list these 13 flavonoids isolated from different *Taxus* species: ginkgetin, sciadopitysin, sequoiaflavone, sotetsuflavone, kayaflavone, amentoflavone, (+)-taxifolin, kaempferol, quercetin, isorhamnetin, 4',7"-di-*O*-methylamentoflavone, 4',7,7"-tri-*O*-methylamentoflavone and 4',4",7,7"-tetra-*O*-methylamentoflavone. Apart from these, the flavonoid catechin has also been isolated from *Taxus* needles (Chattopadhyay et al. 1999, Chen and Chen 1996, Dempsey 2000), stem wood (Kim et al. 1999) and in cell cultures (Agrawal et al. 2003, Kim et al. 2002, Pyo et al. 2004). The major flavonoids in the extract of the *T. baccata* 'Fastigiata' tissue cultures analysed in the present study were catechin and epi-catechin by comparison with standards HPLC. Further isolation and characterization was necessary in order to verify this finding.

8.6.1 Identification of catechin and epi-catechin from the ethyl acetate extract of *Taxus baccata* 'Fastigiata' cell cultures

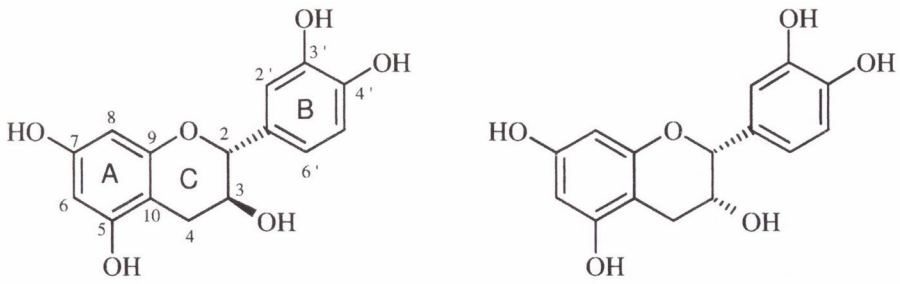


Figure 8.6.1.1 The diastereomers catechin and epi-catechin

Catechin was first isolated from the needles of *T. baccata* 'Fastigiata' by Dempsey (Dempsey 2000). **Figure 8.6.1.2** below shows the ethyl acetate extract of the cell suspension cultures of *T. baccata* 'Fastigiata' compared with the methanol extract and the ethyl acetate extract of the needles from the same tree.

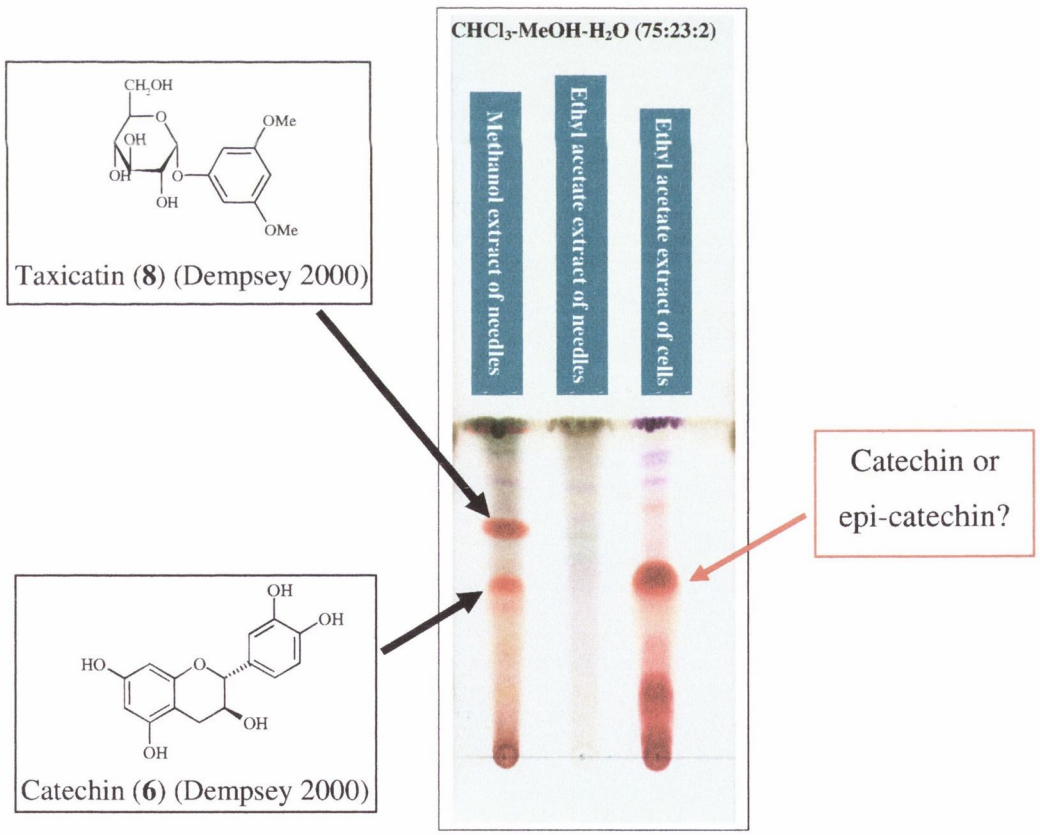


Figure 8.6.1.2 Comparison of needle and cell extracts from *T. baccata* 'Fastigiata'

TLC analysis of both butanol and ethyl acetate extracts of *T. baccata* 'Fastigiata' cells compared with standards of the flavonoids analysed by HPLC (for HPLC chromatogram see **Figure 8.2.2.2**. in section 8.2.2) indicated that catechin and epi-catechin were present (**Figure 8.6.1.3**). TLC plates both turned an intense red colour when sprayed with 1% vanillin-sulphuric spray reagent followed by heating at 110°C for 10 minutes. The Rfs (~0.53) of the two diastereomers were very close in the solvent system used ($CHCl_3$-MeOH-H_2O (75:23:2)).

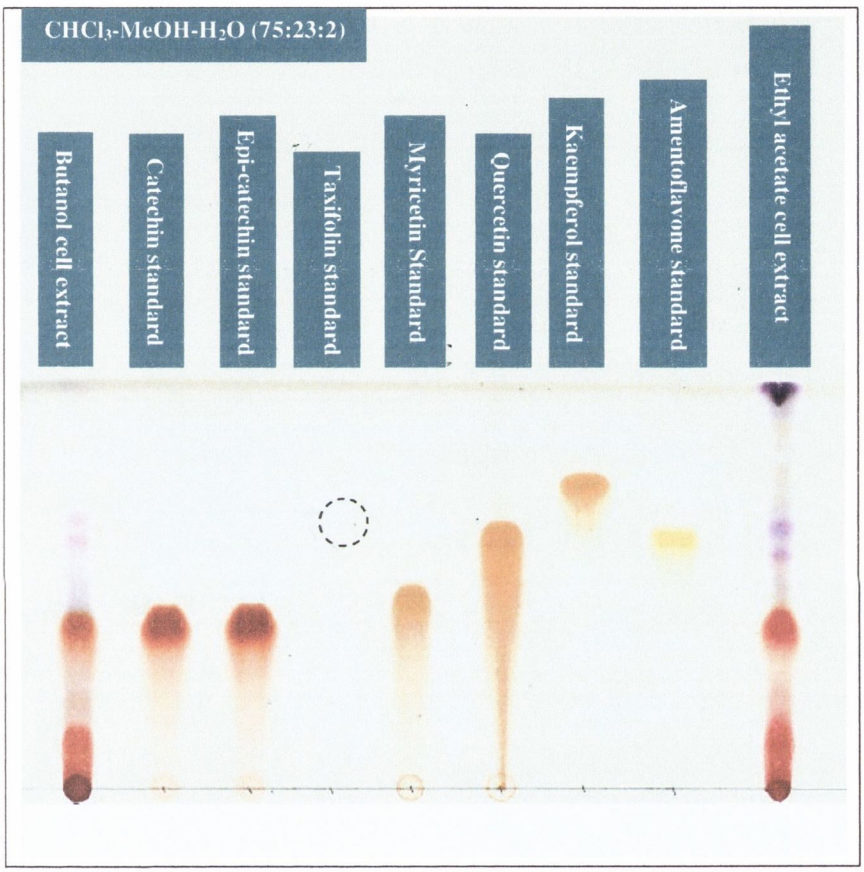


Figure 8.6.1.3 Cell extracts compared to flavonoid standards

Catechin and epi-catechin were first isolated as a mixture by column chromatography (See **Figure 7.7.6.1** in Materials and Methods). Separation was found impossible due to the very close structural similarity, leading to similar elution times of the silica gel column. The ethyl acetate extract was then subjected to preparative TLC (see section 7.7.2 in Material and Methods). From this the major component was isolated as an off-white solid. The solid turned a dark orange colour on standing. The unknown was

further analysed by NMR. The ^{13}C NMR and ^{1}H NMR spectra obtained were compared with ^{13}C NMR and ^{1}H spectra of standards of both catechin and epi-catechin. The ^{13}C NMR data is presented in **Table 8.6.1.1** and the comparison of ^{13}C spectra can be seen in **Figure 8.6.1.4** and **Figure 8.6.1.5**. The full sets of NMR spectra (^{1}H, ^{13}C, DEPT and 2D) of catechin standard, epi-catechin standard and the unknown isolated can be found in Appendix II, III and IV.

Table 8.6.1.1 ^{13}C-NMR of the major component compared to standards

Carbon	Catechin standard (TCD) in DMSO (ppm)	Epi-catechin standard in (TCD) DMSO (ppm)	Literature value of catechin (Shen et al. 1993)	Literature value of epi-catechin (Shen et al. 1993)	Unknown in DMSO (ppm)
C-7	156.49	156.59	156.4	156.3	156.45
C-5	156.21	156.28	156.1	156.5	156.22
C-9	155.39	155.83	155.3	155.8	155.34
C-3'	144.87	144.54*	144.8	144.4*	144.83
C-4'	144.87	144.49*	144.8	144.5*	144.83
C-1'	130.60	130.66	130.7	130.7	130.60
C-6'	118.47	118.01	118.4	118.0	118.40
C-5'	115.10	114.93	115.1	114.8	115.06
C-2'	114.53	114.81	114.5	114.9	114.51
C-10	99.07	98.53	99.2	98.6	99.01
C-6	95.10	95.08	95.3	95.2	95.07
C-8	93.86	94.12	94.0	94.2	93.80
C-2	81.02	78.10	81.0	78.1	80.98
C-3	66.33	64.95	66.4	65.0	66.29
C-4	27.91	28.28	27.7	28.2	27.87

*Assignments may be interchangeable

The ^{13}C- NMR spectra of catechin and epi-catechin show similar carbon signals for the phloroglucnol A-ring and the catechol B-ring, but slight differences in signals for the pyran C-ring. The spectra contained signals of one methylene group, two aliphatic –CH groups, five aromatic –CH groups, one aliphatic –C-OH group, four aromatic –C-OH groups and three quaternary carbons. Catechin differs from epi-catechin by the two downfield signals for C-2 and C-3 (δ 81.02 and 66.33) compared to the more upfield signals of epi-catechin (δ 78.1 and 64.95). In the ^{13}C-NMR spectrum of the unknown the signals for C-2 and C-3 appear at the same downfield position as for catechin (δ 80.98 and 66.29). But, as indicated on the ^{13}C spectrum, there are traces of the signals corresponding to epi-catechin in these positions (δ 78.04 and 64.89).

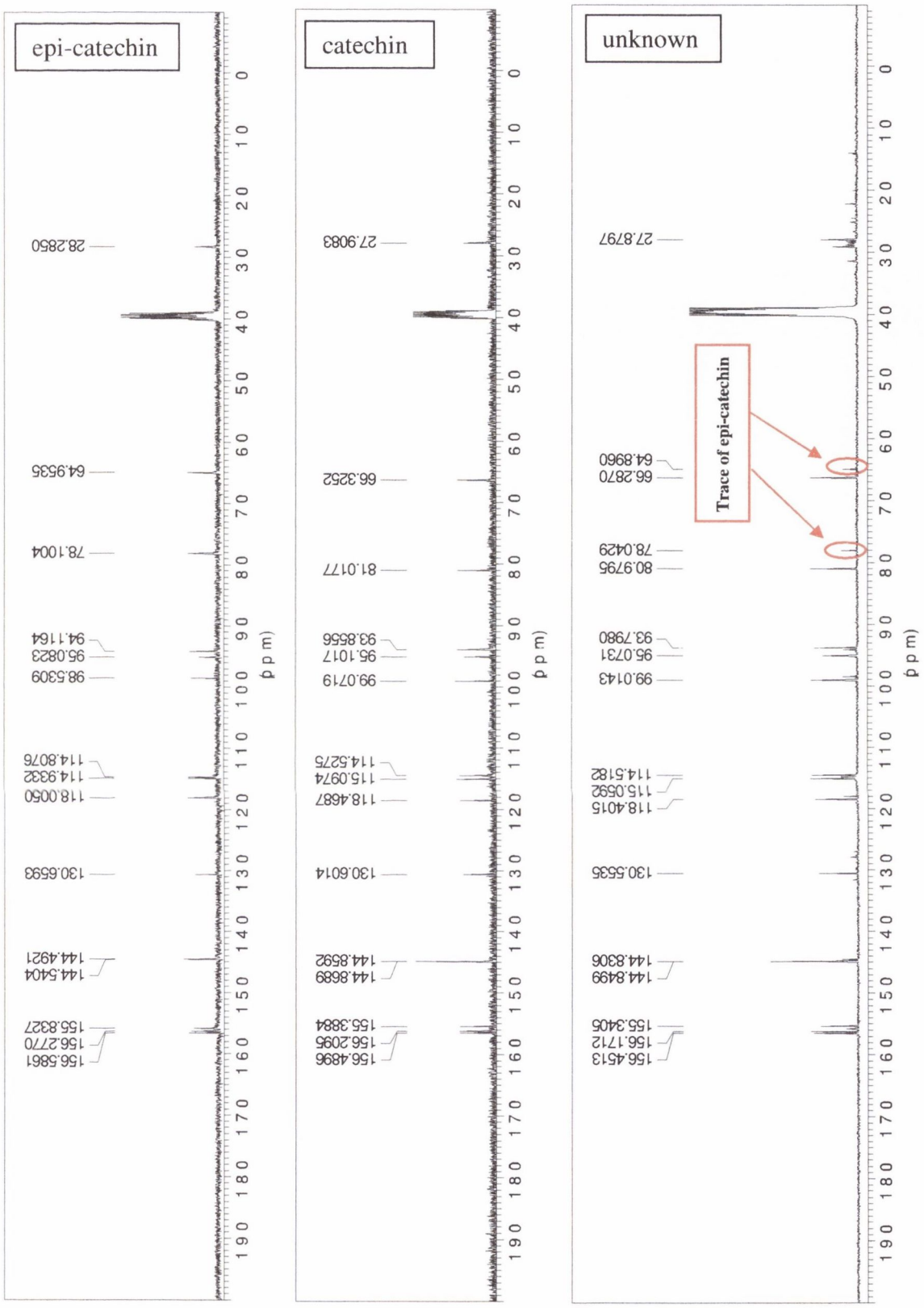


Figure 8.6.1.4 ^{13}C-NMR of catechin, epi-catechin and the unknown (in DMSO)

The ^{1}H-NMR data is presented in **Table 8.6.1.2** and the comparison of ^{1}H spectra can be seen in **Figure 8.6.1.6** and **Figure 8.6.1.7 and Figure 8.6.1.8**.

Table 8.6.1.2 ^{1}H-NMR of "Band 1" compared to standards of catechin and epi-catechin (in DMSO)

Proton	**Catechin** (Shen et al. 1993) **(ppm)**	**Epi-catechin** (Shen et al. 1993) **(ppm)**	**Unknown (ppm)**
H-2'	6.74 (d, *J* = 1.9 Hz)	6.91 (s)	6.89 (d, *J* = 1.0 Hz)
H-5'	6.70 (d, *J* = 8.0 Hz)	6.68 (s)	6.69 (m)
H-6'	6.61 (dd, *J* = 8.0, 1.9 Hz)	6.68 (s)	6.69 (m)
H-6	5.90 (d, *J* = 2.2 Hz)	5.91 (d, *J* = 2.3 Hz)	5.91 (d, *J* = 2.0 Hz)
H-8	5.72 (d, *J* = 2.2 Hz)	5.75 (d, *J* = 2.3 Hz)	5.70 (dd, *J* = 12.6, 2.0 Hz)
H-2	4.51 (d, *J* = 7.3 Hz)	4.75 (s)	4.48 (d, *J* = 7.5 Hz)
H-3	3.84 (m)	4.03 (m)	3.84 (m)
H-4α	2.68 (dd, *J* = 16.0, 5.3 Hz)	2.70 (dd, *J* = 16.4, 4.4 Hz)	2.65 (dd, *J* = 16.1, 5.5 Hz)
H-4β	2.38 (dd, *J* = 16.0, 7.9 Hz)	2.50 (dd, *J* = 16.4, 3.5 Hz)	2.35 (dd, *J* = 16.1, 8.0 Hz)
3-OH	4.75 (d, *J* = 4.7 Hz)	4.56 (d, *J* = 4.7 Hz)	4.88 (d, *J* = 4.0 Hz)
4 x OH	-	-	~9 (m)

The large coupling constants observed for the H-2 signal in the ^{1}H-NMR spectrum of catechin (δ 4.48, *J* 7.53 Hz) indicates a *trans*-orientation between H-2 and H-3 in this molecule, whereas the broad singlet for the corresponding H-2 proton (δ 4.73) in epi-catechin suggests a *cis*-orientation between H-2 and H-3.

In the ^{1}H-NMR spectrum of the unknown the coupling constant for the doublet of the H-2 signal is the same as that of catechin (δ 4.48, *J* 7.53). There is, however, a small singlet at a more downfield position corresponding to that of the H-2 signal of epi-catechin (δ 4.73). For the H-3 signal there is a broad singlet/multiplet at the same position as the corresponding peak in catechin (δ 3.84), but also here there is a smaller singlet at the same position as that found in epi-catechin (δ 4.0).

