Tissue Culture and Phytochemical Studies of *Podophyllum, Diphylleia* and *Passiflora* Species

by

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This thesis is dedicated to my parents
José and Eufrásia

" I have fought the good fight,
I have finished the course,
I have kept the faith ... "

II. Timothy 4: 7.

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Abstract

Lignans are the most important secondary metabolites known in *Podophyllum hexandrum* and *Diphylleia cymosa*. Tissue cultures studies were carried out in order to preserve and increase these germplasms. Somatic embryogenesis in both species was obtained and confirmed histologically. In *D. cymosa*, somatic embryos were induced from leaf and petiole-derived callus. The formation of abnormal embryos, with fused cotyledons, was influenced by the growth regulators used. Embryo maturation was confirmed histochemically through the identification of starch granules in the cotyledons. Regeneration was dependent on the culture media. Somatic embryogenesis in *P. hexandrum* was achieved through embryogenic cell suspension cultures from root-derived callus. Organogenesis via adventitious bud formation led to plant regeneration in liquid medium. Phenotypically normal plants were recovered. The regenerant showed the somatic chromosome number of $2n = 2x = 12$, although some chromosomes were morphologically abnormal. The recalcitrance of *P. hexandrum* towards *Agrobacterium*-mediated transformation was demonstrated with different strains of both *A. tumefaciens* and *A. rhizogenes*. The presence of cytotoxic lignans in *P. hexandrum* may have a role in the inactivation of the bacteria.

Aryltetralin lactone lignans isolated from rhizomes and roots of *P. hexandrum* and characterized by spectroscopic methods were used as standards in phytochemical studies of *D. cymosa*. Enzymic hydrolysis of the lignan glycosides, followed by reverse-phase HPLC, allowed the screening of lignans in leaf tissues, calli and cell suspension cultures. The antitumour lignan, podophyllotoxin was detected in young leaves of cultivated plants and, for the first time, *in vitro* petiole-derived calli.

Flavonoids were investigated in leaf extracts of *Passiflora edulis*, *P. incarnata* and their somatic hybrids. Fractionation of the crude extracts of *P. incarnata* and the somatic hybrid SH1 led to the isolation of compounds with skeletal type of *C*-glycosylflavones. Isoorientin was identified in *P. edulis*, whilst vitexin was found in *P. incarnata* by TLC. All the somatic hybrids showed similar consistant flavonoid banding profiles. Isoorientin and vitexin were detected in the somatic hybrids. HPLC of the parental species revealed a distinct pattern of flavonoids. Isoorientin was clearly detected in *P. edulis*, whereas isovitexin was present in both species. The fingerprint patterns of the HPLC separations of the flavonoids were similar in all the somatic hybrids, probably due to the clonal nature of the plants analysed. They appeared to be more closely related to *P. incarnata* than to *P. edulis*. However, they also exhibited flavonoids intermediate between those of the parental species. This is the first report of the biosynthesis of isoorientin and isovitexin in these novel somatic hybrids and could provide information about their inheritance.

Chapter Three
Establishment of *Diphylleia cymosa* Tissue Cultures

Abbreviations

A	absorbance
A-549	Human lung carcinoma
ABA	abscisic acid
AD	*anno domini* (Latin; in the year of our Lord)
AIDS	acquired immunity-deficiency syndrome
amu	atomic mass units
approx.	approximately
BAP	6-benzylaminopurine
BC	before Christ
C	Central
ca.	*circa* (Latin; around)
cAMP	cyclic adenosine-3′, 5′-monophosphate
CEME	Central de Medicamentos
cm	centimetre
CPPU	N-(2-chloro-4-pyridyl)-N'-phenylurea
cv.	cultivar(s)
d	day(s)
DAD	photodiode array detection
diam.	diameter
DNA	deoxyribonucleic acid
d. wt.	dry weight
E	Eastern
EEC	European Economic Community
e.g.	*exampli gratia* (Latin; for example)
EI	electron impact ionization
et al.	*et alia* (Latin; and others)
f.	formae
FDA	fluorescein diacetate
Fig.	figure
fv.	*filum variatum* (Latin; for natural variation of a species)
f. wt.	fresh weight
g	gram(s)
GA_3	gibberellic acid
gus	β-glucuronidase gene
GUS	β-glucuronidase (gene product)
h	hour

H^+	hydrogen ion
Hand.-Mazz.	Handel-Mazzeti
HIV	Human immunodeficiency virus
^{1}H NMR	proton magnetic resonance spectroscopy
HPLC	high performance liquid chromatography
HPTLC	high performance thin-layer chromatography
HT-29	Human colon adenocarcinoma
Hz	hertz
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
i.e.	*id est* (Latin; for instance)
KB	Human nasopharynx carcinoma
kg	kilogramme
kinetin	6-furfurylaminopurine
l	litre
L.	Linnaeus
Lat.	Latin
LB medium	Luria Bertaini medium
Ltd.	Limited Company
m	metre
M	molar
MCF-7	Human breast adenocarcinoma
MEK	methyl ethyl ketone
mg	milligramme
mg l^{-1}	milligramme per litre
Mhz	megahertz
$[M + H]^+$	protonated aglycone ion (molecular, fragment or product ion)
Michx.	Michaux
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
mp	melting point
MS	mass spectrometry
MS/MS	mass spectrometry/mass spectrometry
MS	Murashige and Skoog (1962) basal medium
MW	molecular weight
m/z	mass-to-charge ratio of ions

n	number of observations
NAA	naphthalene acetic acid
NC	North-Central
ng	nanogram
nm	nanometre
npt II	neomycin phospho-transferase II gene
P	probability
pp	page(s)
P-388	Murine lymphocytic leukemia
PAF	platelet-activating factor
PCV	packed cell volume
pers. comm.	personal communication
pH	negative logarithm of H^+ concentration
picloram	4-amino-3,5,6-trichloropicolinic acid
PKC	protein kinase C
PLC	preparative layer chromatography
ppm	parts per million
P. R. China	People's Republic (of China)
Ro-09-0179	5,4′-dihydroxy-3,7,3′-trimethoxyflavone
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
s	second
S	Southern
SCV	settled cell volume
s.d.	standard deviation
s.e.	standard error
SE	Southern-Eastern
spp.	species
St.	Saint
syn.	synonym
TDZ	thidiazuron [1-phenyl-3-(1,2,3-thidiazol-5-yl) urea]
TLC	thin layer cromatography
tons	tonelads
UK	United Kingdom
UM medium	Uchimiya and Murashige (1974) medium
USA	United States of America

US$	American dollars
UV	ultraviolet
v:v	volume to volume ratio
W	Western
w:v	weight to volume ratio
(x)	magnification
λ	wavelength
µg	microgram
µl	microlitre
µm	micrometre
µM	micromolar
2,4-D	2,4-dichlorophenoxyacetic acid
%	percent/percentage
±	plus and minus
=	equals
<	less than
>	more than
(+)	somatic fusion
°C	degrees Celsius

Chapter One

Introduction

1.1 Overview of medicinal plants research

1.1.1 The role of plants in medicine

Plants are one of the main living needs for human survival. Since prehistoric times plants have been the major source of food and medicine and early man has focussed attention on plants and their uses to treat his illness (He and Cheng, 1991). It has been estimated that the number of higher plant species used as medicines in many cultures range from 35,000 to 70,000 (Farnsworth and Soejarto, 1991). Records from ancient civilizations of Asia, Arabia, Europe and the Americas show the use of herbal medicines. In Mesopotamia, Sumerian medicine listed 1000 species (Principe, 1991) such as *Thymus* and *Cassia* species which were employed to prepare remedies. The early records of plants used for medicines and spiritual purposes in India, are found in the Ayurveda text of Indian healers (Healy and Aslam, 1996). Around 8000 herbal remedies are employed in the Ayurveda Pharmacopoeia (Principe, 1991). The herbal medicine tradition in the People's Republic of China remains strong and has survived dynasties and cultural revolutions. A recent national survey shows that 11,118 species are used medicinally by the Chinese people (Xiao and Shilin, 1996).

In the United States, a survey carried out in the middle 1980s has identified that 119 secondary metabolites, currently used as drugs worldwide, were isolated from only 90 species of higher plants (Farnsworth *et al.*, 1985) found in the wild flora mainly in developing countries (Palevitch, 1991). From *ca.* 250,000 known species only 5 - 15% have been screened for therapeutic potential (Balandrin *et al.*, 1993). Of the totality of flowering plants, around 155,000 species are members of the tropical flora according to Prance (1977) [cited by Cordell *et al.*, 1993]. This shows that more than half of the world's plants species are grown in the tropics. It seems, therefore, that most of the biologically active chemicals will come from tropical forests (Gentry, 1993) such as the anti-glaucoma pilocarpine (from the Brazilian species *Pilocarpus jaborandi*), the anti-malarial quinine and anti-arrhythmic quinidine (both from *Cinchona* species) and other important drugs used in therapy (Farnsworth and Soejarto, 1991). However, little is known about the chemistry of more than 99% of the plants from the vast flora of Brazil according to an estimate made by Brazilian phytochemists (Gottlieb and Mors, 1980). Thus, it is reasonable to assume that higher plants will continue to be a potential source of novel agents (Farnsworth and Kaas, 1981). There are expectations that many other medicinally important plant-derived drugs remain to be discovered and developed (Farnsworth, 1994).