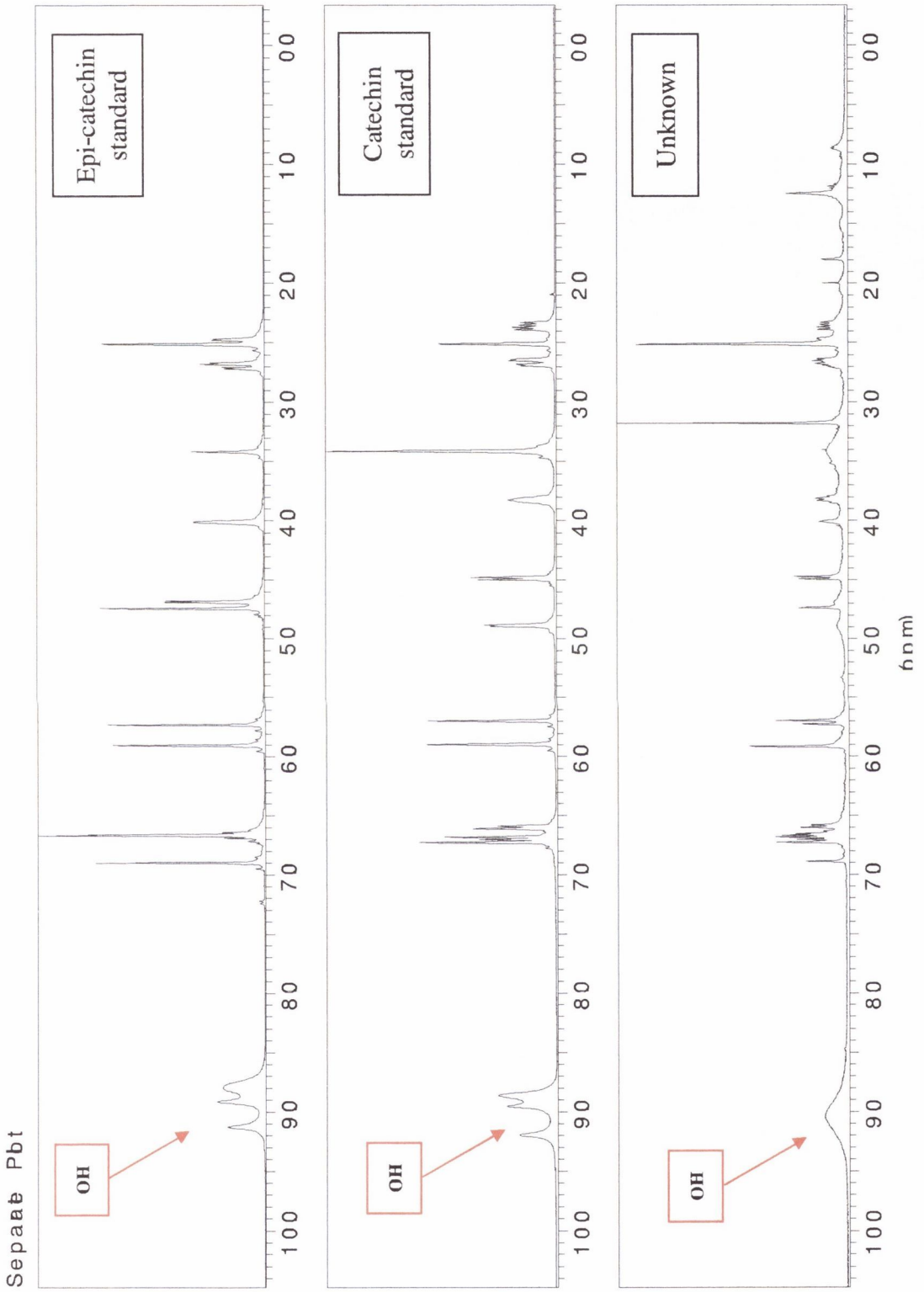


Figure 8.6.1.5 ^{1}H-NMR of the unknown compared to catechin and epi-catechin standards (in DMSO)

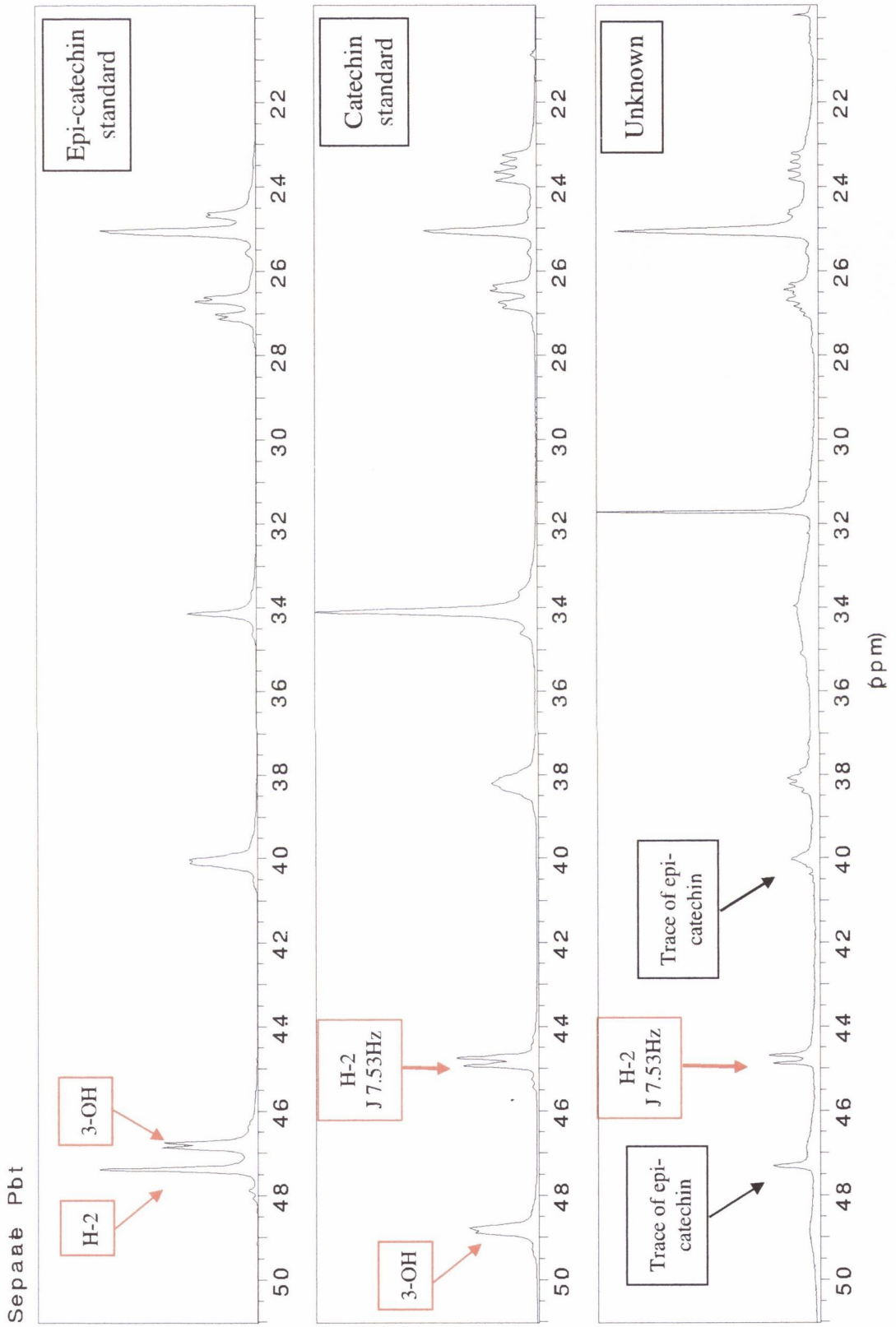


Figure 8.6.1.6 Expansion of ^{1}H-NMR of the unknown compared to catechin and epi-catechin standards (in DMSO)

These observations in conjunction with those of the ^{13}C-NMR suggested the assignment of the unknown as mainly catechin, but there were still traces of epi-catechin present. Because of the close structural relationship between these two diastereomers a complete separation was not possible. Quantification of the ratio of epi-catechin to catechin was done by integration of the catechin and epi-catechin signals in the ^{1}H NMR spectrum of the unknown. It was found that a 2:1 relationship between catechin and epi-catechin was present.

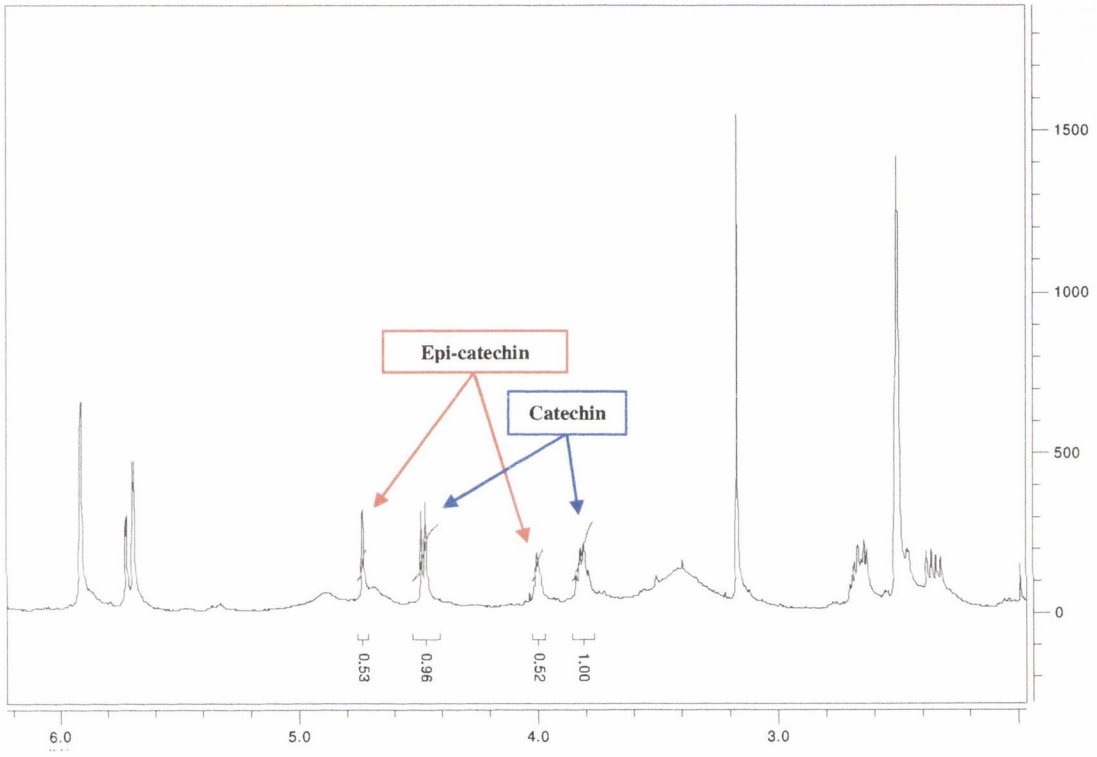


Figure 8.6.1.7 Ratio of epi-catechin to catechin

It was considered that the unknown could be a dimer of the two components with catechin and epi-catechin linked forming a procyanidin. There are various forms of procyanidins commonly present in plants. Procyanidin B1 is an example where the epi-catechin is linked to catechin as shown on **Figure 8.6.1.8**. However, Shoji et al (Shoji et al. 2003) report that the signal for C-10 in the epi-catechin unit will be downfield at 102.1 ppm, and the signal for C-4 will be at 36.6 ppm in a procyanidin B1 type dimer. In the ^{13}C-NMR spectrum of the unknown there are no signals corresponding to these. Shoji et al (2003) also report that C-8 in the catechin unit is shifted downfield to 107.6 ppm in such a dimer. This signal is absent in the spectra of the unknown. The dimeric form of catechin and epi-catechin would also have a more

rigid structure causing broadening of the signals in the NMR spectra. For these reasons the isolate was identified as a mixture of catechin and epi-catechin monomers in ratio of 2:1, where the major component is catechin.

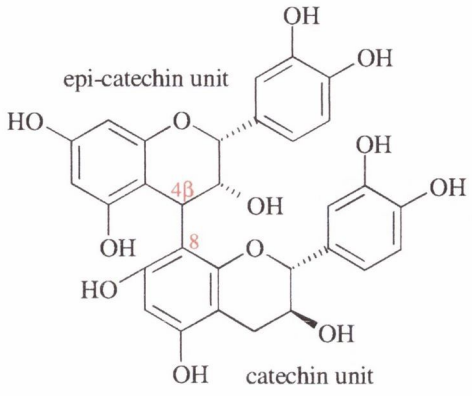


Figure 8.6.1.8 Procyanidin B1 (epicatechin-(4β→8)-catechin)

Catechin (**6**) was first isolated from the needles of Irish yew (*T. baccata* 'Fastigiata') by Dempsey (Dempsey 2000). Catechin has also been isolated from the stem wood of *T. cuspidata* 'Contorta', *T. cuspidata* 'Nana' (Kim et al. 1999) and the needles of *T. yunnanensis* (Chen and Chen 1996) and *T. wallichiana* (Chattopadhyay et al. 1999). During the development of a purification method of paclitaxel from plant cell cultures of *T. chinensis* catechin was found to be the major phenolic constituent present (Kim et al. 2002, Pyo et al. 2004), but no structure elucidation data was presented in these publications. Agrawal *et al* (Agrawal et al. 2003) isolated (+)-catechin in the form of its penta-acetate from the callus cultures of *T. wallichiana*. They found that the cells themselves acetylated the flavonoid. However, they did not isolate catechin from the cell culture. To our knowledge the present study is the only report of isolation of catechin and epi-catechin from cell cultures of *T. b.* 'Fastigiata'.

8.6.2 Isolation and structure elucidation of polar compound from *Taxus baccata* 'Fastigiata' cell cultures

TLC analysis of the ethyl acetate extract of the cell cultures of *T. baccata* 'Fastigiata' highlighted the presence of one (possibly more) very polar compound (R_f 0.19). It can clearly be seen in **Figure 8.6.2.1** where it turned an intense red colour when sprayed with 1% vanillin-sulphuric spray reagent followed by heating at 110°C for 10 minutes. Further investigation was carried out in order to isolate and characterise this compound.

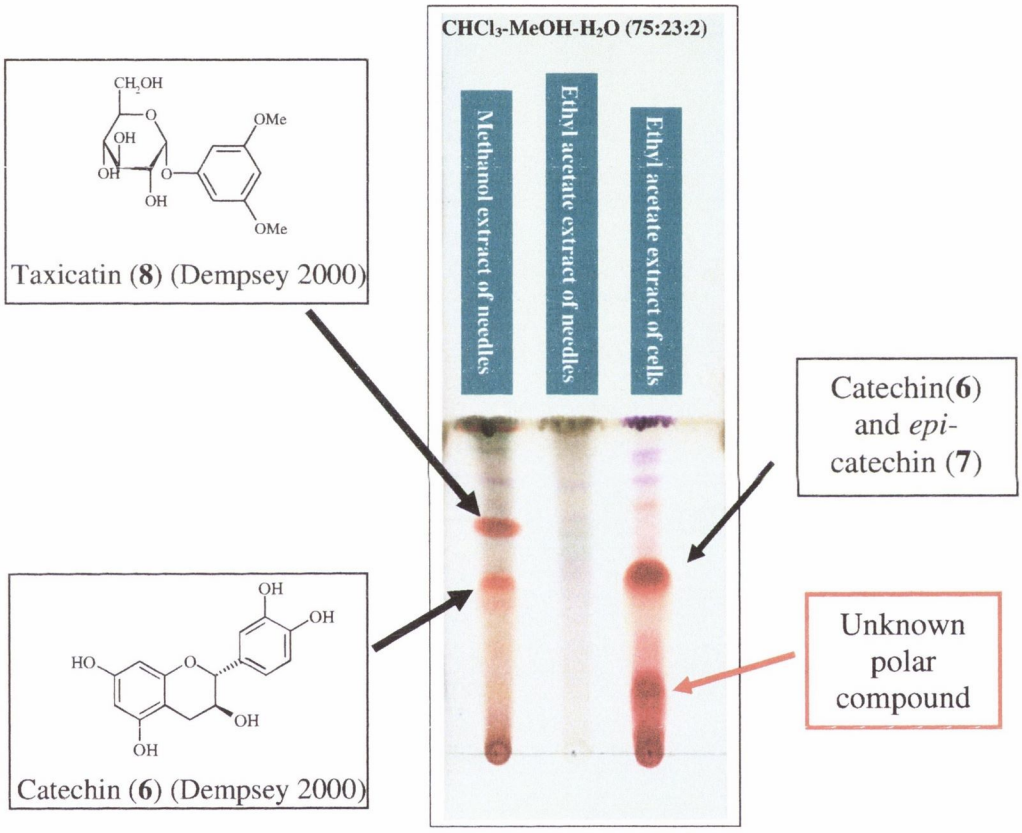


Figure 8.6.2.1 Comparison of needle and cell extracts from *T. baccata* 'Fastigiata'

The ethyl acetate extract of the cells was subjected to column chromatography, and a fraction containing a mixture of the very polar compounds was isolated. In order to separate the compounds the fraction was subjected to preparative HPLC using the HPLC system used for the separation of flavonoids (see Materials and Methods, section 7.7.4). However, the polar compounds were found to be very unstable, and

decomposition occurred. The ethyl acetate extract was also acetylated prior to subjection to column chromatography, but no pure fraction could be obtained.

Due to lack of ethyl acetate residue, the following procedures were conducted using the butanol extract of *T. baccata* 'Fastigiata' cell cultures, which had a similar TLC profile to the ethyl acetate extract. This can be seen in **Figure 8.6.1.3**, where the two extracts were compared to flavonoid standards. A part of the butanol extract was hydrolysed and extracted with ethyl acetate. A sample of that extract was subjected to preparative TLC, but the bands isolated were complex mixtures, again indicating decomposition and instability.