The World Health Organisation (WHO) has emphasized the importance of scientific investigations into herbal medicines (WHO, 1978). A survey carried out by the WHO estimated that 80% of the world's population in developing countries rely on traditional medicines for their basic health needs (Weragoda, 1980). In many cases, people cannot afford to pay high prices for imported drugs used in orthodox medicine. It is cheaper for them to see a traditional physician who uses a medicine from the local flora. Additional to these factors, the socio-cultural aspect is one of the most important since faith in the knowledge of herbal medicine has a long tradition in rural areas (Anand and Nityanand, 1984). On the other hand, cultivation of medicinal plants and their use may help the rural communities in developing countries to improve their local health care and acts as an additional source of income (Houghton, 1995). Thus, WHO has encouraged the governments of developing countries to incorporate the use of medicinal plants in their primary healthcare systems as part of its campaign "Health For All by the Year 2000" (Akerele, 1985). Therefore, conservation of medicinal plants is of particular concern because of social and economic values (Farnsworth, 1988; see Section 1.1.4).

One important aspect in the search for novel plant-derived medicines includes ethnobotanical research linked with indigenous knowledge of healing plants. In fact, the use of some plants by the native peoples works as one form of pre-existing clinical testing (Gentry, 1993). The folklore records of many cultures have provided clues as to plants with potentially useful medicinal properties, although there are those who believe that many traditional folk cures need no proven demonstrable effect.

1.1.2 Ethnobotany: an approach to drug development

According to Jain (1994) "ethnobotany is the best word to define the experience of the first humans, who observed birds and animals and tested leaves, fruits and tubers for their ability to satisfy hunger and/or heal wounds".

Ethnobotanical knowledge was preserved verbally in songs and poems. The pictographs and scrolls of the Egyptian pharaohs contain many records referring to the uses of medicinal plants (Lewis and Elvin-Lewis, 1977). One of the earliest written texts is the *Ebers Papyrus* dated to 1550 BC, which compiled 700 formulae and folk medicines (Cordell *et al.*,1993; Schultes and von Reis, 1995). Some of these ancient beliefs were recorded in texts such as the Chinese herbal *Pen t'sao Ching*, written by the Emperor Shen Nung in 2700 BC, which describes a hundred herbal medicines (Anderson, 1977), and in the scripture of the Hindus known as *Rigveda* (Jain, 1994). European ethnobotanical records also include the Greek writings of Aristotle, Theophrastus and Dioscorides. In *De Materia Medica*, written in 77 AD, Dioscorides gathered information about the botanical and medicinal properties of 600

plants (Davis, 1995). His work was of historic significance for pharmacy for 1500 years (Anderson, 1977). Some of the plants such as *Atropa belladonna* and *Papaver somniferum* are still used as sources of active principles of therapeutic agents. In the New World, the oldest herbal text known is the *Badianus Manuscript* based on the Aztecs' knowledge of the healing properties of plants (Furst, 1995).

In recent years, there has been considerable interest throughout the world in the way that man has utilised plant resources. In South America, particularly in Brazil and Colombia, numerous field studies have concentrated on indigenous peoples' medicinal knowledge (Schultes and Raffauf, 1990; Schultes, 1994; Elisabetsky and Posey, 1994). The present state of ethnobotanical research is multidisciplinary, focusing on the basic knowledge of plant uses, phytochemistry and indigenous cultures (Prance, 1995).

Ethnobotany and ethnomedicine (use of plants by humans as medicines) represent short-cuts to the discovery of useful compounds for modern therapeutic drugs, either directly from the plants themselves, or from synthetic analogue compounds (Farnsworth and Soejarto, 1991). Ethnobotany has emerged as an important interdisciplinary science in drug discovery (Berlin and Berlin, 1994). It has been estimated that 75% of (119) plant-derived drugs were not in fact first discovered through any formula, but were identified by following folklore sources and ethnomedicinal useage (Farnsworth *et al.*, 1985). For example, *Cataranthus roseus* (Madagascar periwinkle) yielded a number of indole alkaloids, which include vincristine and vinblastine, used currently for cancer treatment (Dewick, 1996; Muggia and Von Hoff, 1997). Investigation of this species was stimulated by its reputation as a hypoglycemic agent among the local people from Madagascar (Cordell and Farnsworth, 1976). Many other drugs such as digoxin, morphine and codeine have been introduced into the international pharmacopoeia via ethnobotany and traditional medicinal studies (King and Tempesta, 1994). This has encouraged partnerships involving government funding agencies and private pharmaceutical companies, in the discovery and development of novel pharmaceuticals from plants used in traditional societies in the developing countries.

A recent example of the practical benefits from ethnobotany in drug discovery is the approach used, with success, by Shaman Pharmaceuticals. The American company brought two products, with considerable market potential, into human clinical tests. These are an oral product for the treatment of respiratory viral infections, Provir™, and Virend™ a topical antiviral agent against herpes (King and Tempesta, 1994). The integrated approach used by Shaman Pharmaceuticals involves an ethnobotanist, doctor, local healers (shamans), indigenous and forest-peoples of tropical countries working together in the field. Medical information and plants are

collected and sent to the laboratory for analysis to verify (or question) the ethnomedical claims. The value of such an approach has been stressed by Farnsworth (1994) as being unique in the development of new therapeutic agents. The company has other programmes which are targeted at the identification of novel compounds for treatment of fungal infections and diabetes (Cohen and Tokheim, 1994).

The international interest arising from the applications of ethnobotanical data today, appears in three areas (Cotton, 1996):

1. the identification of novel species with economic potential;
2. the application of traditional techniques in the conservation of species that are rare;
3. natural heritage and the conservation of native germplasm using *in situ* and *ex situ* methods for future plant breeding (see section 1.1.4).

In this context, the extract from *Homalanthus nutans*, a Samoan medicinal species, showed potent *in vitro* activity against human lymphocytic cells infected with the human immunodeficiency virus (HIV-1) (Gustafson *et al.*, 1992). Workers at the American National Cancer Institute (A.N.C.I) isolated a novel antiviral compound, prostratin, from the plant, which is under investigation via clinical trials as a possible candidate for drug development use in AIDS therapy (Cox, 1994). Advances using cell and tissue culture in the selection of plants have been made by using ethnopharmacologic data by the UK company, Phytera (Houghton, 1995).

1.1.3 The potential use of plants; economic aspects

Plants are important to man in many different ways. It is well known that higher plants have the ability to produce a wide range of natural products (phytochemicals). Natural products are sources of drugs, oils, flavours, fragrances, additives in food, resins, latexes, gums and waxes. In addition to their commercial use as fine chemicals by pharmaceutical, food and cosmetics industries they are also involved in the ecological adaptation of plants to their environment (Cotton, 1996; see also Section 1.3.1).

In terms of healing properties, plants can be used as drugs *per se* (in crude form) and in a variety of galenical preparations (advanced forms) for both internal (extracts, tinctures) and external use (lotions, creams). Such drugs have been called phytomedicines (Tyler, 1993) or herbal remedies/medicines (Newall *et al.*, 1996). It was reported that one-quarter of the prescription drugs on the market in the United States contain plant-derived constituents and this situation has remained constant for the last 25 years (Farnsworth and Morris, 1976a; Farnsworth, 1984a). Among the natural products utilized in these prescriptions only nine are produced commercially by total synthesis at a competitive price (Farnsworth, 1984b). Furthermore, the percentage of prescriptions containing plant-derived products increases considerably

to *ca.* 80% when the Chinese and Indian traditional systems of medicine are considered (Husain, 1991).

The European trade for phytomedicines, in 1990, indicated a growth rate of *ca.* 13% per year (McAlpine, 1992). The market value of over-the-counter herbal remedies (i.e. drugs which can be purchased without prescription from a doctor) is estimated at US$2.4 billion (1990 data) at their selling price in seven EEC countries (Keller, 1994). In Germany there is a strong tradition in the use of phytomedicines to treat diseases or symptoms, and it has been considered as one of the complementary therapies (Tyler, 1986). It has been reported that plant-drug preparations made, using crude drugs, remains at *ca.* 15% of all pharmaceuticals on the internal market in Germany. These preparations are mainly in the therapeutic categories of antihypertensives, tranquilizers, hypnotics, laxatives and drugs for arteriosclerosis treatment. The use of phytomedicines or plant chemical substances is also important among the antitussives and cardiotonics (Schumacher, 1991).

Edible plants are used extensively in African medicine, which contrasts with the orthodox medicine where drugs are seen as poisons and they might be effective in healing when used in established doses (Iwu, 1994). In African traditional medicine, plants (fresh/dried) are used as ingredients of remedies (teas and medicinal soups), in the manufacture of phytomedicines and for isolation of pure compounds. For all these purposes, plants are now being cultivated on a large scale in some African countries for local preparations used in health care (Sofora, 1996).

Plants have proven to be a source of active principles of many therapeutic agents. Chemical investigations coupled with the purification of plant extracts with medicinal properties have yielded numerous (purified) bioactive compounds. One example is the enzyme papain isolated from *Carica papaya* and used in the treatment of dispepsia and gastric problems (Husain, 1991) and for cleaning of contact lenses (Dewick, 1997). Natural products are also used as templates (or model compounds) for synthetic modification and structure-function studies in the development of medicinal agents. Because of advances in the last fifty years in organic chemistry, many drugs are being now produced synthetically. The antiasthmatic ephedrine is currently produced in this way rather than by extraction from the Chinese grass, *Ephedra sinica* (Fowler, 1992). Despite the impressive sophistication of synthetic chemistry, the pharmaceutical industry cannot economically synthesize commercially important plant drugs such as atropine, digitoxin and morphine which are still extracted directly from plants (Principe, 1991). Experience has shown that the natural source is one of the best economic sources for plant drug production. For example, the cost of the antihypertensive reserpine commercially extracted from *Rauwolfia*

serpentina roots is only \$0.75 g^{-1} but about \$1.25 g^{-1} via synthesis according to Oldfield (1984) [cited by Plotkin, 1991].

The continued effort to search for novel, clinically effective therapeutic agents from plants and other natural products is indispensable in the face of the need to find a cure for AIDS, tropical and infectious diseases and many other important illnesses for which no effective treatment is available (McChesney, 1993). Likewise, there are serious concerns in the development of new cancer chemotherapeutic agents (Cragg *et al.*, 1997a). The development of useful anticancer drugs such vincristine, camptothecin, etoposide and taxol has renewed and stimulated interest from the pharmaceutical industry in plant research. Camptothecin (from *Camptotheca acuminata*) has led to the development of water-soluble derivatives, irinotecan and topotecan (Wall and Wani, 1993; Potmesil and Pinedo, 1995), which were approved by the Food and Drug Administration (F.D.A) and introduced into cancer chemotherapy in the United States in 1996. There is no doubt that plants remain a reservoir of useful and novel therapeutic agents although large amounts of raw material need to be used for the isolation of small amounts of target compounds. Thus the conservation effort to preserve biological and cultural diversity has to be addressed urgently for present and future generations worldwide.

1.1.4 Conservation of medicinal species

The use of plants has an important role to play in the economics and maintenance of health of the global community (Cragg *et al.*, 1997b) and, in some case towards poverty alleviation. Therefore, preservation of the world's plant genetic resources is worthwhile and of great concern to society. This has been widely discussed in the scientific community and in public policy debates. A conference held in Thailand (1988) was organized by the WHO, the International Union for the Conservation of Nature and Natural Resources (IUCN) and the World Wide Fund for Nature (WWF). For the first time, in the same forum, policy makers in conservation and health care discussed the conservation needs of medicinal plants for sustainable development. It highlighted the importance of medicinal plants to both developing and developed countries and also their rational utilization and conservation for future generations (Hamann, 1991).

Subsequently, another important meeting was held in Rio de Janeiro, Brazil, in 1992; the United Nations Conference on Environment and Development (UNCED) (Garner, 1996). Some of the main points of the Conference were concerned with the conservation of biodiversity, the development of the genetic resources with fair and equitable sharing of the resulting benefits, the promotion of research, training, and technology transfer (Gollin, 1993). It has been emphasized that source countries and

their forest-dwellers (or indigenous people) are the custodians of the majority of the world's genetic resources. It is not surprising then, that 17 articles (of the conference) have addressed a number of issues facing developing countries. Emphasis was also given to the urgent need for an effective international collaboration between scientists and communities of the developing and developed countries (Baker *et al.*, 1995). During the conference, a central theme of debate was an increasing awareness between the participants to ensure environmental protection associated with an efficient economic growth. From this emerged an International Agreement with a set of strategies for promoting sustainable development allied with protection of biodiversity (Agenda 21, 1993).

At a symposium in San Jose, Costa Rica, in 1994 to address the intellectual property rights focusing on the drug development from natural bioactive compounds, scientists of developed (and source) countries presented their points of view. As a result, access to plant genetic resources by foreign collectors now required prior consent by source countries with royalties and sharing mechanisms for the outcomes of research. In Brazil, permission has been handled by the National Council of Scientific and Technological Development and the collectors must have a formal partnership with a Brazilian research institution (Gyllenhaal, 1996). Many pharmaceutical companies, in order to obtain medicinal plants, are now establishing collaborative agreements with individual suppliers or organizations in countries such as Brazil, Costa Rica, Nigeria and China (Baker *et al.*, 1995). Conservation is giving a positive message for the world about plant diversity and has become a global issue for the 1990s and beyond (Given, 1994).

One of the key issues facing plant genetic resource conservation workers is preserving as wide a sample as possible of existing genetic diversity within target species. This requires two basic approaches *ex situ* (or off-site) and *in situ* as complementary strategies to facilitate effective germplasm conservation. The former option has the objective to maintain the integrity, in terms of genetic constitution, of the accessions (Frankel *et al.*,1995). More research is being carried out on the scientific bases of this method. The *ex situ* techniques are subdivided into the storage of seed, storage of DNA, pollen storage, *in vitro* storage, field gene banks and botanical garden conservation (Maxted *et al.*, 1997). The botanic garden option is the best example of *ex situ* conservation, as living plant collections, of rare or endangered plants and wild species with medicinal value (Heywood, 1991; Frankel *et al.*, 1995). However, the method has limitations such as reduced levels of genetic diversity resulting from the long-term storage of seed and, of whole plants or tissues (Heywood, 1991). The resulting germplasm would be potentially unnatural since it

has not been adapted to any environmental changes such as novel biotypes of pests, diseases and climatic changes.

In recent years, attention has been directed towards *in situ* conservation (Maxted *et al.*, 1997). Rather than *ex situ* preservation of individual species, the *in situ* conservation of an ecosystem permits evolution to continue (Prance, 1997). The main techniques include the genetic reserve (for wild species), on-farm (for crops) and home gardens for conservation of medicinal species (Maxted *et al.*, 1997). This approach has been seen as a method of choice for conservation in countries such as Brazil, where there is no complete inventory of medicinal plants. Although, some of its disadvantages are the limited genetic diversity that can be conserved, it requires active supervision and monitoring and needs maintenance of traditional cultural systems (Maxted *et al.*, 1997).

There are several problems regarding genetic conservation of medicinal species resources, often including, little knowledge about their cultivation, breeding systems, harvesting and storage (Ford-Lloyd and Jackson, 1986). It has been reported that for many medicinal species facing over-exploitation (and hence extinction), there is no long-term conservation strategy in protected areas (McNeely and Thorsell, 1991). Among the wild populations of such plants are species which have, or may have, an economic, scientific or social value. Estimates suggest that for every 1,000 extinct species, eight could have yielded useful pharmaceutical products (Jeffries, 1997). Recently, universities, experimental stations and state agriculture and forest departments have set up conservation programmes in response to concerns about biodiversity conservation (Hawtin and Hodgkin, 1997). Even though *ex situ* and *in situ* conservation have their advantages and disadvantages, it is important to ensure conservation of native germplasm through an integration of these approaches (Cotton, 1996). Furthemore, the advances in the use of molecular markers would allow a better analysis of the variation amongst plants at the DNA level using techniques such as RAPD and RFLP analysis. Therefore, the combination of data derived from different fingerprinting techniques with that obtained from biochemical and morphological studies is being used as tools for conservation workers (Karp *et al.*, 1997).

1.1.5 Medicinal plant biotechnology

The rapid development of plant biotechnology and the growing awareness of decreasing plant resources, has helped to focus attention on alternative sources of natural products (Fowler *et al.*, 1990). Plant tissue culture is a promising area for producing plant-derived clinically important natural products especially those which are not readily synthesized or are difficult to obtain in sufficient quantities from whole plants (Phillipson, 1990a). Many biologically active compounds from plants have

complex structures and chiral centres. In these cases, the chemical-synthetic route can be difficult and plant cell cultures thus provide an obvious approach to natural product biosynthesis (Wink, 1990).

Human health is an important concern at the international level. A sustainable supply of high quality material for large scale production required in the pharmaceutical industry has always faced common problems: variation in the concentration of the compounds often depending on season; some species are found in geographically (or politically) restricted areas and often expensive and difficult extraction processing of the desired compound from plants is required. The use of cultured plant cells coupled with chemical methods has advantage over whole plants: optimization of growth parameters; control of growth conditions and less complexity of the extracts facilitating the separation of target compounds (Kutney, 1996). Plant cell culture *per se* could also be exploited for the storage of gene pools of threatened (or rare) medicinal species.