Due to the unstable nature of the isolate on silica gel, gel filtration chromatography using Sephadex LH-20 was explored as a means of separation (section 7.7.8 in Materials and Methods). The gel filtration chromatography of the crude butanol extract led to the isolation of a pure fraction. This fraction was analysed by NMR and Mass spectroscopy (MS). The ^{1}H-NMR is shown in **Figure 8.6.2.2**. The ^{13}C-NMR spectra can be seen in **Figure 8.6.2.3,** and the DEPT 135 and DEPT 90 spectra are shown in **Figure 8.6.2.4**. The isolate was also acetylated and analysed by NMR and MS. However, the resulting mass spectrum showed that the acetylation was incomplete.

The broadness of the peaks in both the ^{1}H-NMR spectrum and in the ^{13}C-NMR spectrum suggested that the isolated compound had a ridged structure. The nature of the compound; its instability on silica gel, its polarity and the broadness of the proton and carbon signals in the NMR spectra led us to believe that the compound was a proanthocyanidin. Comparison the NMR spectra of the isolate with various proanthocyanidins showed similarity between our isolate and the procyanidins, one of the groupings within the proanthocyanidins.

Proanthocyanidins, or more commonly known as condensed tannins, are oligomeric and polymeric end products of the flavonoid biosynthetic pathway. They are present in the fruit, leaves, bark and seeds of many plants, where they provide protection against predation. Proanthocyanidins are responsible for the flavour and astringency of beverages such as wine, beer, fruit juices and teas.

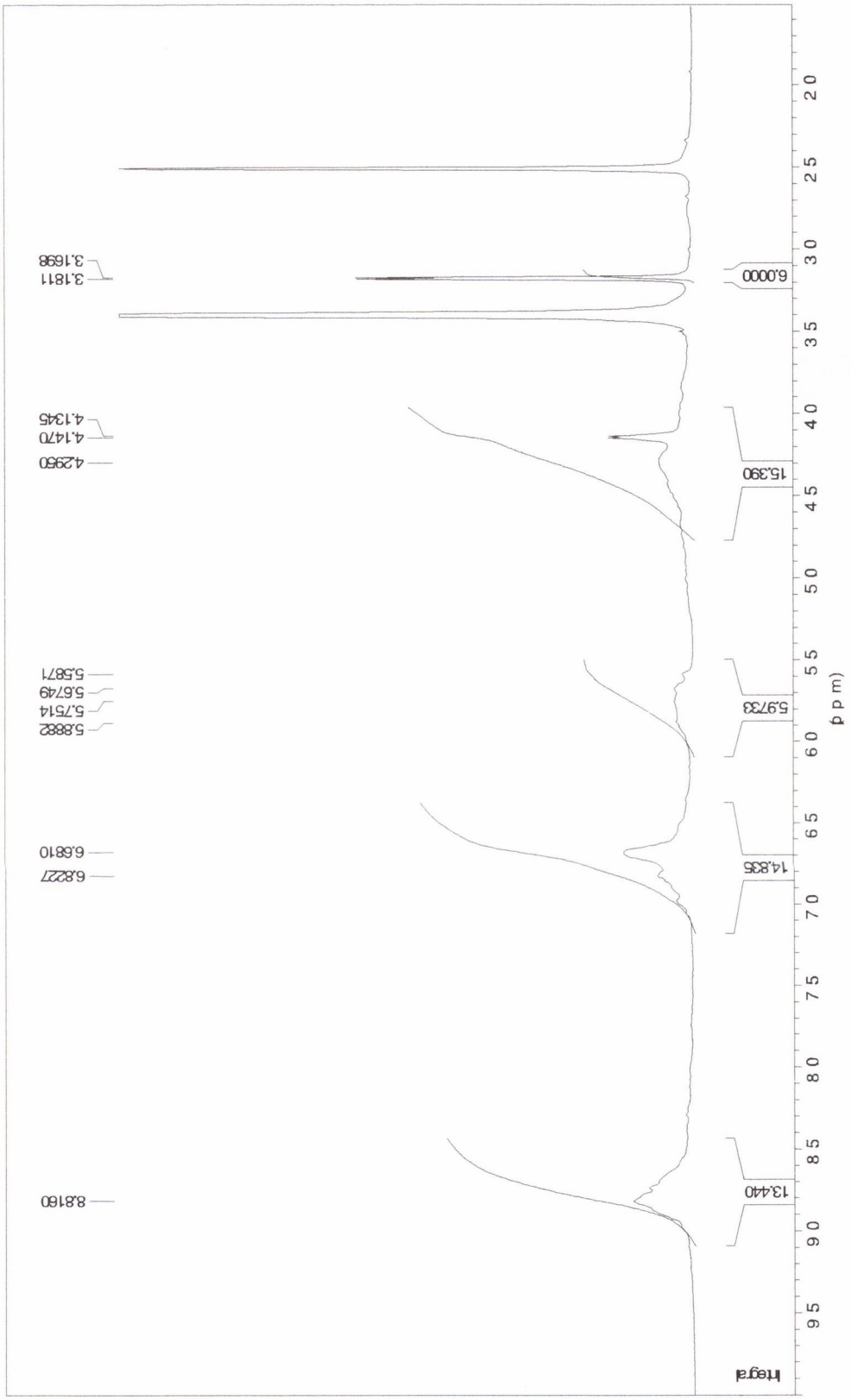


Figure 8.6.2.2 ^{1}H NMR of isolate from Sephadex column (in DMSO)

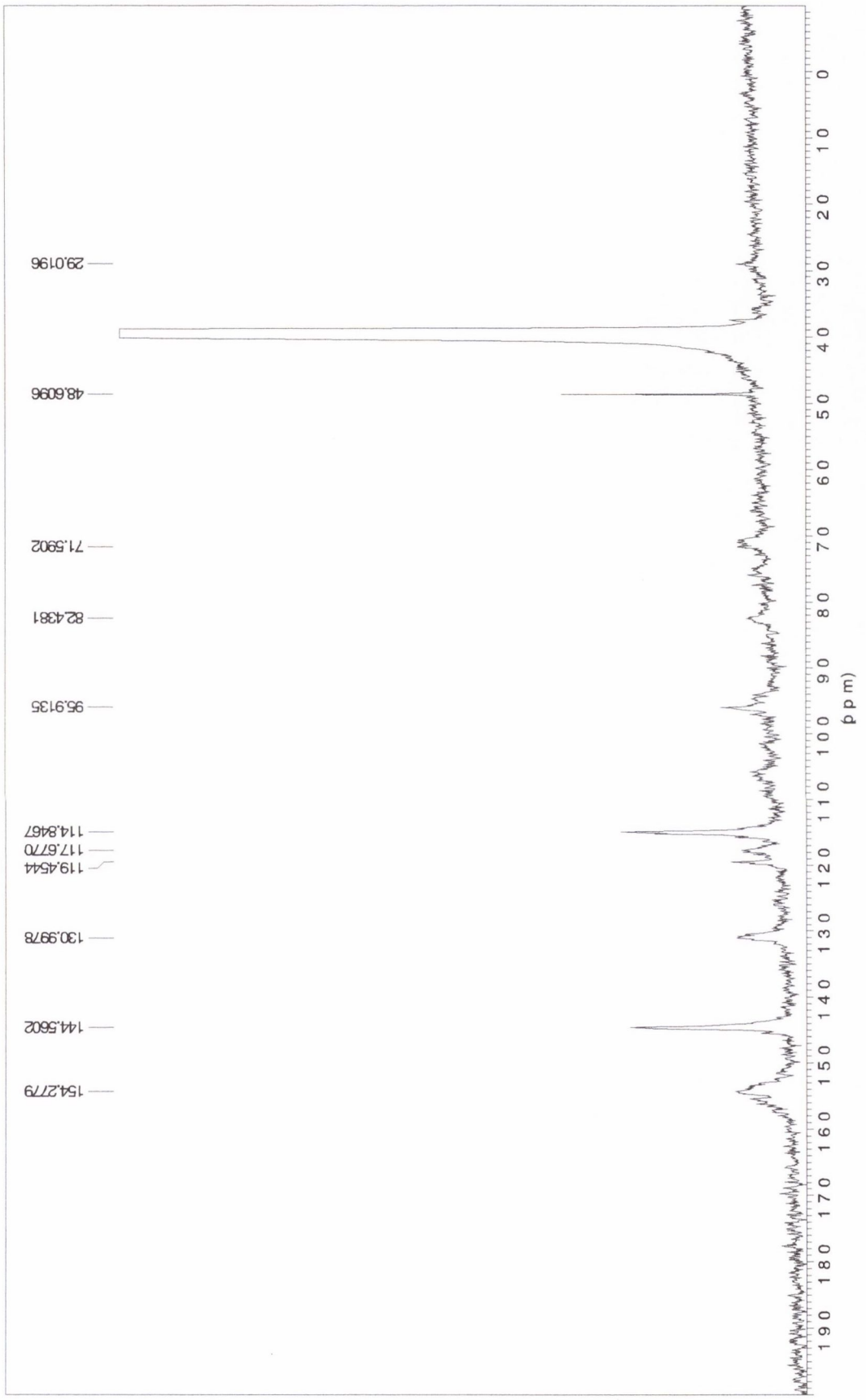


Figure 8.6.2.3 ^{13}C-NMR of isolate from Sephadex column (in DMSO)

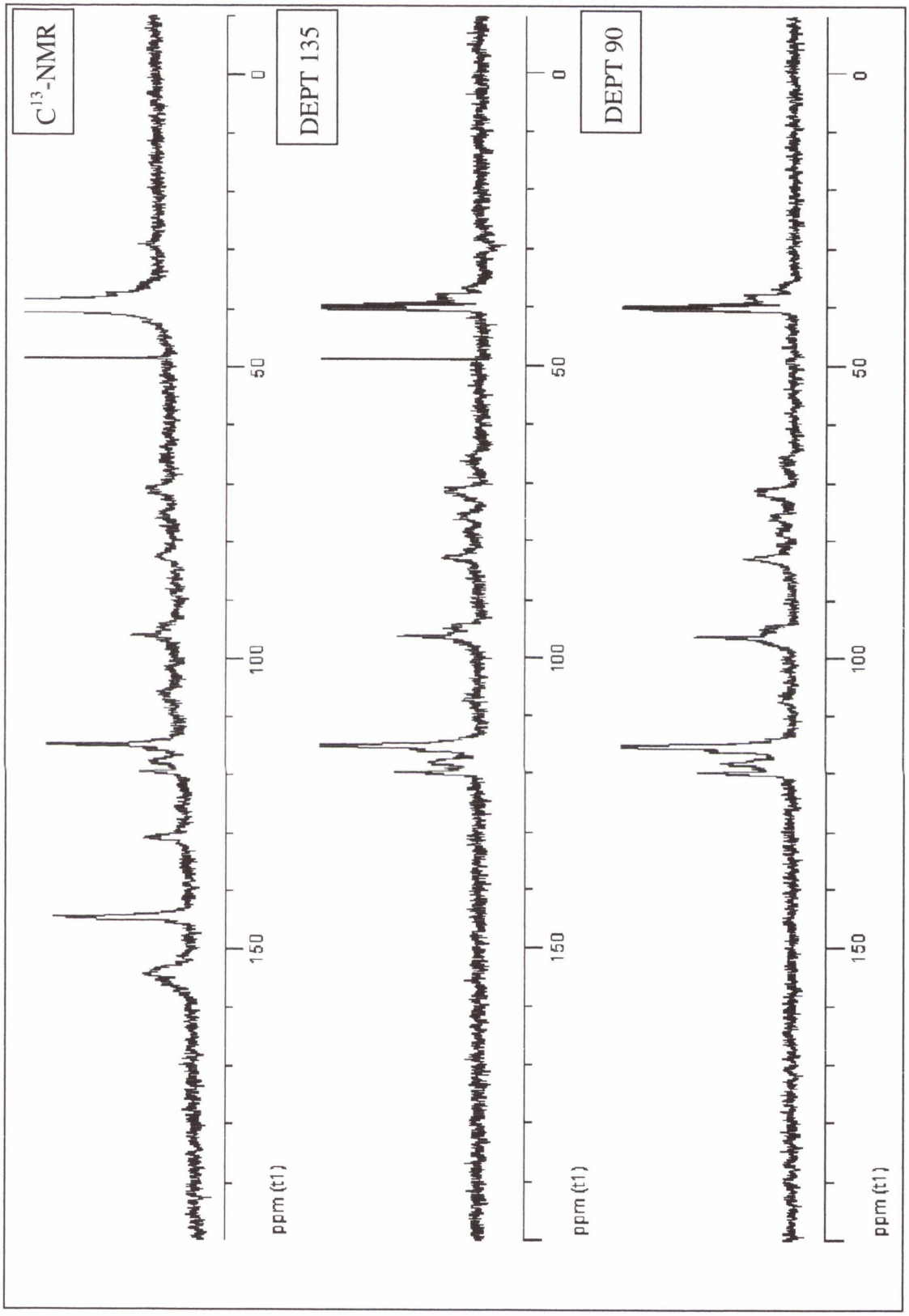


Figure 8.6.2.4 DEPT spectra of isolate from Sephadex column (in DMSO)

Support for the procyanidin nature of the isolate came from the mass spectral analysis. Electrospray Ionisation Mass Spectrometry (ESI MS) and Matrix Assisted Laser Desorption Ionization Time-of-flight Mass Spectrometry (MALDI-TOF MS) are the two mass spectrometry methods mostly used in the analysis of proanthocyanidins at present (Taylor et al. 2003). The isolate was analysed by ESI MS, and the expected and observed $[M + Na]^+$ ions are compared to the ions observed by Taylor and co-workers in their analysis of hops extracts (Taylor et al. 2003) in **Table 8.6.2.1**.

Table 8.6.2.1 Expected and observed $[M + Na]^+$ in ESI mass spectra

Degree of Polymerisation	Expected m/z	Observed m/z of isolate in present study	Observed m/z (Taylor et al. 2003)
2	601.9	601.0	602.7
3	889.8	888.9	888.4, 888.6
4	1178.0	1176.9	1176.9, 1170.0, 1178.5
5	1466.3	1464.9	1464.4, 1465.8, 1466.7
6	1754.6	-	1752.6, 1752.8, 1755.2
7	2042.8	-	2040.5, 2042.4, 2042.8
8	2331.1	-	2328.2, 2331.4

Previous studies on procyanidins by mass spectrometry found that lower molecular weight species produced the most abundant ions (Taylor et al. 2003). This was also observed in the mass spectrum of the isolate where the m/z at 601.0 (2 units) was the most abundant signal. The highest m/z observed was 1464.9, corresponding to five flavon-3-ol units. **Figure 8.6.2.5** illustrates the fragmentation pattern of the dimer procyanidin B1 or B2 (Holt et al. 2002).

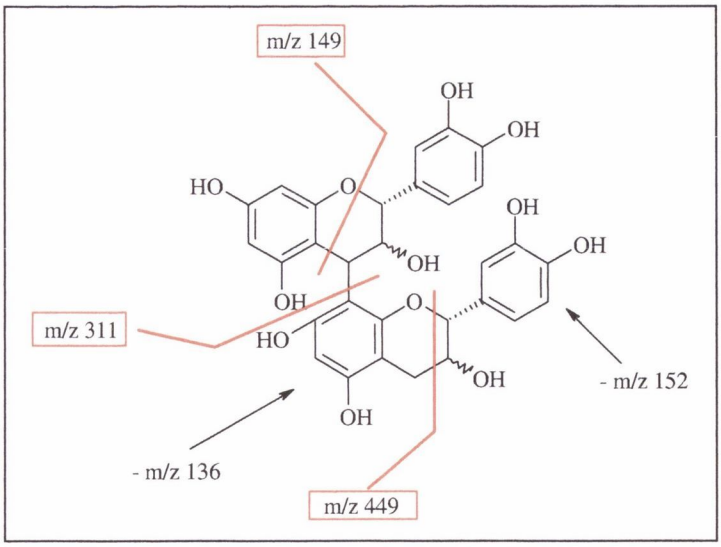


Figure 8.6.2.5 Fragmentation pattern of procyanidin B1 or B2 (Holt et al. 2002)

These ions did show up in the mass spectrum of the isolate, but were not the most abundant signals. No conclusion could be made as to the actual linkage form between the two flanon-3-ol units (4β→8 or 4β→6 interflavonoid bonds (**Figure 8.6.2.6**)) or as to the identity of the two units themselves (catechin or epi-catechin) from the MS.

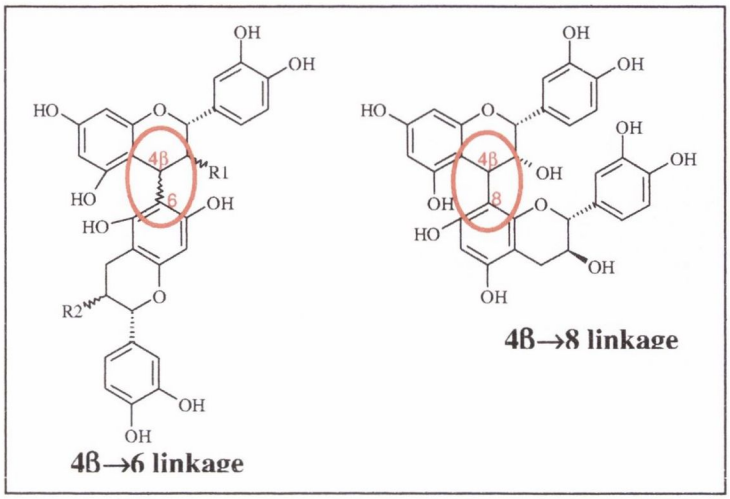


Figure 8.6.2.6 Interflavonoid bonds (4β→8 or 4β→6)

Vivas and Glories (Vivas and Glories 1996) found that the interflavonoid linkage type of the dimer investigated could be assigned from the HMBC experiment, because of the visible correlations corresponding to the long range couplings between the proton linked to C4 and the carbon C8a.

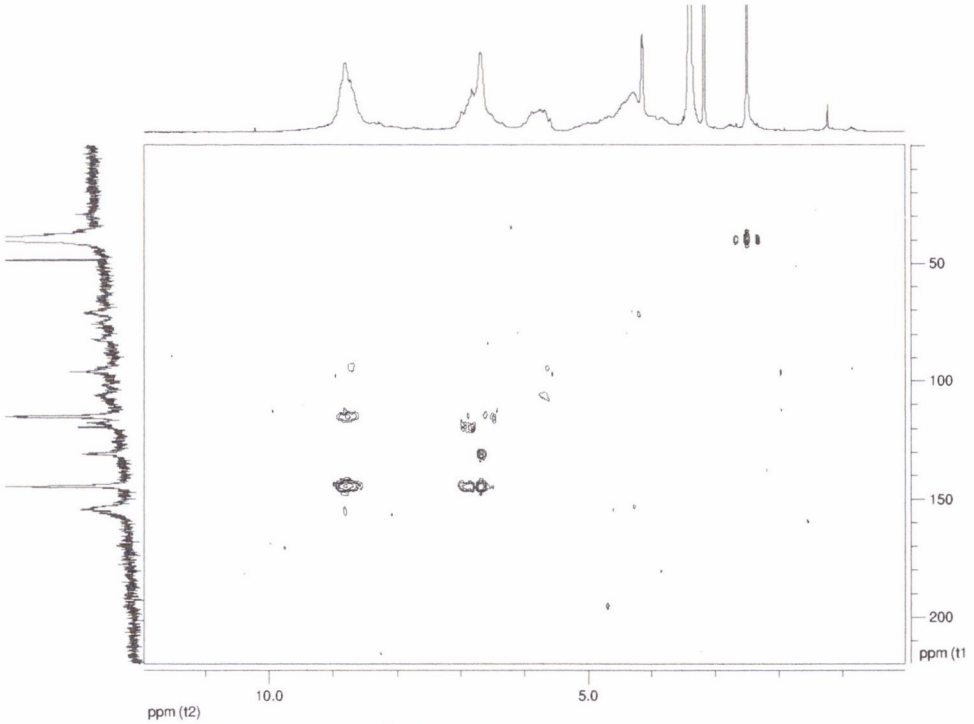


Figure 8.6.2.7 HMBC of isolate (in CD_3OD)

The linkage form could not be determined from the HMBC experiment of the isolate investigated in the present study. This was possibly due to the larger size (pentamer instead of a dimer) causing a more ridged structure and also giving more linkages with similar resonances causing overlap of signals etc. **Figure 8.6.2.7** shows the HMBC spectrum.

The structure of proanthocyanidins varies depending upon the nature of the flavon-3-ol starter and extension unit in the compound, on the position and stereochemistry of the linkage to the "lower unit", the degree of polymerisation and the presence or absence of modifications such as esterification of the 3-hydroxyl group. **Table 8.6.2.2** show some of the groupings of compounds within proanthocyanidins (Harborne 1994), but other variations also occur.

Table 8.6.2.2 Groupings within proanthocyanidins

Proanthocyanidin class	Monomer unit	Substitution pattern						
		3	5	7	8	3'	4'	5'
Procassinidin	Cassiaflavan	H	H	OH	H	H	OH	H
Proapigeninidin	Apigeniflavan	H	OH	OH	H	H	OH	H
Proluteolinidin	Luteoliflavan	H	OH	OH	H	OH	OH	H
Protricetinidin	Tricetiflavan	H	OH	OH	H	OH	OH	OH
Prodistenidin	Distenin	OH	OH	OH	H	H	H	H
Propelargonidin	Afzelechin	OH	OH	OH	H	H	OH	H
Procyanidin	Catechin	OH	OH	OH	H	OH	OH	H
Prodelphinidin	Gallocatechin	OH	OH	OH	H	OH	OH	OH
Proguibourtinidin	Guibourtinidol	OH	H	OH	H	H	OH	H
Profisetinidin	Fisetinidol	OH	H	OH	H	OH	OH	H
Prorobinetinidin	Robinetinidol	OH	H	OH	H	OH	OH	OH
Proteracacinidin	Oritin	OH	H	OH	OH	H	OH	H
Promelacacinidin	Mesquitol	OH	H	OH	OH	OH	OH	H

Figure 8.6.2.8 below shows an overview of the biosynthetic pathway for the formation of proanthocyanidins. The exact mechanism of the condensation reaction in the formation of the dimers, oligomers or polymers is still under investigation (Dixon et al. 2005, Xie and Dixon 2005). There is still debate to whether or not the mechanism in the condensation reaction forming the proanthocyanidins is enzymatic or non-enzymatic.

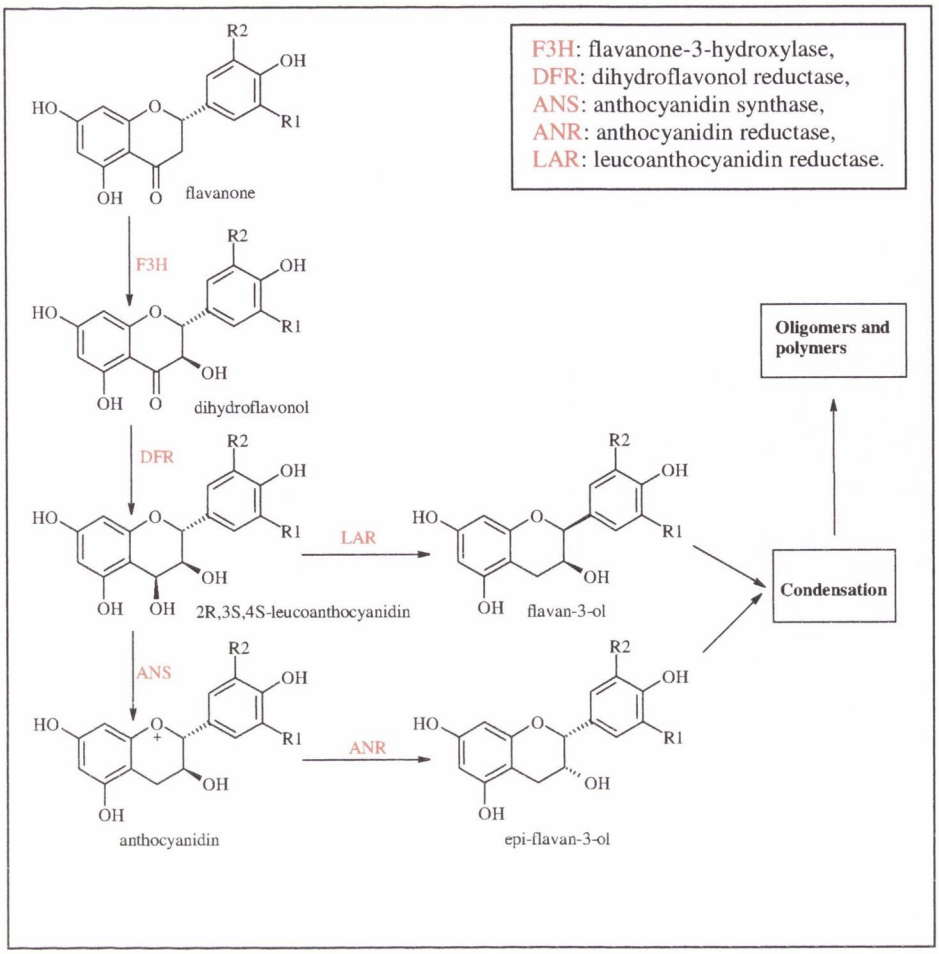


Figure 8.6.2.8 Biosynthetic pathway for the formation of proanthocyanidins

The normal procedure for characterisation of procyanidins would be NMR analysis of their peracetate or methyl ether acetates in conjunction with high-resolution mass spectrometry (Shoji et al. 2003). However, the actual structural determination of procyanidins by NMR has been proved difficult due to the broadening of the ^{1}H NMR signals at ambient temperature. This is due to atropisomerism, which results from steric interactions in the vicinity of the interflavonoid bond about which the flavonoids are free to rotate (Shoji et al. 2003).

Shoji and co-workers (Shoji et al. 2003) overcame this problem by running the NMR analysis at lower temperatures (down to -40°C). The broadening of the NMR signals disappeared, and the assignment of ^{1}H and ^{13}C NMR could be completed. This temperature experiment was performed on the isolate sample and for comparative

purposes on a standard sample of procyanidin B1 (epicatechin-(4β→8)-catechin) in order to obtain narrower ^{1}H NMR signals (**Figure 8.6.2.9** and **Figure 8.6.2.10**).

The change in the ^{1}H NMR spectrum of the procyanidin standard B1 could clearly be seen only by changing the temperature from room temperature to zero degrees (**Figure 8.6.2.9**). The signals were considerably narrower than what they were at room temperature. At -40°C the signals had the resolution necessary in order to completely assign the protons. The ^{1}H NMR data compared to literature values (Shoji et al. 2003) is presented in **Table 8.6.2.3**.

Table 8.6.2.3 ^{1}H NMR spectral data for procyanidin B1 standard in CD_3OD at -40°C

Position	Procyanidin B1 (present study)	Procyanidin B1 (Shoji et al. 2003)
A Unit		
2	5.01 (d, J=4.63Hz)	4.96 (d, J=5Hz)
3	4.20 (m)	4.16 (m)
4	2.53 (dist. d) 2.62 (dist. dd)	2.53 (d, J=17Hz) 2.58 (dd, J=17, 4Hz)
6	5.85 (s)	5.82 (s)
2'	6.84 (br s)	6.82 (br s)
5'	6.73 (br s)	6.69 (d, J=8Hz)
6'	6.92 (dist. d, J=6.18Hz)	6.88 (d, J=8Hz)
B Unit		
2	5.14 (s)	5.10 (br s)
3	3.95 (br s)	3.92 (br s)
4	4.68 (br s)	4.65 (br s)
6	5.92 (br s)	5.92 (br s)
8	5.94 (br s)	5.94 (br s)
2'	6.89 (br s)	6.85 (br s)
5'	6.71 (br s)	6.68 (d, J=8Hz)
6'	6.671 (dist. d, J=2.46)	6.67 (d, J=8Hz)

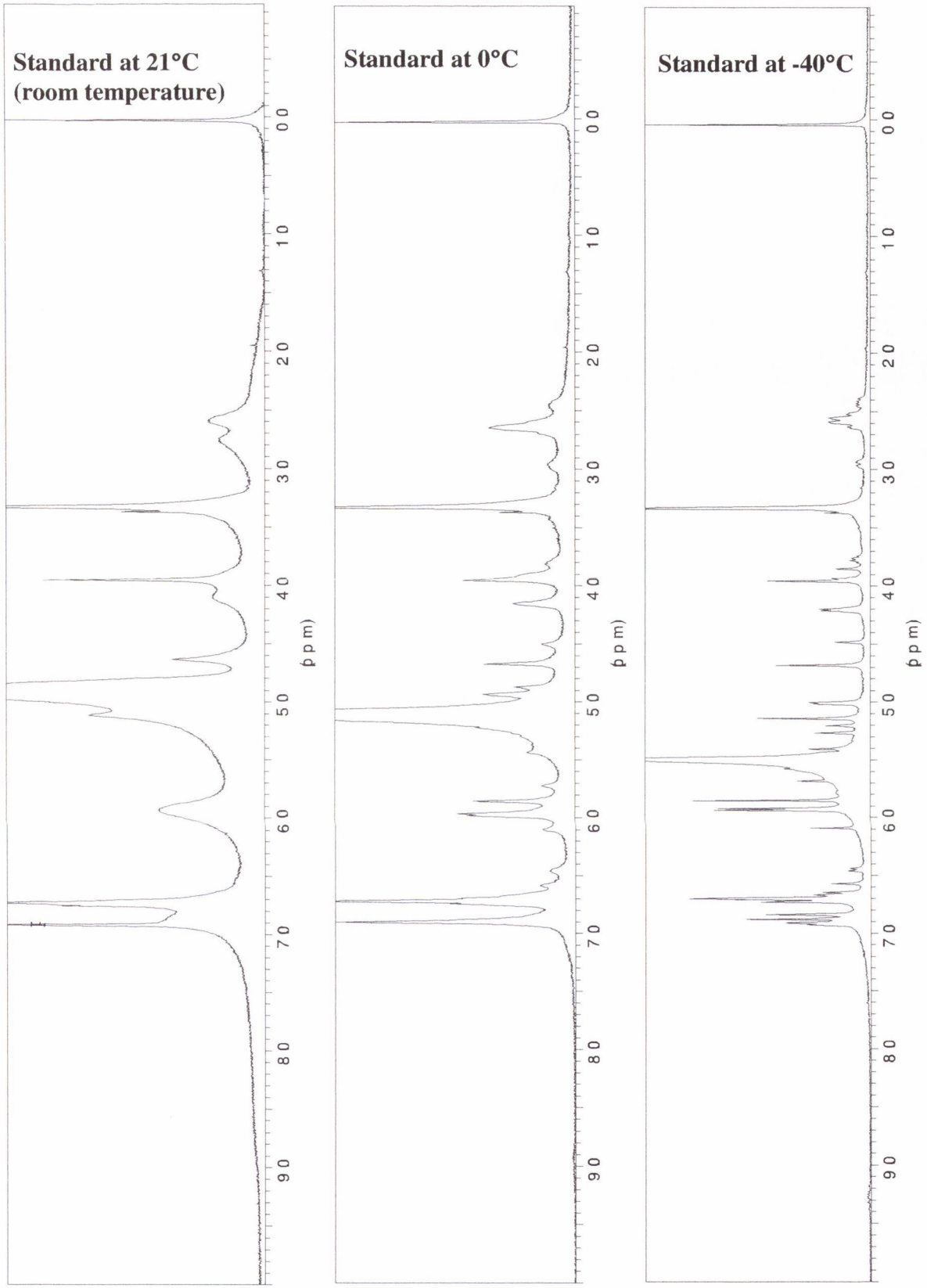


Figure 8.6.2.9 Low-temperature NMR (^{1}H-NMR) of procyanidin B1 standard (CD_3OD)

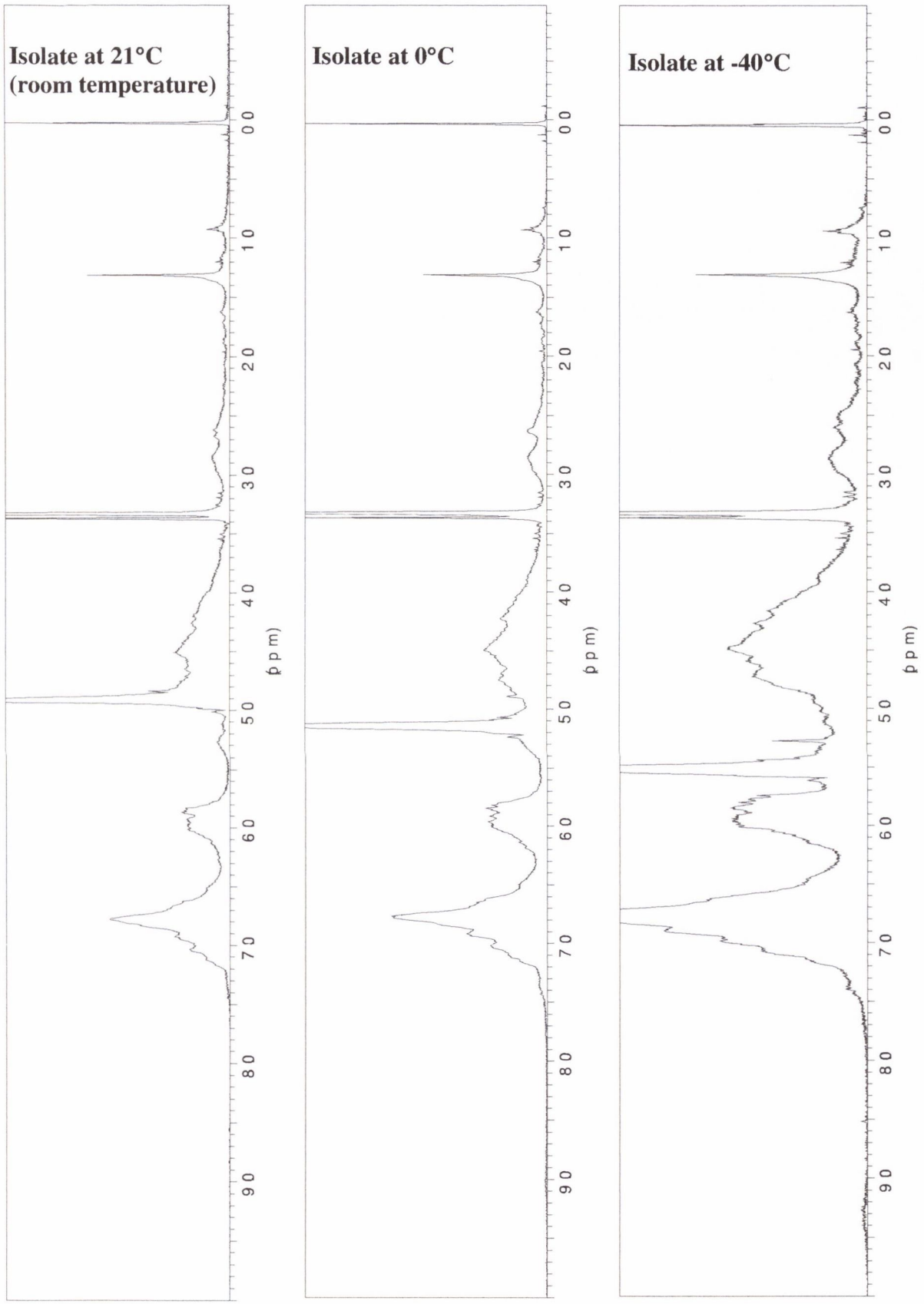


Figure 8.6.2.10 Low-temperature NMR (^{1}H-NMR) of the isolate (CD_3OD)

The low temperature did not have the same effect on the ^{1}H NMR signals of the isolate (**Figure 8.6.2.10**). A small effect could be observed, but not to the same extent as for the standard. This indicated that the unknown was not a dimer, and that it probably had a higher degree of polymerisation.