During the past two decades medicinal species have been of great interest in the context of biotechnology, particularly since the first published reports on secondary metabolite accumulation in cultured plant tissues (Banthorpe, 1994). Many reviews have focussed on a number of biologically active secondary products such as alkaloids, lignans, terpenoids, quinones and novel products which have been synthesised by plant cell cultures (Parr, 1989; Phillipson, 1990b; Fowler, 1992). The production of secondary metabolites has been reported during the post-exponential phase of growth of *in vitro* cultured plant cells (Ravishankar and Venkataraman, 1993). For example, the addition of methyl jasmonate to cell cultures of *Taxus* species has shown promising results in the production of Paclitaxel (Taxol™) and related taxanes (Yukimune *et al.*, 1996). Paclitaxel was approved in 1992 in the United States (Kingston, 1993) for clinical treatments of ovarian and breast cancers but is also effective for other types of cancers (Suffness and Wall, 1995). It is an important example of the production of a chemotherapeutic drug through biotechnological advances involving plant tissue culture.

There are many justifications for mass propagation of medicinal species using *in vitro* techniques rather than conventional methods (Bajaj *et al.*, 1988). Medicinal species can be propagated easily *in vitro* using the same methods frequently established for ornamental plants (Hussey, 1983). Because *in vitro* tissue culture allows long-term conservation for recalcitrant and clonally propagated species, the disadvantage is always the risk of somaclonal variation (Maxted *et al.*, 1997). However from the pharmaceutical point of view, micropropagation from meristem culture has been successfully employed for regeneration of useful genotypes such as

Cinchona succirubra (Koblitz *et al.*, 1983) and some species of *Dioscorea* (yams) which produce steroids.

The cloning of genes for plant enzymes and their expression in cells of yeast and microorganisms have given clues for the potential biosynthesis of desired natural products (Phillipson, 1990b). Plant cell cultures are an excellent source of such enzymes, which are also able to catalyze a broad range of reactions and can also perform modifications that are not easily achieved by chemical synthesis or by the use of microorganisms (Pras, 1990). Plant enzymes have been exploited for the production, by bioconversion, of pharmaceutically important compounds such as the podophyllotoxins (Pras, 1992).

One of the present limitations of producing natural products from tissue culture is the high cost. It has been provoked, partially, by the slow growth rates of cells and the costs of culture media and production facilities which are expensive compared to these required for microbial cells. Extraction of the target compound is often a limiting factor because of lack of release into the media from where it could be easily recovered (Tyler, 1986; Weathers *et al.*, 1990). The low yields of desired metabolites obtained from cultured plant cells is one of the major problems to successful commercial production (Yeoman *et al.*, 1990). Exceptions are the production levels of shikonin, from *Lithospermum erythrorhizon* cell cultures, which is used as a dye and for its antiseptic and anti-inflammatory properties in Japan (Fujita, 1988). Shikonin has been highlighted as the most successful example of drug production by tissue cultured cells (Brownleader and Dey, 1997) approximately 800 times more productive than via plant cultivation and less costly than chemical biosynthesis.

Techniques of genetic engineering are thus now being used in the manipulation of metabolic pathways in order to increase (or decrease) the production of target compounds (Nessler, 1994). Molecular approaches such as *Agrobacterium*-mediated transformation have been reported for yield improvement on classes of useful secondary compounds such as alkaloids either in whole plants or in plant tissue and cell cultures (Yamada and Hashimoto, 1990). Their synthesis is regulated by complex mechanisms and in principle is fixed in the genome of the plant. Hence the first step is in the isolation of the biosynthetic enzymes or those involved in the regulation of different pathways of secondary metabolism. In addition to this it is essential to identify the relevant genes, how they are expressed and the factors involved (Alfermann and Petersen, 1995). Therefore, more research has to be done in plant biochemistry, molecular biology and regulatory aspects of secondary metabolism to improve the production of useful secondary metabolites by plant cell culture especially for an industrial scale operation.

1.2 Botanical features of target species for this study

Part I

1.2.1 The genera *Podophyllum* and *Diphylleia*

1.2.1.1 Aspects of taxonomy

In 1753, Linnaeus described the genus *Podophyllum* when he published *Species Plantarum* in which he gave Latin binomials to some 10,000 species of plants. The name *Podophyllum* is from the Greek describing the leaf shape as like a duck's foot. In 1824, the botanist Nathaniel Wallich created the name *P. emodi* for the species now known as *P. hexandrum* Royle (Senior, 1965). Since then, continued taxonomic work has resulted in the recognition of many other *Podophyllum* species, mostly from China. Several reviews include different classifications of the *Berberidaceae* (Terabayashi, 1982; Loconte and Estes, 1989; Stearn, 1993), and attempts to provide more exact information about the systematics are in progress using molecular analysis integrated with morphological data (Youngdong and Jansen, 1993). This work has used the restriction site mapping of the chloroplast DNA and gene sequence with the aim to provide a broadly-based view of the generic phylogenetic relationships within the family.

Diphylleia was first discovered by André Michaux (1786) in North Carolina, who described and illustrated it in his *Flora Boreali-Americana* in 1803 (Ying *et al.*, 1984). The generic name again comes came from the Greek and refers to its bilobed leaves (Stearn, 1971). *Diphylleia* and *Podophyllum* are among the 120 plant genera with a disjunct distribution between Eastern Asia and Eastern North America (Ying *et al.*, 1984). It is thought that these areas were covered with forest of mixed mesophytic flora that circled the globe during the Tertiary period. The existence of several of these genera in the Tertiary forest flora has been confirmed through paleobotanical data (Wood, 1972), although there is no evidence so far from fossil records of *Diphylleia* or *Podophyllum* (Ying *et al.*, 1984).

The genus *Diphylleia* is taxonomically close to *Podophyllum*. While they have characteristics in common such as habit, morphology, karyotype and chemistry, both are a source of lignans; the differences are related to floral biology. These include pollination, mode of anther dehiscence and pollen structure (Meacham, 1980; Shaw, 1996a).

1.2.1.2 Classification and distribution

The placement of the genus *Podophyllum* within the *Berberidaceae* has been the subject of numerous systematic investigations. Some authors such as Jafri (1974) have followed the classification by Hutchinson (1969) in which the herbaceous species

from the *Berberidaceae* are put into a new family *Podophyllaceae*. As recognized by Stearn (1993), *Berberidaceae* is a family of shrubs and perennial herbs with approximately 600 species, 2 subfamilies, 5 tribes and 13 to 16 genera (Table 1.1). The genera *Podophyllum* and *Diphylleia* consist of perennial herbaceous species, with thick, fleshy rhizomes, adapted to the undergrowth in temperate deciduous forests (Foster, 1989). According to taxonomic classifications there is though a close link between *Podophyllum* and *Diphylleia* (Meacham, 1980; Loconte, 1993). A recent taxonomic revision of the genus *Podophyllum* reports that 10 species of *Podophyllum* and 3 species of *Diphylleia* comprise the subfamily *Podophylloideae* (Shaw, 1996a). The current location of *Podophyllum* and *Diphylleia* in the subfamily *Podophylloideae* is given in Table 1.1.

Podophyllum L. is a single genus in the *Berberidaceae* distributed in North America (1 species) and Sino-Himalayan area (9 species) (Shaw, 1996a). Within the genus *Podophyllum* four sections are recognized: *Podophyllum*, *Dysosma*, *Paradysosma* and *Hexandra* (Table 1.2). Within the sections *Podophyllum* and *Hexandra*, the species *P. peltatum* L. and *P. hexandrum* Royle have been the most utilised for their medicinal value (Section 1.2.1.5).

Table 1.1: Systematic positioning of *Podophyllum* and *Diphylleia* within the *Berberidaceae*

Subfamily	Tribe	Genus
Berberidoideae	Berberideae	*Berberis* *Mahonia*
	Nandineae	*Nandinia*
	Epimediae	*Epimedium* *Vancouveria* *Jeffersonia*
	Achlyeae	*Achlys* *Bongardia* *Leontice* *Gymnospermium* *Caulophyllum*
Podophylloideae	Podophylloideae	*Podophyllum* (*Dysosma*) *Diphylleia*

Data from Stearn, in Heywood (1993).

P. peltatum is widespread and common in Eastern North America (Meijer, 1974). In Asian countries, *P. hexandrum* (syn. *P. emodi* Wall) is found in Bhutan (Grierson and Long, 1984), Nepal (Hara, 1979), Tibet and Northern Yunnan (China) (Anon, 1980), Afghanistan (Browicz, 1973) and the Himalayan areas of Pakistan (Jafri, 1974) and India (Rao and Hajra, 1993). Before over-collection, it was common in the Alpine Himalayas growing at an altitude of 3000 to 4000 m (Rao and Hajra, 1993).

The genus *Diphylleia* is a perennial, herbaceous group of three medicinal species (*D. cymosa* Michx., *D. grayi* Schmidt and *D. sinensis* Li) (Table 1.2). *D. cymosa* is indigenous to Eastern North America, distributed at higher altitudes on moist, organic, mineral soils of woodlands and mountain stream banks in the Appalachians (Ying *et al.*, 1984).