Upon acid hydrolyis (5% HCl) of the isolate in the presence of *tert*-butanol, cyanidin was formed. This was verified by comparison with cyanidin standard by TLC and HPLC (see procedure in Materials and Methods, section 7.3.4). The formation of cyanidin showed that the compound was a procyanidin, i.e. the flavon-3-ol units where catechin and/or epi-catechin units (Foo and Lu 1999). The mechanism for this formation is shown in **Figure 8.6.2.11**.

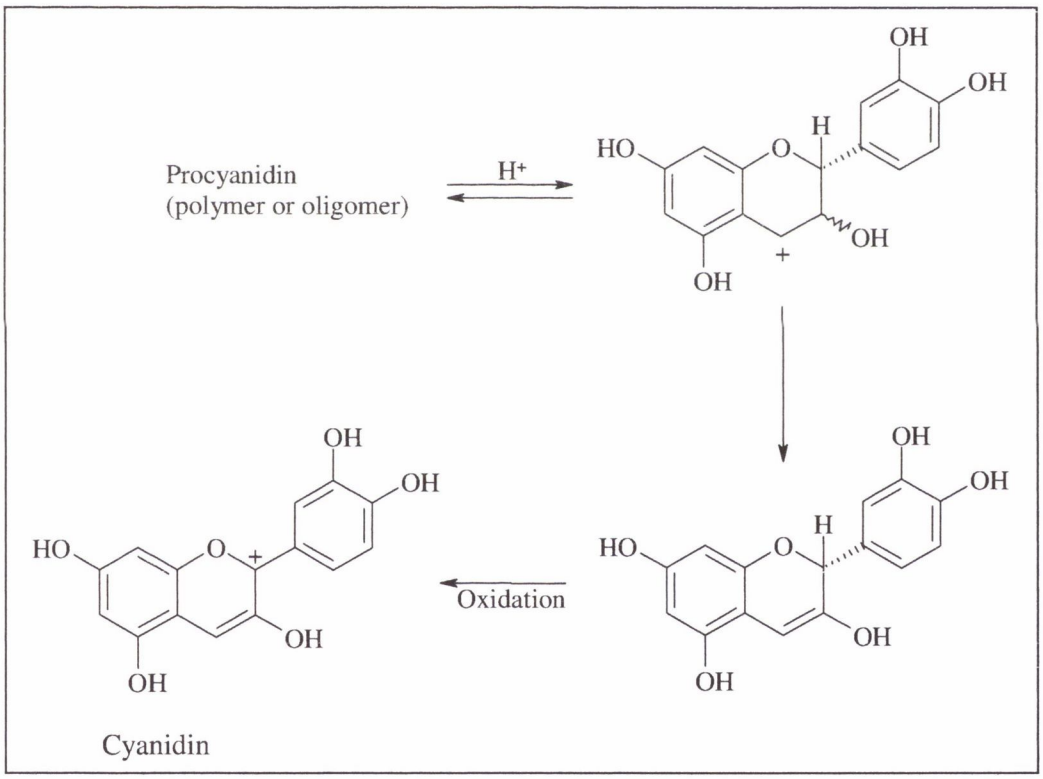
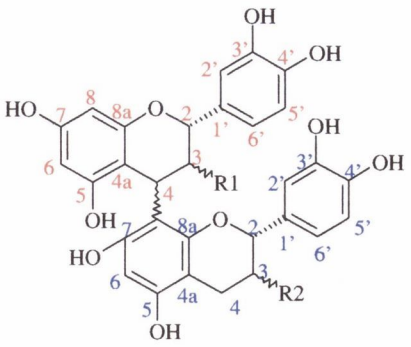
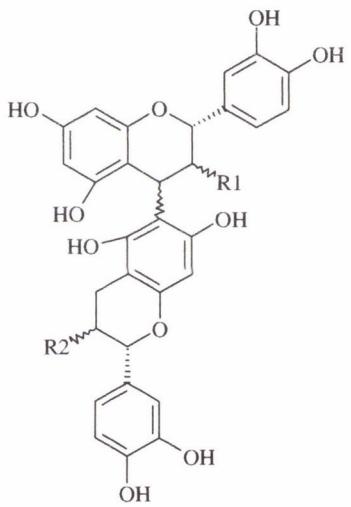


Figure 8.6.2.11 Scheme for formation of cyaniding from procyanidin

Various procyanidins containing catechin and epi-catechin units are shown in **Figure 8.6.2.12 - 4.6.2.14** below. Procyanidins also exists as larger polymers, and for example procyanidins with more eight units have been isolated and structurally identified (Taylor et al. 2003) from hops (*Humulus lupulus* L.).



R1	R2	Interflavan bond	
····OH	—OH	—	Procyanidin B1 or epicatechin-(4β→8)-catechin
····OH	····OH	—	Procyanidin B2 or epicatechin-(4β→8)-epicatechin
—OH	—OH	·····	Procyanidin B3 or catechin-(4β→8)-catechin
—OH	····OH	·····	Procyanidin B4 or catechin-(4β→8)-epicatechin



R1	R2	Interflavan bond	
····OH	····OH	—	Procyanidin B5 or epicatechin-(4β→6)-epicatechin
—OH	—OH	·····	Procyanidin B6 or catechin-(4β→6)-catechin
—OH	····OH	·····	Procyanidin B8 or catechin-(4β→6)-epicatechin

Figure 8.6.2.12 Procyanidin B1-B8

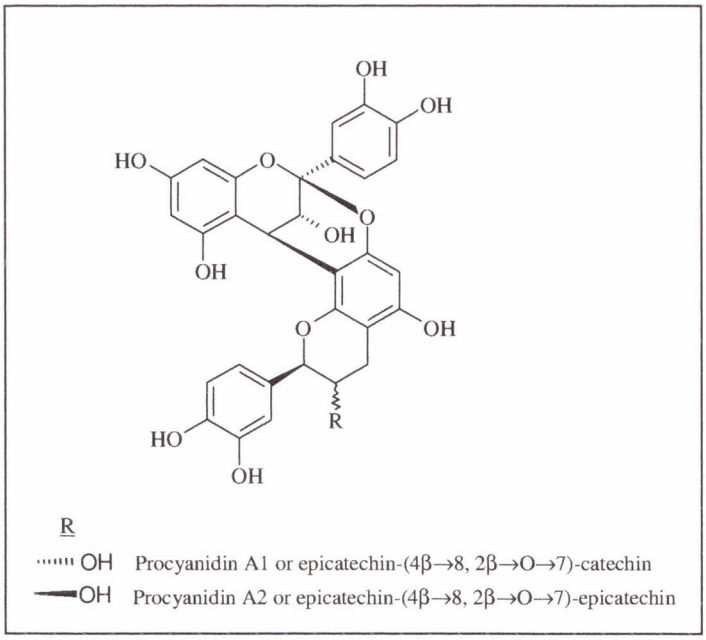


Figure 8.6.2.13 Procyanidin A1 and A2

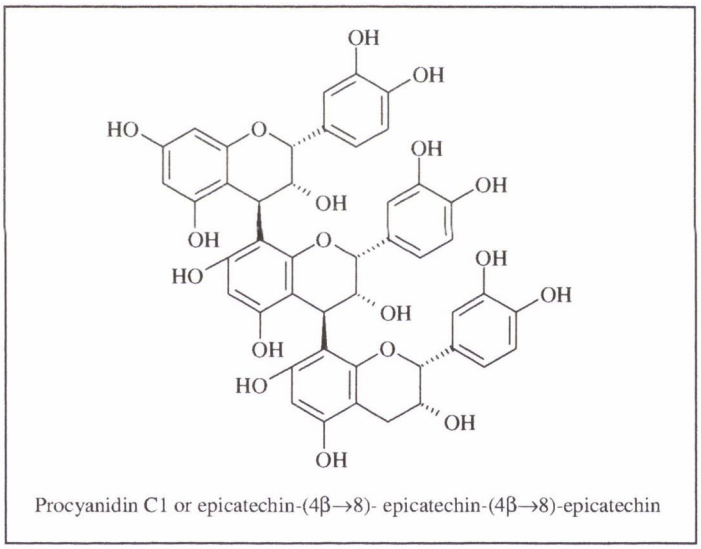


Figure 8.6.2.14 Procyanidin C1

The ratio of catechin (**6**) to epicatechin (**7**) units may be obtained from direct integration of the respective C-2 signals in the ^{13}C NMR spectrum. The ridged nature of the compound makes this possible, but peak heights can not be used, as the signals are not coincident when resolved. The C-2 has a chemical shift around δ84 in catechin and δ77 in epi-catechin. Czochanska et al. (Czochanska et al. 1980) compared the ratio obtained using this method with the ratio obtained using the more traditional and

more time-consuming methods such as acid degradation and thiolysis, and found that the ratio obtained by integrating the C-2 signals of the *cis* and *trans* unit gave a self-consistent value.

The integration of the signals at ~δ84 ppm and ~δ77 ppm gave a ratio of 4:1 on average (n=5), indicating that there is a higher percent of catechin than epi-catechin present in the isolate. **Figure 8.6.2.15** shows the integration of the signals in the ^{13}C NMR spectrum.

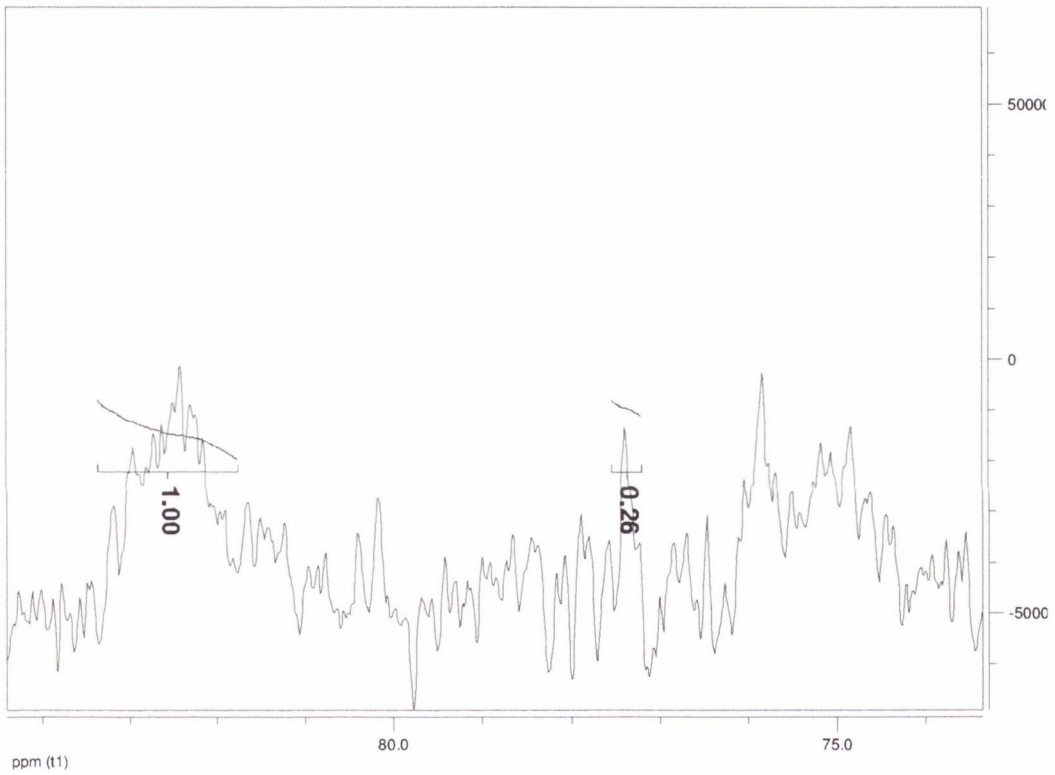


Figure 8.6.2.15 Integration of C-2 signals in ^{13}C NMR of isolate

Conclusion

The presence of a procyanidin has been confirmed for the first time in cell cultures of *Taxus*. The procyanidin is formed by coupling five units composed of catechin and epi-catechin in a ration of 4:1. The site of interflavan bonds and characterisation of the terminal unit could not be determined at present, but work is ongoing in those areas.

8.7 Fatty Acid profile of *Taxus* Tissue Cultures Compared to Needle and Seed

The fatty acid composition of the seeds and leaves of conifers has been found to contain "unusual" fatty acids that are not found in angiosperms (Mongrand et al. 2001, Takagi and Itabashi 1982, Wolff et al. 1997a). These fatty acids contain a Δ5-ethylenic bond arranged in a non-methylene interrupted manner, frequently referred to as Δ5-olefininc acids or Δ5-unsaturated polymethylene-interrupted fatty acids. The structures of these "unusual" acids are 5,9-18:2 (taxoleic), 5,9,12-18:3 (pinolenic), 5,9,12,15-18:4 (coniferonic), 5,11-20:2, 5,11,14-20:3 (sciadonic) and 5,11,14,17-20:4 (juniperonic) (Berdeaux and Wolff 1996, Takagi and Itabashi 1982).

A chemotaxonomic grouping of the main conifer families, including Pinaceae, Taxodiaceae, Cupressaceae and Taxaceae, was established by multivariate analysis of the seed fatty acid composition of 82 conifer species (Wolff et al. 1997b). In this study they found that the *Taxus* analysed, including *T. canadensis*, *T. cuspidata* and *T. baccata* formed a homogenous group that was completely isolated from all the other conifer families. *Torreya nucifera* (Taxaceae) was excluded from this group. Wolff et al. (Wolff et al. 1998) continued this study and found that the greatest difference between *Taxus* and *Torreya* was in the distribution profile of the Δ5-olefininc acids. In *Taxus*, the main Δ5-olefininc acid was 5,9-18:2 (taxoleic) in the range 9.7-16.2%. This acid was absent in *Torreya*. In *Taxus* pinolenic acid (5,9,12-18:3) accounted for 0.3-3.3%, whereas in *Torreya* it was less than 0.1%. In *Torreya* the main Δ5-olefinic acid was 5,11,14-20:3 (sciadonic) acid.

The main fatty acids in the seeds of *T. baccata*, *T.cuspidata*, *T. canadensis* and *T. chinensis* were found to be oleic acid (9-18:1) with 54.8-34.3%, linoleic acid (9,12-18:2) with 23.1-34.2% and taxoleic acid (5,9-18:2) with 9.5-16.2%. There was also a presence of palmitic acid (16:0) accounting for 2.35-3.23%, linolenic acid (9,12,14-18:3) accounting for 1.3-2.1%, and pinolenic acid (5,9,12-18:3) accounting for 0.33-3.31% (Wolff et al. 1998).

Mongrand et al. (Mongrand et al. 2001) reports that the fatty acid composition of gymnosperm and angiosperm photosynthetic tissues are similar in that the major fatty acids are linolenic (18:3), linoleic (18:2) and palmitic (16:0) acids. However, the gymnosperm profile contains a much higher number of fatty acids, with sometimes as many as 26 different fatty acids detected. In their study they established four chemotaxonomic groups based on the multivariate analysis of the needle fatty acid composition of 132 species of gymnosperms belonging to 14 families. From this study they found that the major fatty acids in *Taxus* leaves from the species *T. baccata*, *T. baccata* 'Lutea', *T. baccata* 'Fastigiata', *T. brevifolia* and *T. cuspidata* were palmitic acid (16:0) with 14.1-25.9%, linolenic acid (9,12,15-18:3) with 25.6-52.1%, linoleic acid (9,12-18:2) with 5.4-13.4%. The taxoleic acid (5,9-18:2) was only present in trace amounts, and likewise with pinolenic acid (5,9,12-18:3). However, sciadonic acid (5,11,14-20:3) accounted for 3.3-.8% and likewise juniperonic acid (5,11,4,17-20:4) accounted for 3.3-6.8% of the fatty acid composition. This differs from the fatty acid composition of the seed, where only 1.6-2.1% sciadonic acid and only trace amounts of juniperonic acid were present (Wolff et al. 1998).

These studies show that the fatty acid composition can in fact be used as a chemotaxonomic marker to differentiate between conifer families. However, within the species the differences are smaller, and could be due to geographical differences. As an example Wolff et al. (Wolff et al. 1998) found that the fatty acid composition of *T. cuspidata* and *T. chinensis* were hardly distinguishable, and they proposed that these two species might only be two geographical varieties. It is clear that for these kinds of conclusions to be made other means of differentiation must be taken into consideration, such as morphological, molecular and chemical variation combined.

Based on the differences in the fatty acid composition between seeds and leaves in *Taxus*, the full fatty acid profile of the cell cultures of *Taxus baccata* 'Fastigiata' was compared to that of the seeds and needles of the same species. The four cell lines maintained in our laboratory (see section 8.1.3) were all analysed. The extraction method followed was first described by Browse et al. (Browse et al. 1986) and used hot methanolic HCl to digest fresh photosynthetic tissue and simultaneously convert fatty acids to methyl esters. The same digestion and extraction method was used for the seeds, leaves and cell culture material. The hexane extracts containing the FAMEs

were then analysed by capillary GC (see Materials and Methods, section 7.8.3, for more details).

The extracts were compared to standard FAMEs injected into the same GC system. The relative retention times are reported as equivalent chain lengths as well as the actual retention times. Equivalent chain lengths (ECL) is the most established method of reporting relative retention times of fatty methyl esters. ECL are based on the linear relationship between the logarithm of the adjusted retention times and the number of carbons in the homologous series (James and Martins 1952). The ECL concept uses the saturated straight chain FAME as reference compounds.

Equivalent chain lengths (ECL) were calculated for the standard and the extract signals according to the formula below (Mjøs 2003) with myristic (14:0; ECL 14), palmitic (16:0; ECL 16), stearic (18:0; ECL 18) and arachidic (20:0; ECL 20) acid as standards:

$$ECL_{(x)} = z + \frac{logt'_{R(X)} - logt'_{R(z)}}{logt'_{R(z+1)} - logt'_{R(z)}}$$

where **z** is the carbon number in the saturated FAME eluting immediately before the analyte of interest, **x**, and **z + 1** is the number of carbons in the saturated FAME eluting immediately after analyte x. t'_R is the adjusted retention times. The results are presented in **Table 8.6.2.1** (standards) and **Table 8.6.2.2** (extracts) below.

Table 8.6.2.1 Retention times and equivalent chain lengths of FAME standards

Standard FAME	t'_R	$Logt'_R$	ECL	Fatty Acid Structure
Myristate	10.00	1.00	14.00	14:0
Palmitate	13.30	1.12	16.00	16:0
Stearate	15.70	1.20	18.00	18:0
Oleate	16.39	1.21	18.11	18:1 Δ9
Taxolate	17.07	1.23	18.19	18:2 Δ5,12
Linoleate	17.69	1.25	18.30	18:2 Δ9,12
Pinolenate	18.25	1.36	18.36	18:3 Δ 5,9,12
Linolenate	19.28	1.29	18.51	18:3 Δ9,12,15
Arachidate	23.44	1.37	20.00	20:0
Coniferonate	23.81	1.38	19.04	18:4 Δ 5,9,12,15

Table 8.6.2.2 Retention times and equivalent chain lengths of extracts

Sample	t'_R	$Logt'_R$	ECL	Fatty Acid Structure	Fatty Acid
Cells from cell line 19 (from *T. baccata* 'Fastigiata' seed)	12.48	1.10	17.43	*NI**	*NI**
	16.23	1.21	18.11	Oleate	18:1 Δ9
	17.41	1.24	18.26	Linoleate	18:2 Δ9,12
	18.97	1.28	18.47	Linolenate	18:3 Δ9,12,15
	23.17	1.36	18.97	Arachidate	20:0
Cells from cell line 3 (from *T. baccata* 'Fastigiata' seed)	12.40	1.09	17.41	*NI**	*NI**
	15.86	1.20	18.02	Stearate	18:0
	16.13	1.21	18.07	Oleate	18:1 Δ9
	17.29	1.24	18.24	Linoleate	18:2 Δ9,12
	18.83	1.27	18.45	Linolenate	18:3 Δ9,12,15
	21.90	1.34	18.83	*NI**	*NI**
	23.10	1.36	18.96	*NI**	*NI**
	23.73	1.38	19.03	Coniferonate	18:4 Δ 5,9,12,15
Cells from cell line 8 (from *T. baccata* 'Fastigiata' seed)	12.38	1.09	17.41	*NI**	*NI**
	15.84	1.20	18.02	Stearate	18:0
	16.13	1.21	18.07	Oleate	18:1 Δ9
	17.30	1.24	18.24	Linoleate	18:2 Δ9,12
	18.84	1.28	18.45	Linolenate	18:3 Δ9,12,15
	23.13	1.36	18.97	Arachidate	20:0
Cells from cell line *H (from *T.baccata* 'Fastigiata' leaf)	11.78	1.07	17.22	Myristate	14:0
	12.39	1.09	17.41	*NI**	*NI**
	16.14	1.21	18.07	Oleate	18:1 Δ9
	17.31	1.24	18.24	Linoleate	18:2 Δ9,12
	18.06	1.26	18.35	Pinolenate	18:3 Δ 5,9,12
T. baccata 'Fastigiata' Leaf	11.84	1.07	17.30	Myristate	14:0
	12.34	1.09	17.40	*NI**	*NI**
	13.37	1.13	17.60	Palmitate	16:0
	15.23	1.18	17.92	*NI**	*NI**
	17.24	1.24	18.23	Linoleate	18:2 Δ9,12
	18.83	1.27	18.45	Linolenate	18:3 Δ9,12,15
	21.85	1.34	18.82	*NI**	*NI**
	23.67	1.37	19.02	Coniferate	18:4 Δ 5,9,12,15
T. baccata 'Fastigita' Seed	12.51	1.10	17.43	*NI**	*NI**
	13.12	1.12	17.55	Palmitate	16:0
	15.69	1.20	18.00	Stearate	18:0
	16.39	1.21	18.11	Oleate	18:1 Δ9
	16.82	1.23	18.17	Taxolate	18:2 Δ5,12
	17.51	1.24	18.27	Linoleate	18:2 Δ9,12
	18.97	1.28	18.47	Linolenate	18:3 Δ9,12,15
	19.94	1.30	18.60	*NI**	*NI**
	22.02	1.34	18.84	*NI**	*NI**
	23.02	1.37	18.97	Arachidate	20:0

* *NI*=Not Identified

From these result we can see that only the extract from cell line 3, originated from seeds of *T. baccata* 'Fastigiata', and cell line *H, originated from leaves of *T. baccata* 'Fastigiata', had a presence of a Δ5-olefinic acid. The three other cell lines either did not have any presence of this type of fatty acids or only contained trace amounts of them. Cell line 3 contained a small amount of coniferonic acid whereas cell line *H contained a small amount of pinolenic acid. The main fatty acids in the cell extracts were oleic acid, linoleic acid and linolenic acid.

The results of the leaves extract were closely comparable with the results from Mongrand et al.'s study (Mongrand et al. 2001). The main fatty acids were palmitic, linoleic and linolenic acid. Mongrand et al. only detected small amounts of stearic and oleic acid, whereas in this study only trace amounts of those fatty acids were present. They also reported the presence of the Δ5-olefinic acids sciadonic and juniperonic acids whereas those acids were not present in measurable amounts in the needles of *T. baccata* 'Fastigiata'. However, the needles extracted did contain a small amount of coniferonic acid. Mongrand et al. only detected small amounts of this Δ5-olefinic acid in *T. brevifolia* (0.3%) and *T. cuspidata* (0.2%) needles.

The results of the seed extract was similar to the results reported by Wolff et al. (Wolff et al. 1998). The main fatty acids were oleic acid, taxoleic acid and linoleic acid. Small amounts of palmitic, stearic, linolenic and arachidic acid were also present. Wolff et al. also reported the presence of pinolenic and sciadonic acid, but those two fatty acids were not detected in a measurable amount in the seeds of *T. baccata* 'Fastigiata'.

A compound present in all the samples analysed with the retention time of ~12.45 and the ECL of ~17.4 could not be identified. However, this compound was a relatively major peak in the fatty acid profile of the six samples analysed. A few other rather minor compounds also remain un-identified.

From this study it can be concluded that the same fatty acids that are the major components in the seeds are not all present in the cell cultures originating from the seeds of the Irish yew. Oleic and linoleic acid are present in both the seeds and the

cells, but the cells do not contain any measurable amount of the Δ5-olefininc acid taxoleic acid, which is a main fatty acid in the seeds. Linolenate is a main fatty acid in the cells, but the seeds only contain a small amount of this acid.

The extract of the cell culture developed from the needles of *T. baccata* 'Fastigiata' does not compare with the actual needle extract. The needles does not contain measurable amounts of oleic acid, but this is present in the cells. The cells do not contain palmitic and linolenic acid, whereas in the needles these are major fatty acids. However, the cells do contain a small of pinolenic acid, which was reported by Mongrand et al. (Mongrand et al. 2001) to be present only in small amounts in *T. baccata* 'Lutea'(0.4%), *T. brevifolia* (0.3%), and *T. cuspidata* (0.2%), but not in *T. baccata* 'Fastigiata'. Wolff et al. (Wolff et al. 1998) reported a larger amount of this acid present in the seeds they analysed.

The reason for this variation can not be clarified based on these results. But it could be speculated that the reason for the simpler fatty acid composition of the cell culture could be due to the more protected environment created for the cells. The role of the Δ5-olefinic acid remains unknown, but Wolff et al. (Wolff et al. 1997a) reported that pine species restricted to warm-temperate regions had a low content of Δ5-olefinic acids. Their hypothesis being that these acids are in some way related to temperature acclimation. Maybe the low content or near lack of Δ5-olefinic acids in the cell cultures analysed is related to this theory.

9 Conclusion

The carbon source in the growth medium of *T. b.* 'Fastigiata' cell cultures was changed from sucrose to inulin, resulting in a medium with reduced osmotic pressure. This hypoosmotic medium led to a dramatic decrease in cell growth, where the growth was reduced by 40% when the medium contained 3% inulin and by 70% when the medium contained 1% inulin. It was therefore concluded that inulin could not sustain cell growth in the same way as the original carbon source sucrose. However, inulin had a positive effect on taxane accumulation, where 1% inulin resulted in a significant increase in 10-DAB III production compared to the control (60.08 ± 2.15 mg/kg vs. 30.20 ± 5.00 mg/kg, respectively ($P<0.001$)). The mechanical stress the cells were put under as a result of a reduced osmotic pressure elicited the accumulation of taxanes. The mechanical stress was not found to induce the accumulation of flavonoids as a result of the plant defence mechanism. However, initiation of the phenyl propanoid biosynthetic pathway, and thereby the accumulation of flavonoids, is an immediate response to stress. The flavonoid analysis was conducted in the end of the growth cycle, and therefore the immediate response to the stress could have been missed.

An attempt at eliciting the taxane production by addition of molybdenum to the growth medium was un-successful. Although molybdenum plays an essential role in the metabolism of carbon, nitrogen and sulphur cycles in the plant, it was found that an increased level was unfavourable to cell growth, and an increased level of extracellular taxanes indicated cell lysis or possible cell structure change or damage. Molybdenum, being a transition metal, could participate in the production of reactive oxygen species, and the induction of an oxidative burst could elicit taxane accumulation. However, the concentrations used did not have a positive effect on secondary metabolism.

It has been suggested that NO plays a regulatory role in activating the oxidative burst in cells (Wang and Wu 2004). In vertebrates nitric oxide (NO) acts as a key signalling molecule, and is formed directly from the guanidine nitrogen of the *L*-arginine by nitric oxide synthase (NOS) (Rockel et al. 2002). The addition of *L*-arginine at three different concentrations, to the growth medium of *T. b.* 'Fastigiata' cell suspension cultures resulted in a reduction of growth, especially at the highest concentration, and a

decreased taxane production. The high concentration of *L*-arginine was probably toxic to the cells, inducing the hypersensitive response, which in turn would lead to cell death. The high concentration of *L*-arginine could also have resulted in overproduction of NO leading to cell damage or ultimately cell death.

Methyl jasmonate was found to stimulate the synthesis and excretion of the taxanes 10-DAB III, baccatin III and cephalomannine in the suspension cultures of *T. b.* 'Fastigiata'. However, the biosynthesis of paclitaxel itself was not enhanced. The elicitation of paclitaxel by methyl jasmonate has been reported to be cell line specific (Bonfill et al. 2003, Ketchum et al. 1995), and location in the biosynthetic pathway could also influence its time of elicitation (Naill and Roberts 2005). Methyl jasmonate did have a slight negative effect on cell growth in the cultures, however, the effect was not damaging, and an increase of taxanes often results in a decrease in biomass (Laskaris et al. 1999, Yukimune et al. 1996).

Tert-butyl hydroperoxide (tBH) in conjunction with hematin was used in order to induce an oxidative burst in the suspension cultures, where the oxidative burst was expected to induce the plant defence mechanism leading to accumulation of flavonoids and finally enhanced taxane production. An immediate burst of flavonoids (after 10 minutes) was indicative of the induction of the cells defence system, but also indicated that tBH had an effect on the production of secondary metabolites in the cultured cells. The induction of an oxidative burst leading to oxidative stress in the plant cells also affected the taxane production in the *T. b.* 'Fastigiata' cells. The concentration of both 10-DAB III and baccatin III increased after 48 hours compared to the controls at this time of harvest. The increase in total taxane biosynthesis in the test cells was 45% greater than that in the control at 48 hours after treatment with tBH and hematin. The results show that accumulation of taxanes is a downstream event of signal transduction resulting from the oxidative burst. The activation of defence genes is a more upstream and immediate response to the treatment.

I*n situ* product removal using a liquid-solid culture system consisting of the aqueous growth medium and Amberlite resin, a solid polar absorbent, resulted in a significant increase in overall taxane production in *T. b.* 'Fastigiata' suspension cultures. It could be concluded that the Amberlite acted as a secondary metabolite sink, adsorbing the

taxanes that were excreted into the medium from the cells. However, bags had a negative effect on the cells growth during the experiment, reducing growth and resulting in less aggregation of the cells. This was probably due to an increased level of mechanical stress in the form of shear. The bags could also have affected or altered the hydrodynamic of the cultures. The presence of the Amberlite resin bags led to increased flavonoid production, probably as a response to mechanical stress.

The major flavonoids in the ethyl acetate extract of *T. b.* 'Fastigiata' cell suspension cultures were found to be catechin and epi-catechin. This was established by comparison with reference data of standards using HPLC and verified by phytochemical isolation and structure characterisation. To our knowledge this is the only report of the isolation of catechin and epi-catechin from cell suspension cultures of *T. b.* 'Fastigiata'. The presence of a procyanidin consisting of five units composed of catechin and epi-catechin (ratio of 4:1) was also confirmed for the first time in cell cultures of *Taxus*.

The fatty acid composition of the seeds and leaves *Taxus* (and other conifers) has been found to contain "unusual" fatty acids, Δ5-olefininc acids, that are not found in angiosperms (Mongrand et al. 2001, Takagi and Itabashi 1982, Wolff et al. 1997a). The full fatty acid profile of the cell suspension cultures of *T. b.* 'Fastigiata' was compared to that of the seeds and needles of the same species. It was found that the same fatty acids that are the major components in the seeds are not all present in the cell cultures originating from the seeds. The cells do not contain any measurable amounts of the Δ5-olefininc acid taxoleic acid, which is a major fatty acid in the seeds. Instead the main fatty acid in the cells is linolenate, only found in small amounts in the seeds. The extract of the cell culture developed from the needles of *T. baccata* 'Fastigiata' did not compare with an actual needle extract, where the major fatty acids in needles were palmitic and linolenic acid. The role of the Δ5-olefinic acid remains unknown, but Wolff et al. (Wolff et al. 1997a) reported that pine species restricted to warm-temperate regions had a low content of Δ5-olefinic acids. It could be speculated that the reason for the simpler fatty acid composition of the cell culture could be due to the more protected environment created for the cells in the warmer temperature of the tissue culture room (25°C).

10 Thesis Conclusion

As part of the continuing research into *Taxus* species, a major objective of the current work was to further investigate the use of morphological characteristics, such as needle and seed dimensions for identification purposes. Instead of manual measurements this investigation was carried out using WinSEEDLE™, an image analysis software-package specifically developed for needle and seed analysis. The dimension analysis was combined with classical morphological characteristics, such as tree shape and foliage colour. The significant variation in needle and seed dimensions within the species analysed, together with major differences in tree shape and foliage colour, suggests that the value of morphological characteristics for taxonomic purposes is limited. This was especially apparent in *T. baccata* and its cultivars where, even when outliers were excluded from the analysis, the intraspecific variation was very large. Tree shape varied from pyramidal large trees to bun-shaped shrubs and bushes, and foliage colour was either dark green, light green or golden. No single parameter was found that could distinguish between the species. Even when needle and seed analysis were combined with tree shape and foliage colour for each species no conclusive identification key could be determined. The great diversity in terms of the appearance of *Taxus* makes the correct identification difficult and results question the accuracy of nomenclature of many samples supplied by Botanical Gardens. This was illustrated by the samples of *T. canadensis*, where not only morphological characters varied greatly, but also the taxane specific to *T. canadensis* (9-DHAB III) was not present in all 'authentic' *T. canadensis* samples.

The morphological analysis of needle dimensions also highlighted the necessity of a suitable collection protocol as significant variation in needle sizes were found, depending on from where on the tree samples were taken. Needles collected from lower-situated branches were longer than collections made from higher branches. In addition two of the four sides of the tree also had slightly longer needles possibly due to light/shade effects. The reduction of needle surface-area upon drying was also shown, and it was observed that the difference in surface-area of last year's and this year's growth, was significant. Drying reduced leaf size, so therefore dry and fresh samples cannot be compared directly to each other and results must be kept separately.

These findings question the usage of herbarium samples for identification of *Taxus* species, since the herbarium sample usually consists of one twig and location of the twig on the tree (side, distance from the ground etc) is not always included in description.

Another objective of this research was to determine if taxanes or other constituents could be used taxonomically to identify the *Taxus* species. This study was based on the presence of 10-DAB III, 9-DHAB III, cephalomannine and paclitaxel. The variable inter- and intra-specific concentration of the taxanes suggested that the value of taxanes as taxonomic markers was limited. The seasonality of the taxane constituents and the variation due to geographical location and environmental factors suggest the same. The variability of taxanes within a species also made it difficult to suggest which species would be best suited for direct isolation of paclitaxel. The most abundant taxane was found to be 10-DAB III in all *Taxus* samples analysed. Species-specific taxanes are recognised, brevifoliol in *T. brevifolia* and 9-DHAB III in *T. canadensis*, and can be used as chemotaxonomic markers for these species. In the present study phloroglucindimethylether was isolated from *T. baccata* and the precence of a high concentration of this constituent in a sample is suggested as indicative of this species.

Overall the results obtained from morphological and chemical examination illustrated the difficulties in making conclusive identification of species in the genus *Taxus*. Elwes and Henry suggested that "*the genus Taxus is mono-specific, consisting of a large number of varieties which exhibit enormous diversity in terms of their appearance but which are essentially taxonomically indistinguishable*". The results from the present study tended to support this opinion, although further work especially in the field of molecular systematics is needed so that relationships between the species can be clarified.

Needles of *Taxus baccata* 'Fastigiata' were previously found to yield high levels of taxanes and seeds had been used to initiate suspension cultures. The second part of the present work was to determine the profile of taxane constituents of these cell cultures, including 10-DAB III, baccatin III, cephalomannine and paclitaxel. Of these taxanes

10-DAB III, baccatin III and cephalomannine were found to be most abundant, whereas paclitaxel was absent or only produced in minor amounts.