1.2.1.3 Cytology

Cytologically the genus *Podophyllum* is uniform with a diploid chromosome number $2n = 2x = 12$ for those species so far examined (Kosenko, 1979). Swanson and Sohmer (1976a) also reported $x = 6$ for *P. peltatum*. Karyotype studies are described for *P. peltatum* (Newman, 1959, 1966; Kosenko, 1979), *P. hexandrum* (Heyenga, 1989; Siddique *et al.*, 1990), *P. pleianthum* and *P. mairei* (Li, 1986; Zhang *et al.*, 1991).

A chromosome number of $2n = 2x = 12$ was reported for *D. cymosa* by Langlet (1928). The closely related japanese species *D. grayi* is better studied cytologically and there are a number of published chromosome counts and karyotype studies (Kuroki, 1967; Noda and Fujimura, 1970). A karyotype study has been reported for the species *D. sinensis* (Shao-Bin and Zhi-Hao, 1996).

1.2.1.4 Economic importance

The species *P. hexandrum* and *P. peltatum* have received considerable attention in relation to their phytochemistry since the early 1960's when they were screened for anti-tumour activity. Some other species have been investigated in the past 20 years, mainly by Chinese workers. As a result, several lignans and their glycosides with anti-tumour activity were isolated and characterized (Dewick, 1996). Since then, *Podophyllum* species have become economically important as a source of such lignans.

There are few data on market prices for these species and the drugs derived from them so far. In the United States (in 1991), the consumption of *P. peltatum* was approximately 100 tons even before the anticancer properties were reported [Duke, in Principe, (1991)]. In the same year, the annual commercial value of *P. peltatum* was

Table 1.2: Intra-generic classification of *Podophyllum* and *Diphylleia*

Genus	Section	Species	Distribution	Reference
Podophyllum	Podophyllum	*P. peltatum* L.	Canada (S), North America (E)	Ying, 1979
	Dysosma	*P. pleianthum* Hance	China (E), Taiwan	Hance, 1883
		P. versipelle Hance	China (S), Vietnam	Ying, 1979
		P. mairei Gagnepain	China (W, NC)	Gagnepain, 1938
		P. difforme Hemsley & Wilson	China (W, C)	Hemsley, 1906
		P. x majoense Gagnepain[1]	China (C)	Gagnepain, 1938
		P. sp. A[2]	China (Omei Shan, Sichuan)	Zhuang *et al.*, 1993
		P. delavayi Franchet	China (W, C)	Franchet, 1895
		P. delavayi var. *longipetalum*[3]	China (Omei Shan, Sichuan)	Zhuang *et al.*, 1993
	Paradysosma	*P. aurantiocaule* Hand.-Mazz.	China,Tibet (SE), Himalaya	Handel-Mazzeti, 1924
		P. furfuraceum (Bao) Shaw	China (Yunnan)	Bao, 1987
	Hexandra	*P. hexandrum* Royle	Afghanistan, India, Nepal (Bhutan), Pakistan, Tibet-Yunnan (China)	Selivanova-Gorodkova, 1975; Shaw, 1998
Diphylleia		*D. cymosa* Michx.	North America (E)	Li, 1947; Ying *et al.*, 1984
		D. grayi Schmidt	Siberia (E, C), Japan	Li, 1947; Ying *et al.*, 1984
		D. sinensis Li	China (Yunnan)	Li, 1947; Ying *et al.*, 1984

[1] A putative hybrid between *P. delavayi* and *P. mairei* (Shaw, 1996a); [2] *Dysosma emiense* Wu & Zhuang *ineditus*; [3] Wu & Zhuang *ineditus*.
C: Central; E: Eastern; NC: North-Central; S: Southern; SE: Southern-Eastern; W: Western.

estimated at about $200,000 based on the market value of raw material at $ 1.00 per pound (Principe, 1991).

The anticancer drug market between 1980 to 1990 has been growing at 25% annually [Anon, in Principe (1991)]. In this context, *Podophyllum* has played an important role in the development of two novel potential chemotherapeutic agents, etoposide and teniposide (Cragg *et al.*, 1993; see also Chapter 4). Etoposide was introduced in 1984 and has been used in the chemotherapy of cancer having annual sales around $15 million in the United States (Fellows, 1992).

1.2.1.5 Ethnobotanical approaches for *Podophyllum* and *Diphylleia*

Many drugs were scientifically investigated by an examination of published ethnobotanical reports and from field studies, such as the cytotoxic effects of *Podophyllum* (Schultes and von Reis, 1995). The species *P. hexandrum*, *P. peltatum* and *D. cymosa* are included in Hartwell's survey (Hartwell, 1971) which is a compilation of over 3,000 different species and their ethnomedical uses against cancer.

Podophyllum plants were used over 2,000 years ago in China as an anti-tumour drug (Dewick, 1996). They have a long and extensive folklore use in American and Asian cultures for the treatment of skin cancers and warts (Cordell *et al.*, 1993). There is a record of the use of *P. peltatum* as a drastic purgative in constipation resulting from hepatic disorders (Fahmy, 1932). The use of podophyllin, the resin of *P. peltatum* (commonly known as the American Podophyllum, wild Mandrake, Mayapple root, mayweed, wild lemon or devil's apple) (Leung, 1980), goes back to folk medicine of the Maine Penobscot Indians, who applied it topically to treat poisonous snakes bites. The natives also used the roots as a suicide agent and poison (Kelly and Hartwell, 1954). For the American Indians the resin was utilized to treat cancer (Hartwell, 1967) and as vermifuge (Sanecki, 1996). The ripe fruit of *P. peltatum* is known to be consumed by the Menomini and Meskwaki tribes (Smith, 1923; 1928) and is also used medicinally by the Iroquois (Moerman, 1986). In the medical practices of the Cherokee Indians the rhizome and whole plant were used as a slow-acting purgative and an antihelmintic; the juice was dropped into the ear for ear ailments; it was also used as a dermatological dressing for ulcers and sores and as an antirheumatic (Hamel and Chiltoskey, 1975). The native Americans used extracts of the roots for skin disorders and tumours (Simpson and Ogorzaly, 1995). According to a report on the Chinese herbal use of *Podophyllum*, resin was used to treat snake bites, lymphadenopathy and tumours (Bracchi and Routledge, 1996).

P. hexandrum, known as Indian Podophyllum, is one of the medicinal plants cited in the Pharmacopoeia of India (1955). It was probably used many years ago in

the ancient Indigenous System of Medicine, the Ayurveda, based on the fact that this species has many vernacular names in linguistic regions not adjacent to its normal habitat. "Rasa", "tikta guna", "snigdha", "vipaka" and "madhura Virya" are some of the names associated with the plant according to Ayurvedic use (Karnick, 1994). The resin was administered in small doses for chronic constipation while in overdose it acts as a poison (Dymock *et al.*, 1890). It has been reported that its roots were ground and used as a purgative (Chatterjee, 1952). A decoction of roots was employed in the treatment of diarrhoea and liver problems. The ripe fruit is eaten in some parts of the Indian Himalayas (Rao and Hajra, 1993), where it has been used to promote conception (Kapahi, 1990) The combination of ethnomedical information and ethnobotanical data referring to this species is summarised in Table 1.3.

Table 1.3: Ethnomedical and ethnobotanical data on *P. hexandrum*

Plant part	Main uses	Region	Reference
Rhizome	Eye treatment	Kedernath	Dymock *et al.*, 1890
	Bile expellent	Punjab	Chatterjee, 1952
	Hepatic stimulant	Nepal	Suwal, 1979
	Antitumour	China	Evans, 1996
Root	Unspecified	West	Chaudhri, 1956
	Cholagogue, purgative	India, Pakistan	Krishnamurthy *et al.*, 1965
Fruit (immature)	Unspecified	Lahoul	Dymock *et al.*, 1890
	Fertility agent	Nepal	Lancaster, in Shaw, 1996a
Fruit (dried, ripe)	Treatment of amenorrhea	China	Xiao, 1989

Vernacular names are important in the ethnobotanical approach because they are indicators of the means of communication with the people from the villages (Jain, 1994). Earlier reports of the vernacular names such as "papra", "papri" and "bakra" for *P. hexandrum* suggested that the plant shows a bile expelling property (Chatterjee, 1952). Some of the key vernacular names for *P. hexandrum* are given in Table 1.4.

Diphylleia cymosa is morphologically similar to *P. peltatum* and it has been called by the common names of Southern Mayapple and umbrella leaf (Lloyd and Lloyd, 1887). The roots of the related *D. sinensis* were used for the treatment of cancerous sores in China (Hartwell, 1968).

The American Cherokee Indians used an infusion of *D. cymosa* as a diuretic, antiseptic and for treatment of smallpox (Moerman, 1986). Earlier clinical investigations reported that the resin of *D. cymosa* demonstrated none of the

biological actions associated with *Podophyllum* (Lloyd and Lloyd, 1887). However, substances with an action similar to colchicine and podophyllin, showing an antimitotic effect on cancer cells, were identified in extracts of *D. grayi* (Kimura, 1963). These findings were used as the basis for the division of the family *Podophyllaceae* into two subfamilies: *Podophylloideae*, including the genera *Diphylleia* and *Podophyllum* (with anti-tumour properties), and *Glaucidioideae* containing the genus *Glaucidium* (Toyokuni and Toyokuni, 1964). Since then *Glaucidium* has been shown to belong to the *Paeoniaceae/Ranunculaceae* families (Melville, 1983; Jury, 1993a). Phytochemical work has identified *Podophyllum* lignans in *D. grayi* rhizomes and *D. cymosa* leaves (Broomhead and Dewick, 1990) but at low concentrations in the rhizome of *D. cymosa*. This may account for the negative report referring to biological activity by Lloyd and Lloyd (1887).

Table 1.4: Reported vernacular names for *P. hexandrum*

Local name	Language / Region	Reference
Banbaigan	Hindi	Jain and DeFilipps, 1991
Bankari	Punjabi	Nadkarni, 1976
Banwagan	Kashmiri	Nadkarni, 1976
Hsiao-yeh-lien	Chinese	Xiao, 1989
Indian Podophyllum	English	Chatterjee, 1952
Mámirán	Kedernath	Dymock *et al.*, 1890
Padeval	Maharastra	Jain and DeFilipps, 1991
Papra	Hindi	Krishnamurthy *et al.*, 1965
Papra	Bengali	Nadkarni, 1976
Papri	Hindi	Chopra *et al.*, 1958
Rikhpeta	Jaunsar (Chakrata)	Krishnamurthy *et al.*, 1965
Vakra	Sanskrit	Nadkarni, 1976
Venivel	Gujarati	Jain and DeFilipps, 1991

1.2.1.6 History of cultivation of *Podophyllum* species

References to cultivation of *P. peltatum* date back 1664 when it was introduced into Britain for cultivation in woodland gardens (Sweet, 1830; Sanecki, 1996). It has been reported that the species was collected in the Appalachian mountains rather than being cultivated for medicinal use (Krochmal, 1968). The interest in cultivation was stimulated by the demand in the drug trade for dried rhizomes (Meijer, 1974).

The related species *P. hexandrum* has been found in the wild since 1820. It was one of the 41 Indian species listed in the British Pharmacopeia as growing on a limited scale but being capable of cultivation and exploitation in India (Hooper, 1913). Earlier work reported that the species can be propagated easily either from sections of rhizomes or seeds (Troup, 1915). However, plants raised from rhizome cuttings needed at least twelve years to produce a marketable rhizome. It has been found growing naturally in West Pakistan but there was no cultivation on a commercial scale (Chaudhri, 1956). Cultivation was recommended in India and its inclusion was considered in the Indian and Colonial Addendum to the British Pharmacopeia (Ellis and Fell, 1962). There are a number of reports referring to vegetative propagation through rhizome cuttings and seeds (Badhwar and Sharma, 1963; Krishnamurthy *et al.*, 1965). These studies revealed that those cuttings containing the apical portion of the rhizome bearing leaf buds gave the best results in relation to the percentage of successful propagation and growth of shoots. In adition to these results, the most economical harvesting of roots and rhizomes was at five years for plants raised by vegetative propagation, and six years in the case of plants raised from seed.

It is also noteworthy that *Podophyllum*, like many other species in the *Berberidaceae*, has been cultivated as an ornamental (Stearn, 1993). There is only one record of *P. hexandrum* as an alien species in Britain. This was based on a single occurrence; the plant though at Charmouth did not flower. (Shaw, 1996b). The species shows a solitary pink or white flower and pigmented foliage. The fruit is oval in appearance and becomes red when ripe (Dewick and Shaw, 1988).

1.2.1.7 Conventional cultivation of *P. peltatum* and *P. hexandrum*

P. peltatum can be grown from small sections of rhizomes and seeds. However, seed production in the wild and in cultivation is rare. Seedlings are quite uncommon. Some of the many problems with breeding of the species are related to sterility, incompatibility and low fertility. Studies using artificial pollination methods reported that *P. peltatum* is self-incompatible, non-agamospermic, cross-pollination between clones was required for optimum seed production (Swanson and Sohmer, 1976b; Rust and Roth, 1981). Fruits from naturally pollinated flowers produced two and half times as many seeds than those derived from hand pollinations (Whisler and Snow, 1992). In the Netherlands, plants in cultivation are known to develop leaf blotch disease caused by the fungus *Septotinia podophyllina* which infects *P. peltatum* and also other species in Europe (Gremmen, 1987; Shaw, 1996b).

Propagation of *P. hexandrum* is commonly carried out by seed. Even though cultivation seems easy, the germination of seeds needs appropriate conditions (see

Chapter 2) and has been studied with the aim to increase yield (Nautiyal *et al.*, 1987; Nautiyal, 1988).

Studies of *P. hexandrum* lines have shown that plants cultivated at the University of Nottingham are long-lived and produce large clonal colonies (Shaw, 1996b). The rhizomes have been kept growing for about six years after which the growth rate declined. Vegetative propagation through rhizome cuttings has been found to be an inefficient but slow method compared with seedlings.

It has been suggested that *P. hexandrum* is of hybrid origin, probably from *P. aurantiocaule* x *P. delavayi* Shaw (1996a). The species shows different morphological characters throughout its range such as leaf shape and flower colour. Such changes are thought to indicate that this species has a hybrid origin. In an investigation carried out by Shaw (1996a), interspecific crosses between *P. peltatum* with pollen from *P. hexandrum* resulted in seedless F_1 hybrid fruits. At the same time, artificial intraspecific crosses between different lines of *P. hexandrum* produced plants with aborted anthers and few seeds. It appears that variation in anther length in the wild may lead to variation of fertility (Shaw, 1996a).

1.2.1.8 Cultivation of *Diphylleia cymosa*

While the species *D. cymosa* is collected in the wild (Krochmal, 1968), the literature does not report commercial cultivation for pharmaceutical use. However, it has been introduced as an ornamental plant in woodland gardens (Stearn, 1989; Griffiths, 1994). The sterility of seeds occurring in *Diphylleia* species was discussed by Noda and Fujimura (1970), who suggested that asynchronization of mitosis observed in the endosperm tissue contributed to abortion of the embryos. Plants of *D. cymosa* cultivated at the University of Nottingham were self-compatible and fruit was set by self-pollination producing a globular blue berry, containing one or two seeds; most were abortive (Shaw, 1996a).

An alternative method of mass propagating *Diphylleia* is through rhizome cuttings (Shaw, 1999). This species has been shown to contain lignans (for details see Chapter 4) therefore it has been suggested as suitable for commercial cultivation (Broomhead and Dewick, 1990). The main advantage of such culture is the possibility of harvesting the leaves several times in a year and thus to provide an accessible source of the plant for pharmaceutical use. This would be more efficient than digging up the rhizome system from *Podophyllum* species. However, like other sub-alpine plants, more studies are needed for domestication and cultivation on a commercial scale. The appropriate cultivation of the plant should be encouraged, avoiding collection from the wild, because of its very localized geographical distribution.

Part II

1.2.2 The genus *Passiflora*

1.2.2.1 The family *Passifloraceae*

The *Passifloraceae* is a medium-sized family of trees, vines, shrubs and herbs, usually climbers with axillary tendrils, having attractive flowers, foliage and edible fruits (Perry, 1972). It is divided into 20 genera mainly *Passiflora*, *Adenia* and *Tetrapathaea* (Evans, 1996). In Brazil, the family is represented by only 2 genera *Dilkea* and *Passiflora* (Leitão Filho and Aranha, 1971), whilst 13 genera have been found in Africa (Jury, 1993b). The family is native to the tropics and subtropics (Jury, 1993b). The number of species is not clear, ranging from over 300 (Gilg and Schürhoff, 1967) to 580 - 600 (Leitão and Aranha, 1971; Jury, 1993b).

Engler and Diels placed the family in the order *Parietales* and into the suborder *Flacourtiineae* (Lawrence, 1963). However, subsequent evidence showed that *Parietales* was not a phylogenetic taxon and all its families were transferred to other orders. As a result, *Passifloraceae* was reclassified into the order *Violales* (Cronquist, 1988). However, in the classification adopted by Hutchinson (1973) *Passifloraceae* belonged to the order *Passiflorales*, together with the families *Malesherbiacease* and *Achariaceae*. *Passifloraceae* plants are characterized by leaves with small deciduous stipules.

In evolutionary terms, the genera *Smeathmannia*, *Soyauxia* and *Barteria* are the most primitive within the *Passifloraceae* (Hutchinson, 1973). The family is closely related to the *Cucurbitaceae*, *Loasaceae* and especially to the *Flacourtiaceae*, although *Passifloraceae* is considered the most advanced because of its perigynous calyx, corolla, corona and tendrils (Jury, 1993b). There are indications that climbing plants depend for their defence, against insects predators, on toxic compounds with low molecular weights (Frankel *et al.*, 1995). These compounds tend to be biodynamically active and thus potentially medicinal. These plants are therefore promising sources of substances of medicinal value. The roots of *Adenia lobata* were reported to be used for the treatment of nasal cancer in Gabon (Walker and Sillans, 1961) [cited in Hartwell, 1970] while species of the genus *Passiflora* have a number of applications in herbal medicine (see Sections 1.2.2.6, 1.2.2.7 and 1.2.2.8). The species *P. incarnata* is the only one mentioned with alleged hallucinogenic properties in the *Passifloraceae* family (Schultes and Hofmann, 1980). Amino acids and secondary metabolites such as flavonoids have been identified in this family but still require more chemical study (Evans, 1996). The identification of flavonoids in *Passiflora* species is given in more detail in Chapter 6.