Induction of taxane biosynthesis could be of commercial value and another aspect of the present research was to influence the taxane production in these suspension cultures by various cell culture techniques. In the previous work some optimisation of the cell culture had been carried out. Further work showed that a change of carbon source from sucrose to inulin resulted in reduced osmotic pressure causing mechanical stress in the cell culture system. The hypoosmotic medium led to a dramatic decrease in biomass and this mechanical stress that the cells were put under elicited the accumulation of taxanes and resulted in a significant increase in 10-DAB III production compared to the control (60.08 ± 2.15 mg/kg vs. 30.20 ± 5.00 mg/kg). The overall result led to the conclusion that although the taxane production was enhanced, inulin as carbon source could not sustain cell growth in the same way as sucrose and would therefore not be a suitable carbon source for the cultures.

Methyl jasmonate was found to stimulate the synthesis of the taxanes 10-DAB III, baccatin III and cephalomannine in the cell suspension cultures. The elicited cells had a five times higher content of 10-DAB III. There was also doubling in both baccatin III and cephalomannine content. However, the biosynthesis of paclitaxel itself was not enhanced. Methyl jasmonate was also found to enhance the excretion of taxanes to the culture medium. The results showed a statistically significant increase in 10-DAB III and baccatin III excretion (three-fold and two-fold, respectively) in the elicited cultures compared to that of the control cultures.

Oxidative stress has been suggested as a factor influencing taxane yields. Another objective of this research was to examine the effect of the induction of oxidative stress using oxidative burst inducers and elicitors on the production of taxanes by the cell suspension cultures. Attempts of eliciting taxane production by addition of the transition metal molybdenum and the NO-production inducer *L*-arginine were unsuccessful. Both molybdenum and *L*-arginine in the concentrations used were found to be detrimental to the cells. The induction of an oxidative burst using *tert*-butyl hydroperoxide (tBH) in conjunction with hematin resulted in 45% enhancement of

total taxane production (mainly 10-DAB III and baccatin III) in the cell culture after 48 hours. A short-term experiment was also carried out over one hour, but no significant enhancement of taxane production was observed immediately after oxidative burst induction. The flavonoid profile of the cells was also investigated and an immediate burst of flavonoids was observed indicating an induction of the cells defence system by the oxidative burst. The activation of defence genes was an upstream and immediate response to the treatment, whereas the accumulation of taxanes was a downstream event of signal transduction resulting from the oxidative burst.

In situ product removal using a liquid-solid culture system consisting of the aqueous growth medium and the solid polar absorbent Amberlite resin, resulted in a significant increase in overall taxane production in the suspension cultures. Amberlite was found to adsorb the taxanes that were excreted into the medium from the cells, acting like a secondary metabolite sink. However, the presence of the Amberlite bags had a negative effect on the growth of the cells during the experiment. This was probably due to an increased level of mechanical stress in the form of shear. The presence of the Amberlite resin bags also led to increased flavonoid production, probably as a response to mechanical stress.

Examination of the flavonoid profile of the cell cultures found the major flavonoids to be catechin and epi-catechin. This finding was established by HPLC and verified by isolation and structure elucidation. To our knowledge this is the only report of isolation of catechin and epi-catechin from cell suspension cultures of *Taxus baccata* 'Fastigiata'. Other flavonoids present in various concentrations were taxifolin, kaempferol, quercetin and amentoflavone. Further phytochemical analysis of the *T. b.* 'Fastigiata' cell suspension culture resulted in the isolation of a procyanidin from cell cultures of *Taxus*. This is the first report of the isolation of a procyanidin from *Taxus* cultures. It was deduced to be a polymer with five units composed of catechin and epi-catechin (ratio of 4:1). Further work is ongoing in order to confirm exact molecular conformation.

Fatty acid profiles of *Taxus* seed oils had been determined in a previous study and had indicated a high degree of unsaturation. As oxidative burst generates free radicals detrimental to unsaturated fatty acids of cell membranes, the fatty acid profile of the

cell suspension cultures of *T. b.* 'Fastigiata' cell lines were compared to that of the seeds and needles of the same species. The cell cultures analysed were found to have a simpler fatty acid profile than the needles and the seeds, where the unusual Δ5-olefininc acids were the major fatty acid constituents. The main fatty acid in the cell cultures was linolenate, only found in a small amount in the seeds themselves. The fatty acid profile of the cell culture developed from the needles of *T. b.* 'Fastigiata' also did not compare with the profile from the actual needles, where the major fatty acids were palmitic and linolenic acid. The fatty acid profiles of cultures developed from both seeds and needles therefore differed from the original plant organs.

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Appendix I Cell Culture Medium

Table ApI.1 Formulation of cell culture media

Component (mg/l)	Westvako3 (WV3)	Westvako5 (WV5)	Rugini's Olive	McCown Woody Plant
Macro Elements	mg/l	mg/l	mg/l	mg/l
Ammonium nitrate (NH_4NO_3)	-	700	412	400
Potassium nitrate (KNO_3)	910.1	1084.06	1100	-
Potassium chloride (KCl)	656.79	718.67	500	-
Potassium dihydrogen phosphate (KH_2PO_4)	270	270	340	170
Calcium chloride ($CaCl_2$)	452.88	452.88	332.16	72.50
Calcium nitrate x $6H_2O$ ($Ca(NO_3)_2$ x $6H_2O$	-	-	416.92	471.26
Potassium sulphate (K_2SO4)	-	-	-	990
Micro Elements				
Boric acid (H_3BO_3)	31.0	31.0	12.4	6.20
Magnesium sulphate x $7H_2O$ ($MnSO_4$ x $7H_2O$)	-	-	-	-
Magnesium sulphate x H_2O ($MnSO_4$ x H_2O)	15.16	15.16	16.9	22.30
Magnesium sulphate ($MnSO_4$)	903.79	903.79	732.6	180.54
Zinc sulphate x $7H_2O$ ($ZnSO_4$ x $7H_2O$)	8.6	8.6	14.3	8.60
Potassium iodide (KI)	0.83	0.83	0.83	
Sodium molybdate (Na_2MoO_4)	0.25	0.25	0.25	0.25
Copper sulphate ($CuSO_4$)	0.25	0.25	0.25	0.25
Cobalt chloride ($CoCl_2$)	0.025	0.025	0.025	-
Sodium EDTA (FeNaEDTA)	36.71	36.71	36.71	36.70
Ferrous sulphate ($FeSO_4$)	-	-	-	-
Vitamins				
Myo-inositol ($C_6H_{12}O_6$)	1000	1000	100	100
Casein amino acid	500	500	500	-
Nicotinic acid ($C_5H_5NO_2$)	-	-	5.0	0.50
Pyridoxine hydrochloride ($C_8H_{11}NO_3HCl$)	-	-	0.5	0.50
Thiamine hydrochloride ($C_{12}H_{17}CIN_4OSHCl$)	0.4	0.4	0.5	1.00
Biotin	-	-	0.05	-
Folic acid	-	-	0.5	-
Glycine ($C_2H_5NO_2$)	-	-	2.0	2.00
L-glutamine ($C_5H_{10}N_2O_3$)	1000	1000	1000	-
2,4-Dichlorophenoxyacetic acid ($C_8H_6Cl_2O_3$)	3.0	3.0	3.0	-
6-Benzylaminopurine ($C_{12}H_{11}N_5$)	0.5	0.5	0.5	-
Kinetin ($C_{10}H_9N_5O$)	-	-	-	-
Sucrose ($C_{12}H_{22}O_{11}$)	30000	30000	30000	30000
pH	5.7	5.7	5.7	5.7