1.2.2.2 Taxonomy of the genus *Passiflora*

1.2.2.2.1 Historical background

The botanic name *Passiflora* is derived from the Latin *Flos passionis* and the generic name passion flower refers to the religious symbolism which was ascribed to this plant (Grigson, 1974). It is thought that European travellers to the New World were attracted by the exotic and intricate flowers of the vines and not for the fruits. The morphology of the flower led to its association with the crucifixion of Christ (Simpson and Ogorzaly, 1995). It has been reported that the passion flower was first discovered by the Spanish Monardes in Peru (1569) and was used by the people living in the mountains (Mowrey, 1986). According to legend, in the Sixteenth Century the Spanish conquistadors and Jesuits in South America saw a semblance of Christ's passion represented in each part of the flower (Gledhill, 1989). For them the five anthers represented the five wounds of Christ on the cross, they saw in the triple style the three nails (one for the feet and the other two for each hand), the central receptacule seems the pillar of the cross, the filaments of the corona represented the crown of thorns on the head, and the calyx symbolized the glory surrounding the sacred head (Friend, 1884) [cited by Morley, 1970]. In another interpretation, the ten sepals and petals of the flowers were the apostles (less Judas and Thomas but for some Peter was omitted) (Perry, 1972).

1.2.2.2.2 Botany and classification

Members of the genus *Passiflora* are vigorous climbers (Bailey, 1973), and are described as perennials or rarely annuals (*e.g. P. gracilis*) (Vanderplank, 1996). The leaves are alternate, entire, lobed or parted, petioles usually being nectariferous gland-bearing. The flowers are bisexual, often large and showy, and are arranged singly or in small cymes, axillary or pedunculate. Flowers consist of five sepals and petals, the latter being rarely absent, with five stamens which are either free or basally united and fused in a tube. The ovary is superior, unilocular, and with many ovules on each placenta; three styles free or united with (3 - 5) stigmas. The fruit is a berry or capsule containing a fleshy endosperm involving numerous seeds (Bailey, 1973; Jury, 1993b; Turland, 1994). In Brazil, the flowers open up during the first hours of the afternoon in the months of the dry season and the whole process needs from 5 - 20 min (Cavalcante, 1976).

Formerly, some *Passiflora* species with elongated and cylindrical calyx tubes were classified as *Tacsonia* (Perry, 1972). Later these species were reclassified and are now grouped in the genus *Passiflora*. This genus is the largest within the family *Passifloraceae* (Salomão and Andrade, 1987), and belongs to the subfamily *Passifloraeae*. The genus *Passiflora* has been reported as the only one of importance

in the family (Jury, 1993b) this clearly being associated with its commercial and medicinal values.

1.2.2.3 Distribution of *Passiflora* species

There are estimates that the number of species of *Passiflora* ranges from 300 - 400 (Leitão and Aranha, 1971), (including *Tacsonia*; Fouqué, 1972; Bailey, 1973), or up to 500 (Jury, 1993b). Members of the genus *Passiflora* are mainly distributed in the tropics with 95% found in South America (Vanderplank, 1996); around 200 species are native to Brazil (Rocha and Silva, 1981). One species has been reported in Madagascar (Bailey, 1973) and a few species are found in Asia, Australia, Africa, USA and Hawaii (Salomão and Andrade, 1987).

1.2.2.4 Cytology

Karyotype studies have shown a diploid chromosome number, $2n = 2x = 18$ in many species of *Passiflora* such as *P. edulis* (Snow and MacDougal, 1993), although there are species, *P. cumbalensis* and *P. manicata*, for example, where the chromosome numbers are still unknown (Oliveira, 1987; d'Utra Vaz, 1992). There are some accounts referring to chromosome variations within the genus *Passiflora*. Of these, regenerants obtained from protoplast cultures of *P. edulis* fv. *flavicarpa*, *P. amethystina* and *P. cincinnata* showed polyploidy [$2n = 4x = 36$ (Dornelas and Vieira, 1993)]. In another study, chromosome analysis of somatic hybrid plants *P. edulis* fv. *flavicarpa* and five wild species, all diploids, have the expected $2n = 4x = 36$ chromosome number (Dornelas *et al.*, 1995).

1.2.2.5 Economic importance

There are 50 - 60 *Passiflora* species that produce edible fruits (Jury, 1993b) which are economically important for the production of soft drinks, juices, ice creams, sorbets and sweets (Oliveira, 1987). Only a small proportion of the fruits are used in desserts or eaten fresh (Vanderplank, 1996). In South America, the fruits are eaten raw or used to prepare a blended drink with sugar and water known as "refrescos". It is one of the tropical fruits worthy of attention in the Amazonian forest together with guava, açaí and others and thus is receiving attention due to its potential for the domestic and foreign markets (Gilbert, 1997). *P. edulis* Sims is the most important and widely cultivated species for the fruit juice industry (Kantharajah and Dodd, 1990). Such use relies on the commercial varieties *P. edulis* Sims f. *edulis* (purple passion fruit) and, *P. edulis* Sims fv. *flavicarpa* Degener (yellow passion fruit) and

their hybrids. Some of the species of *Passiflora* which have edible and aromatic fruits are shown in Table 1.5 but few of them have been commercially cultivated to date.

There has been a considerable increase in demand for passion fruit in international markets even though no formal data is available referring to global production. The major importers of fruit and juice are Belgium, Canada, France, Germany, Italy, Japan, Switzerland, The Netherlands, UK and the USA (Drew, 1997). In the American market around 30,000 tonnes per year are consumed as pure juice or blended juice made with other tropical fruits (Bruckner, 1994). In Australia, Indonesia, Kenya, New Zealand, South Africa, Sri Lanka, USA (Hawaii), Zaire and the West Indies the production is on a large scale (Suzuki and Lins, 1987). It has been reported that there is an annual growth in terms of cultivated area (hectares) in Colombia, Surinam,Venezuela, Cook Islands, Norfolk Islands, Solomon Islands and the Philippines (Vanderplank, 1996).

Brazil is considered the biggest worldwide producer of passion fruit with over 12,000 ha in cultivation (in 1985) and with production capacity varying from 8 - 10 tonnes per hectare (Ruggiero, 1987). Recent figures show that the Brazilian company Maisa have produced 3,310 tonnes of processed passion fruit annually giving 221 tonnes of frozen juice for the European market (News Maisa Letter, 1993).

Approximately 20 species of *Passiflora* are cultivated specifically for the ornamental market (Jury,1993b). Of these, *P. elegans*, *P. picturata* and *P. raddiana*, natives in Brazil, are the most popular species (Oliveira, 1987). Others, including *P. ligularis*, *P. manicata* and *P. quadrangularis*, have been introduced and cultivated as ornamental species and for their edible fruits in Madeira (Turland, 1994).

Being tropical/subtropical species, they are worthy of cultivation in the glasshouse and conservatory (Swithinbank, 1994). However, there is a limited literature describing their use as houseplants. There are fragrant species suitable for growing in pots, for example, *P. antioquiensis*, *P. coerulea* and *P. rubra*. The species *P. capsularis*, *P. foetida*, *P. mollissima* and *P. subpeltata* and others can be grown in a cold conservatory or glasshouse (Vanderplank, 1996).

1.2.2.6 Ethnomedicine of *Passiflora* species

The use of *Passiflora* dates back to the Mayan culture where *Passiflora* was employed to treat dysentery (Griggs, 1997). The first report on the sedative action of *Passiflora* leaves has been attributed to Phares (1870) [cited by Leclerc, 1927]. It has been used as a tranquilliser in many European countries, the United States and Canada for over 200 years. For example, in Germany in 1979, there were 42 sedatives and 6 cardiotonics; commercially available products containing passion flower derivatives (Mowrey, 1986).