Appendix II NMR Spectra of Catechin Standard

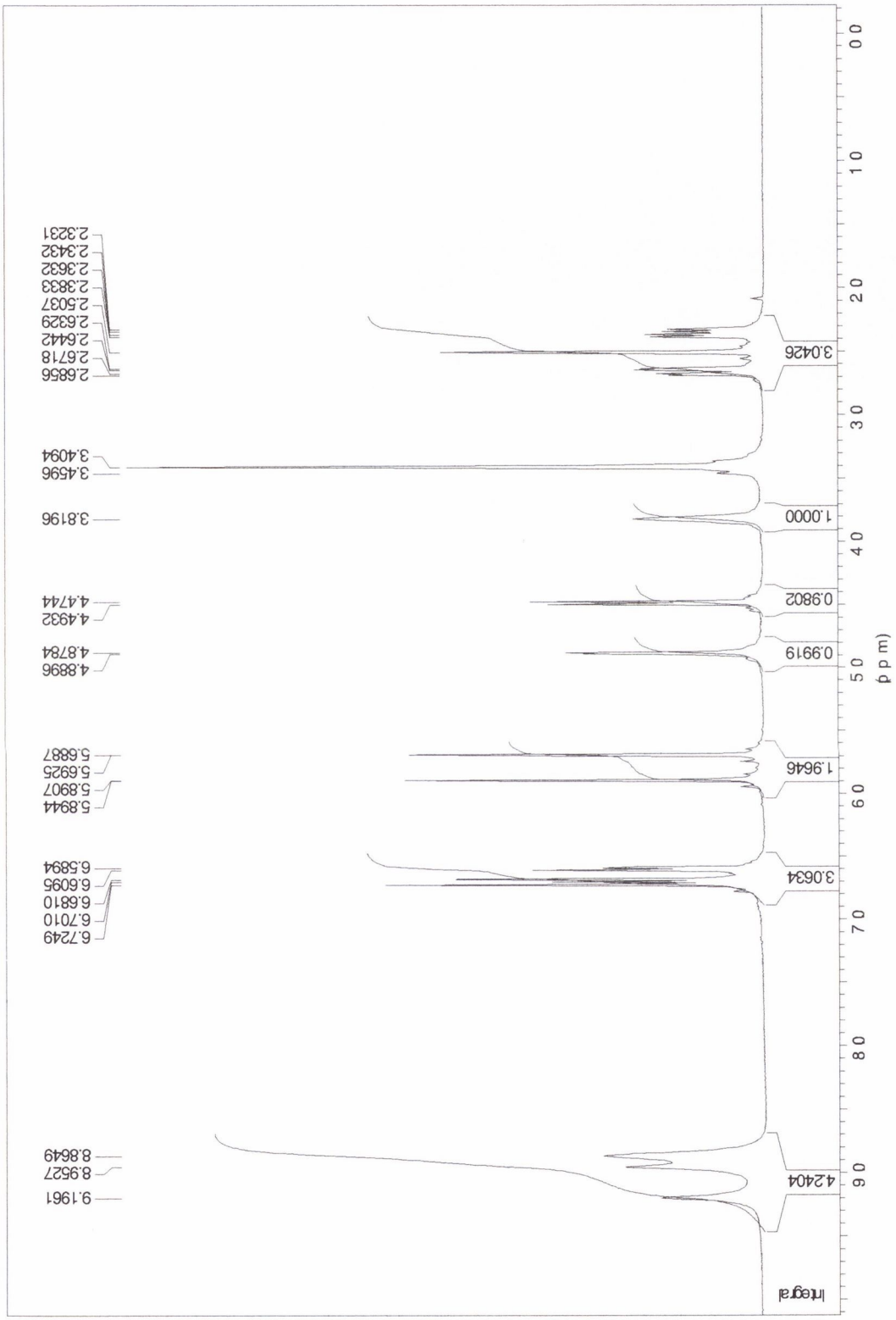

Figure ApII.1 ^{1}H-NMR of catechin standard in DMSO

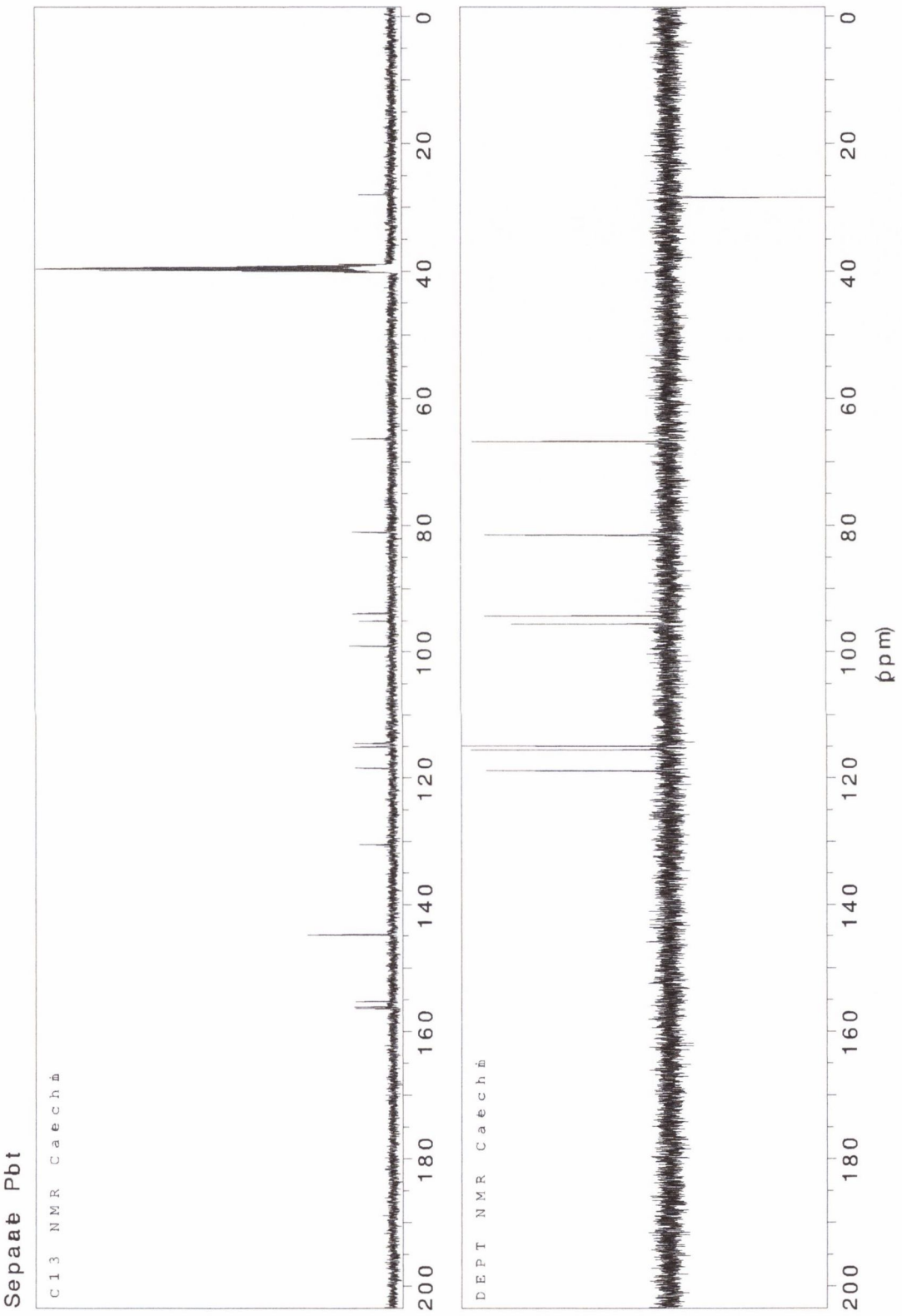

Figure ApII.2 ^{13}C- and DEPT 135 NMR of catechin standard in DMSO

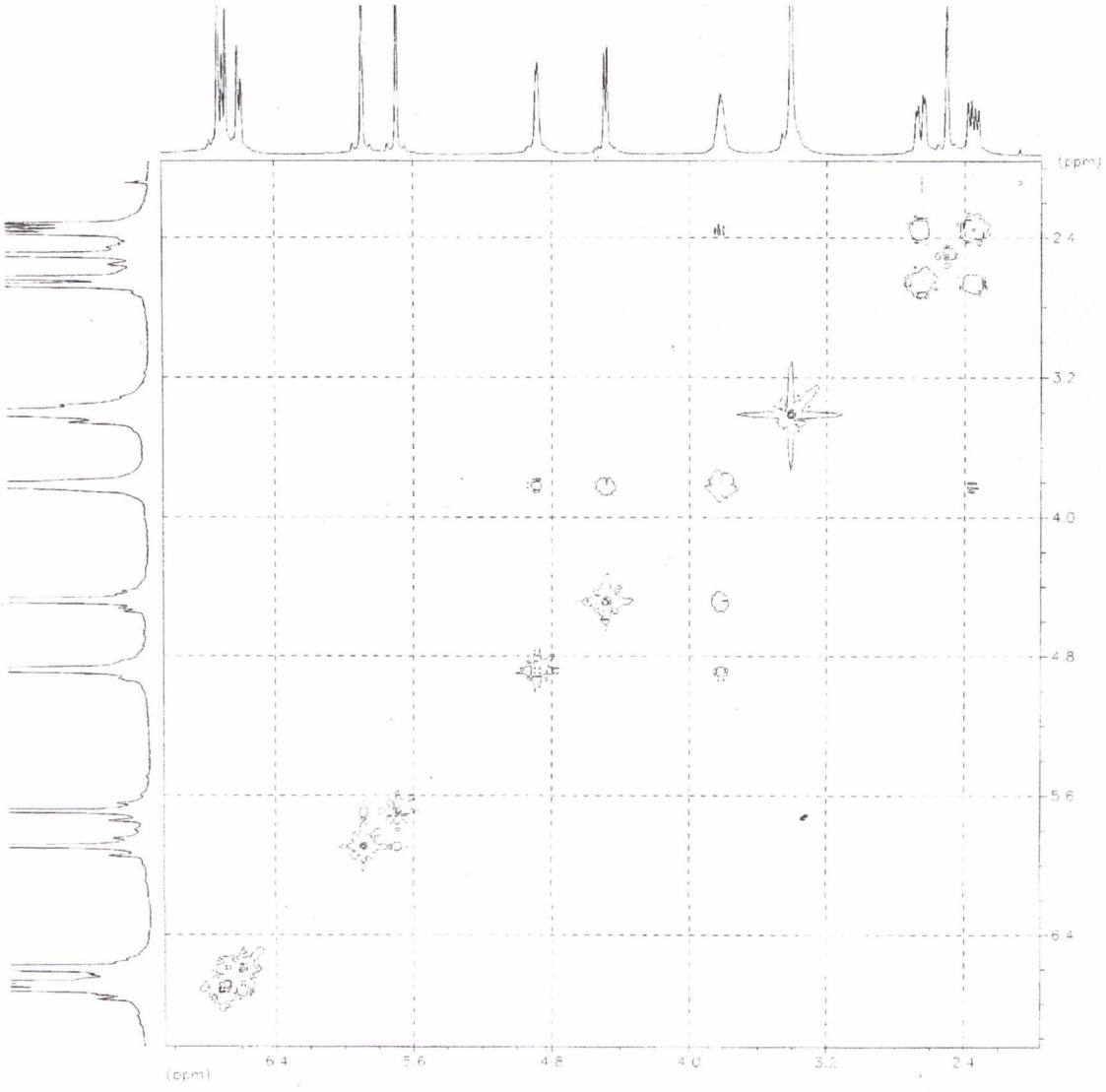

Figure ApII.3 HH-COSY of catechin standard in DMSO

Appendix III NMR Spectra of Epi-catechin Standard

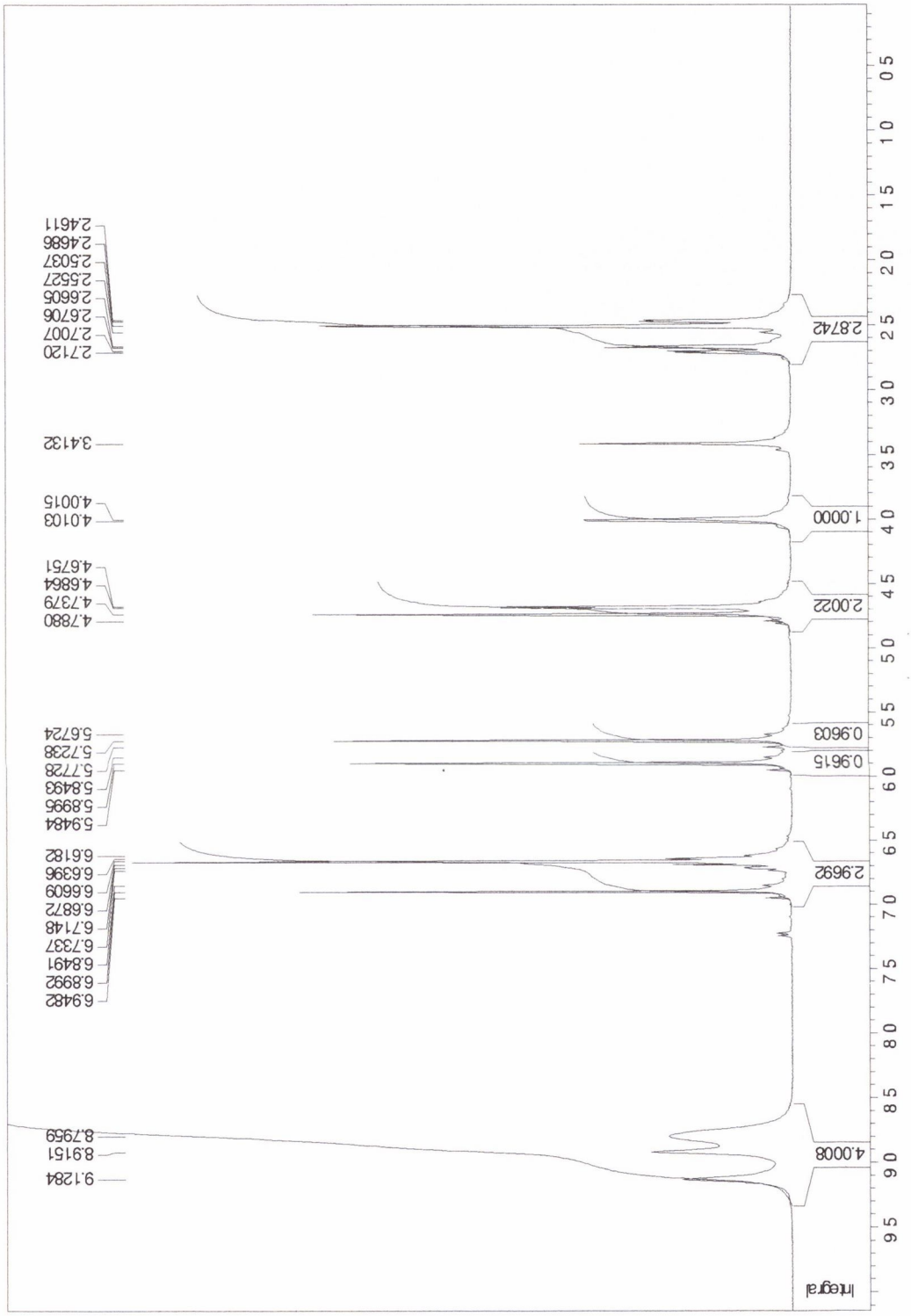

Figure ApIII.1 ^{1}H-NMR of epi-catechin standard in DMSO

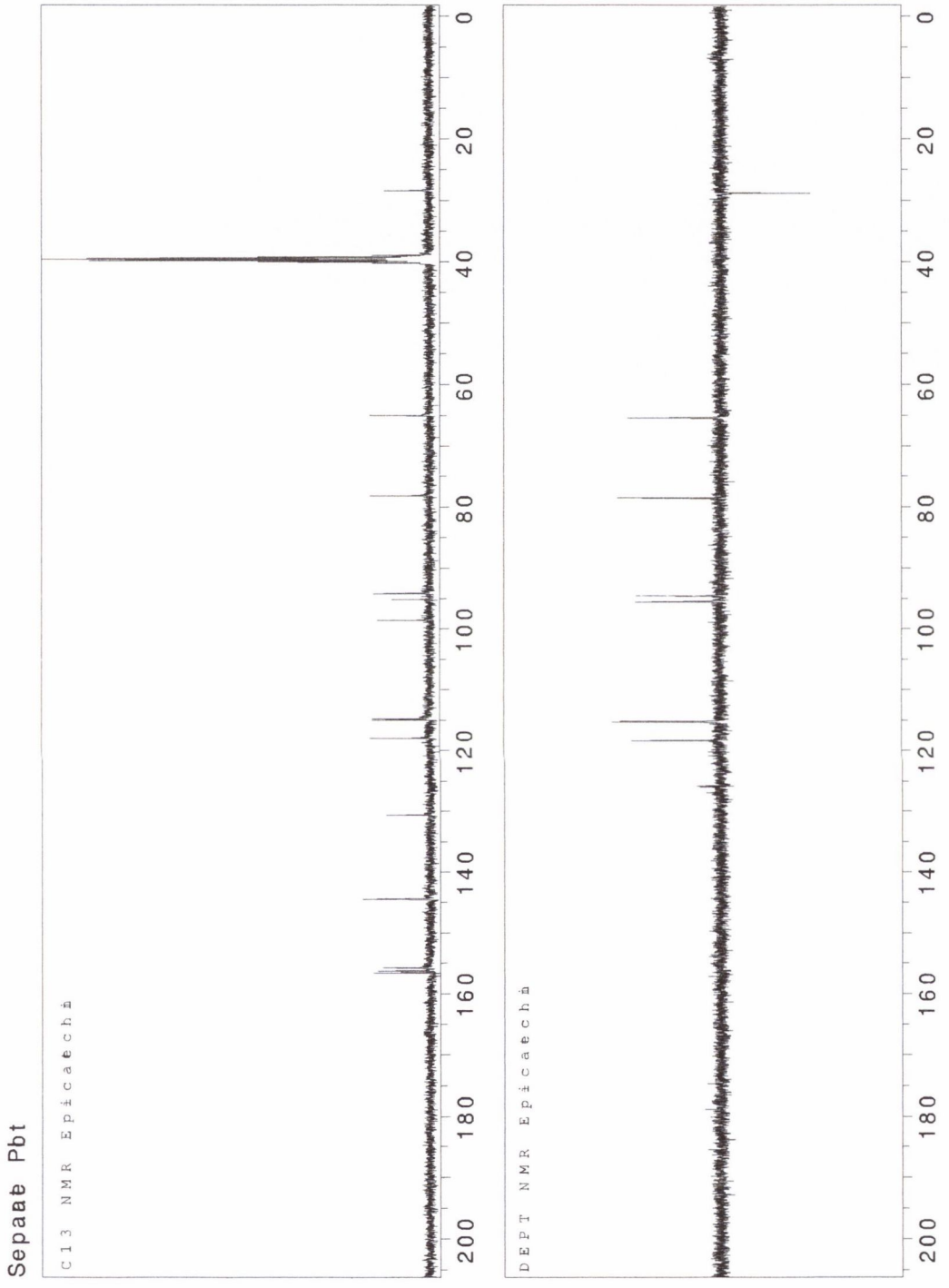

Figure ApIII.2 ^{13}C- and DEPT 135 NMR of epi-catechin standard in DMSO

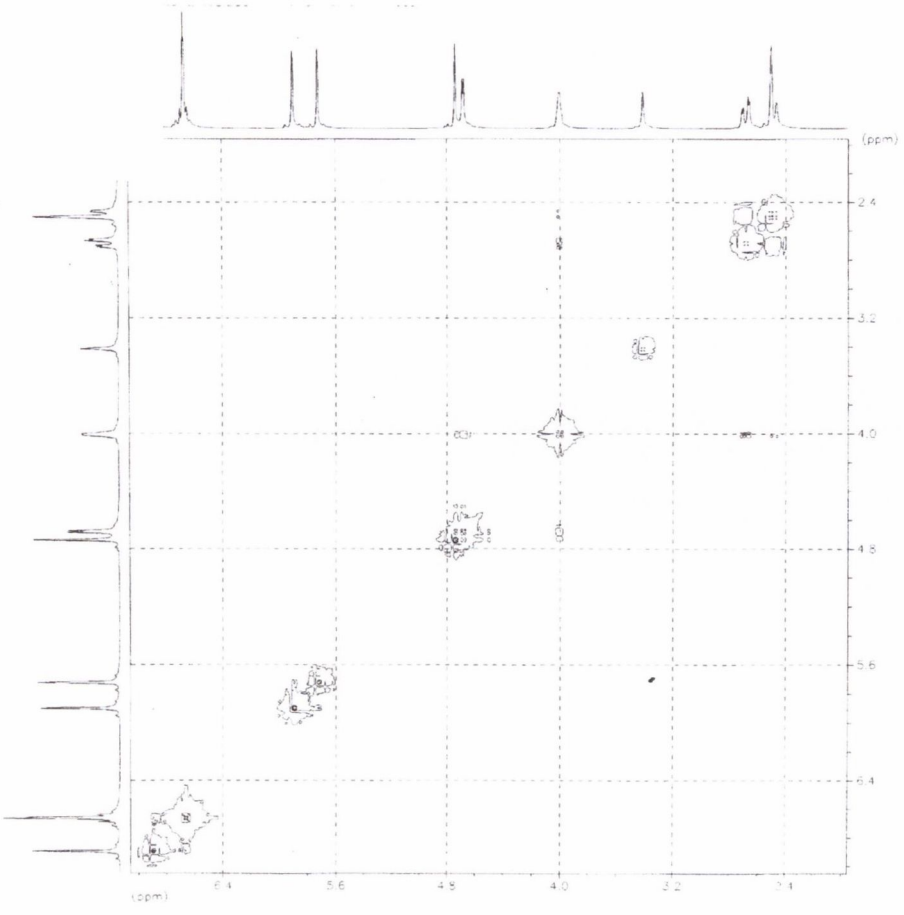

Figure ApIII.3 HH-COSY of epi-catechin standard in DMSO

Appendix IV 2-D NMR Spectra of Unknown (catechin)

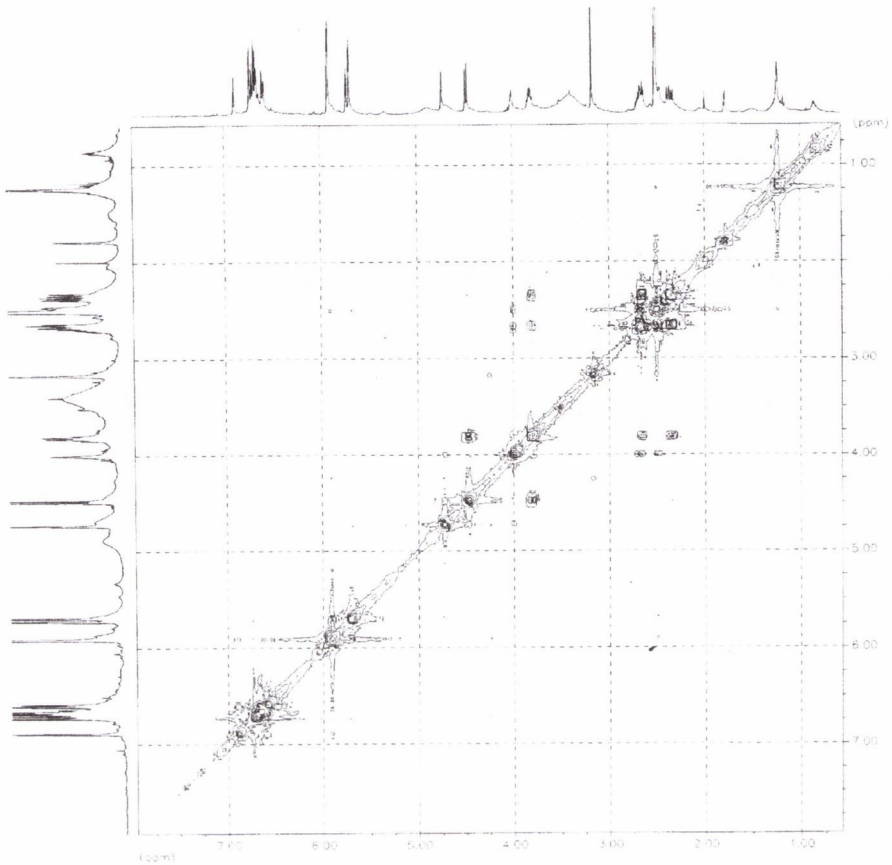

Figure ApIV.1 TOSCY of unknown (catechin) in DMSO

Appendix V Validation of Taxane HPLC System

10.1.1 Appendix V.1 Linearity of concentration vs. response

Data from Instat: Linear Regression

Number of points = 5

	Best-fit	Standard	95% confidence interval	
Parameter	Value	Error	from	to
Slope	4.861E+07	529821	4.693E+07	5.030E+07
Y intercept	-154936	184759	-742839	432966
X intercept	0.003187			

Correlation coefficient (r) = 0.9998. r squared = 0.9996

Standard deviation of residuals from line (Sy.x) = 254038

Test: Is the slope significantly different from zero?

The P value is < 0.0001, considered extremely significant.

This result was obtained from the following ANOVA table.

Source of variation	Degrees of freedom	Sum of squares	Mean square
Linear regression (Model)	1	5.433E+14	5.433E+14
Deviations from linearity (Residual)	3	1.936E+11	6.454E+10
Total	4	5.435E+14	

F = 8418.9

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Appendix V.2 Linearity of LogConcentraion vs. response/concentration

Data from Instat: Linear Regression

Number of points = 5

	Best-fit	Standard	95% confidence interval	
Parameter	Value	Error	from	to
Slope	3.819E+07	1.967E+07	-2.442E+07	1.007E+08
Y intercept	6.526E+07	1.574E+07	1.515E+07	1.153E+08
X intercept	-1.709			

Correlation coefficient (r) = 0.7461. r squared = 0.5567

Standard deviation of residuals from line (Sy.x) = 1.645E+07

Test: Is the slope significantly different from zero?

The P value is 0.1476, considered not significant.

This result was obtained from the following ANOVA table.

Source of variation	Degrees of freedom	Sum of squares	Mean square
Linear regression (Model)	1	1.019E+15	1.019E+15
Deviations from linearity (Residual)	3	8.121E+14	2.707E+14
Total	4	1.832E+15	

F = 3.767

* * *

Appendix VI Validation of Flavonoid HPLC System

10.1.2 Appendix VI.1 Linearity of concentration vs. response

Data from Instat: Linear Regression

Number of points = 5

	Best-fit Value	Standard Error	95% confidence interval from	to
Slope	1.907E+07	452321	1.764E+07	2.051E+07
Y intercept	343939	153056	-143084	830962
X intercept	-0.01803			

Correlation coefficient (r) = 0.9992. r squared = 0.9983

Standard deviation of residuals from line (Sy.x) = 206290

Test: Is the slope significantly different from zero?

The P value is < 0.0001, considered extremely significant.

This result was obtained from the following ANOVA table.

Source of variation	Degrees of freedom	Sum of squares	Mean square
Linear regression (Model)	1	7.568E+13	7.568E+13
Deviations from linearity (Residual)	3	1.276E+11	4.256E+10
Total	4	7.581E+13	

F = 1778.4

* * *

Appendix VI.2 Linearity of LogConcentraion vs. response/concentration

Data from Instat: Linear Regression

Number of points = 5

	Best-fit	Standard	95% confidence interval	
Parameter	Value	Error	from	to
Slope	1.400E+07	9481428	-1.617E+07	4.417E+07
Y intercept	2.691E+07	7805711	2076862	5.175E+07
X intercept	-1.922			

Correlation coefficient (r) = 0.6488. r squared = 0.4209

Standard deviation of residuals from line (Sy.x) = 8310390

Test: Is the slope significantly different from zero?

The P value is 0.2362, considered not significant.

This result was obtained from the following ANOVA table.

Source of variation	Degrees of freedom	Sum of squares	Mean square
Linear regression (Model)	1	1.506E+14	1.506E+14
Deviations from linearity (Residual)	3	2.072E+14	6.906E+13
Total	4	3.578E+14	

F = 2.181

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