Table 1.5: Species of *Passiflora* that produce edible fruits

Species	Distribution	Reference
P. alata	Peru to Brazil	Killip, 1938; Martin and Nakasone, 1970
P. antioquiensis	Colombia	Martin and Nakasone, 1970
P. bahiensis	Brazil	Salomão and Andrade, 1987
P. coerulea	Brazil, Argentina	Salomão and Andrade, 1987; Turland, 1994
P. coccinea	South America (Northeast)	Martin and Nakasone, 1970
P. edulis[1]	Brazil to Argentina	Martin and Nakasone, 1970
P. edulis f. *flavicarpa*[1]	Hawaii	Jury, 1993b
P. foetida	Tropical America, Galapagos Islands	Perry, 1972; Salomão and Andrade, 1987
P. incarnata	USA	Bailey, 1973
P. laurifolia[2]	Brazil, South America (North)	Killip, 1938; Martin and Nakasone, 1970
P. ligularis[1]	Mexico to South America (North), Bolivia	Martin and Nakasone, 1970; Salomão and Andrade, 1987
P. macrocarpa	Brazil	Salomão and Andrade, 1987
P. manicata	Venezuela to Peru	Martin and Nakasone, 1970; Leitão and Aranha, 1971
P. mollissima[1]	Andes, South America	Martin and Nakasone, 1970
P. mixta	Ecuador	Martin and Nakasone, 1970
P. nigradenia	Bolivia (North)	Martin and Nakasone, 1970
P. ovata	French Guyana	Killip, 1938
P. perfoliata	Jamaica	Killip, 1938
P. pinnatistipula	Colombia to Chile	Martin and Nakasone, 1970
P. platyloba	Guatemala to Costa Rica	Martin and Nakasone, 1970
P. popenovii	Brazil, Peru	Martin and Nakasone, 1970
P. quadrangularis[1]	Brazil	Killip, 1938
P. racemosa	Brazil	Bailey, 1973
P. riparia	Brazil, Peru	Martin and Nakasone, 1970
P. seemannii	Panama to Colombia	Martin and Nakasone, 1970
P. serrato-digitata	Bolivia	Martin and Nakasone, 1970
P. suberosa	Brazil	Salomão and Andrade, 1987
P. vitifolia	Nicaragua to South America (North)	Martin and Nakasone, 1970

[1] Indicates the most important cultivated species (Martin and Nakasone, 1970) [cited by Salomão and Andrade, 1987]; [2] An additional cultivated species according to Vanderplank (1996).

In the UK, the aerial parts of *Passiflora* were the most popular ingredient incorporated in several pharmaceutical preparations such as extracts, tablets and tinctures marketed as herbal sedatives (Tyler, 1994).

Species of the genus *Passiflora* are commonly known in Brazil as "maracujá". This is derived from the word "mara-cuiá" meaning "food prepared in gourd" (Hoehne, 1946), or from the Tupi native language and meaning "fruit drunk by sucking" (Rizzini, 1978). *P. alata* Ailton (syn. *P. alata* Dryand) is the only species officially registered as a medicinal species in the Brazilian Pharmacopoeia (see Pharmacopeia dos Estados Unidos do Brasil, 1929). This species is one of 19 used in traditional healing among the Kayapó, one of the major Jê-speaking groups in Brazil (Elisabetsky and Posey, 1994). The Kayapó healers have been using the whole plant to treat "tep kanê", a gastrointestinal disorder that is associated with diarrhoea, yellow body and general pain.

P. coccinea is used as a contraceptive by the Yawalapiti Indians, a tribe of natives that live at the Xingu reserve in the Amazonia forest (Valle, 1978) [cited in Rizzini, 1978]. It is called (by them) "ma-uan-tapa" that literally means "root that leaves a woman without child". The Indian women daily drink a cold tea made from these roots to avoid pregnancy. The same species has also been used in Guyana for the treatment of conjunctivitis (Grenand *et al.*, 1987).

P. tetrandra, known as "kohia" or passion fruit in New Zealand, is used medicinally by the Maori aborigines. The oil from the seeds was employed in the treatment of chronic sores, wounds, cracked nipples and as skin ointments while a mixture of the oil with a decoction from roots was used for flatulence (Perry *et al.*, 1991). Other *Passiflora* species are claimed to be astringents, depuratives, vermifuges and are used in the treatment of arthritis, gout, haemorrhoids, skin inflammations and for stress reduction (Caribé and Campos, 1991; Griggs, 1997). Some of the medicinal uses of species of *Passiflora* are summarised in Table 1.6. The species *P. edulis* and *P. incarnata* are the most popular as used in herbal medicine and thus are treated separately in the following sections.

There are indications of poisonous metabolites such as prussic acid in the leaves, pericarp and immature seeds of *P. quadrangularis* (Blohm, 1962). It has been reported that ingestion of immature fruits of some species, for example *P. adenopoda*, can be fatal (Spencer and Seigler, 1983). The "maracujá" has also been associated with death in Brazilian folklore, particularly in the Northeast of Brazil, where it is known as plant which brings bad luck (Anonymous, 1978) [cited in Rizzini, 1978).

Table 1.6: General uses of *Passiflora* species

Species	Indications for use	Reference
P. alata	Anthelmintic and diuretic, for treatment of anxiety, asthma, fever, hyterism, insomnia and migraine, discomforts during menopause, and as a hypnotic	Coimbra, 1958; Simões *et al.*, 1986; Sanguinetti, 1989
P. capsularis	Abortive and emmenagogue	Corrêa, 1978
P. coerulea	Sedative	Speroni *et al.*, 1996
P. cumbalensis and *P. killipiana*	For fever	Schultes and Rauffauf, 1990
P. laurifolia	Vermifuge	Grenand *et al.*, 1987
P. ligularis	Used for diarrhoea, dysentery, stomach pains and indigestion	Caceres *et al.*, 1990
P. macrocarpa	Aphrodisiac, eases childbirth, for chronic alcoholism, nervousness and as a sedative	Sangirandi, 1981
P. phaeocaula	Treatment of conjunctivitis	Schultes and Rauffauf, 1990
P. quadrangularis	Anthelmintic, narcotic and used as an emollient poultice	Killip, 1938; Blohm, 1962
P. serratodigitata	For eye inflammation	Schultes and Rauffauf, 1990
Unspecified	Analgesic and sedative useful during menses, coronary disease, for children with problems of concentration in school, weak circulation, sleep disorders	Mowrey, 1986

1.2.2.7 *Passiflora edulis*

1.2.2.7.1 Medicinal uses and vernacular names

The traditional use of *P. edulis* for its sedative properties is well known in South America particularly in Brazilian popular medicine. Such traditional use is based on leaves even though roots have also been used (Table 1.7). The fruits are also used against stomach cancer in Madeira (Hartwell, 1970).

Table 1.7: Traditional uses of "maracujá" (*P. edulis*) in Brazilian medicine

Plant part	Preparation	Use	Dose	Indications for use	Reference
Leaves	Bruised	Topical	Not specified	Haemorrhoids (tumours)	Almeida, 1993
	Decoction	Oral	Not specified	Diuretic	Almeida, 1993
	Infusion[1]	Oral	1 cup, at night	Induces sleep	Matos, 1991
		Oral	2 - 3 cups, daily	Tranquilliser	Matos, 1991
	Tea[2]	Oral	1 - 2 cups, at night	Anxiety, insomnia, short temper and nervousness	Matos, 1989; Martins *et al.*, 1994
	Not specified	Oral	Not specified	Treatment of colds, hysteria and insomnia, perturbations during the menopause, as a hypotensor	Almeida, 1993
Roots	Not specified	Oral	Low[3]	Anthelmintic	Almeida, 1993
			High[4]	Emetic, provokes spasms	Almeida, 1993

[1] The infusion is prepared with 3.0 - 5.0 g/dried leaves or 6.0 - 10.0 g/fresh leaves in 150 ml of boiling water; it should not be taken for longer than one week.
[2] The tea is prepared using 4.0 - 6.0 g/leaves (dried or fresh); [3, 4] Terms used according to Almeida (1993) without specifying the dosage.

1.2.2.8 *Passiflora incarnata*

1.2.2.8.1 Medicinal uses and vernacular names

Since the beginning of the century, *P. incarnata* has been recommended for insomnia and nervous manifestations during the menopause (Leclerc, 1920). This species is native to the USA and has long been used by the American indians as a tonic (Ody, 1996). Its aerial parts (bearing flowers, fruits and stems) have been traditionally used in popular medicine (in the USA) for anxiety and neuralgia (Brasseur and Angenot, 1984).

The common names of this species include apricot vine, grenadille, maypop, passion flower, passion vine and wild passion flower (Leung, 1980; Newall *et al.*, 1996).

P. incarnata was introduced into Western herbal medicine in the Nineteenth Century, formerly to treat epilepsy and latterly for the cure of insomnia (Ody, 1996). It is cultivated in Europe and has been extensively used in homoeopathy and phytotherapy (Rehwald *et al.*, 1995; Newall *et al.*, 1996). Since *P. incarnata* is a mild sedative, it may be used in combination with other sedative species such as *Valeriana officinalis*, *Humulus lupulus* and *Piscidia piscipula* for insomnia (Hoffmann, 1999). The traditional medicinal uses for *P. incarnata* are summarised in Table 1.8. In addition to these uses it has been ascribed helpful for tension headaches, for irritable bowel syndrome and as a pain killer (Ody, 1996).

It has been reported in the pharmaceuticals trade that stems and leaves of *P. edulis* and *P. coerulea* are often used to falsify *P. incarnata* (Bruneton, 1995). Authentication of *P. incarnata* is now based on methods such as HPLC, TLC and microscopy (Bradley, 1992; Bisset, 1994).

1.3 Plant biochemistry

1.3.1 Classification of plant-derived compounds

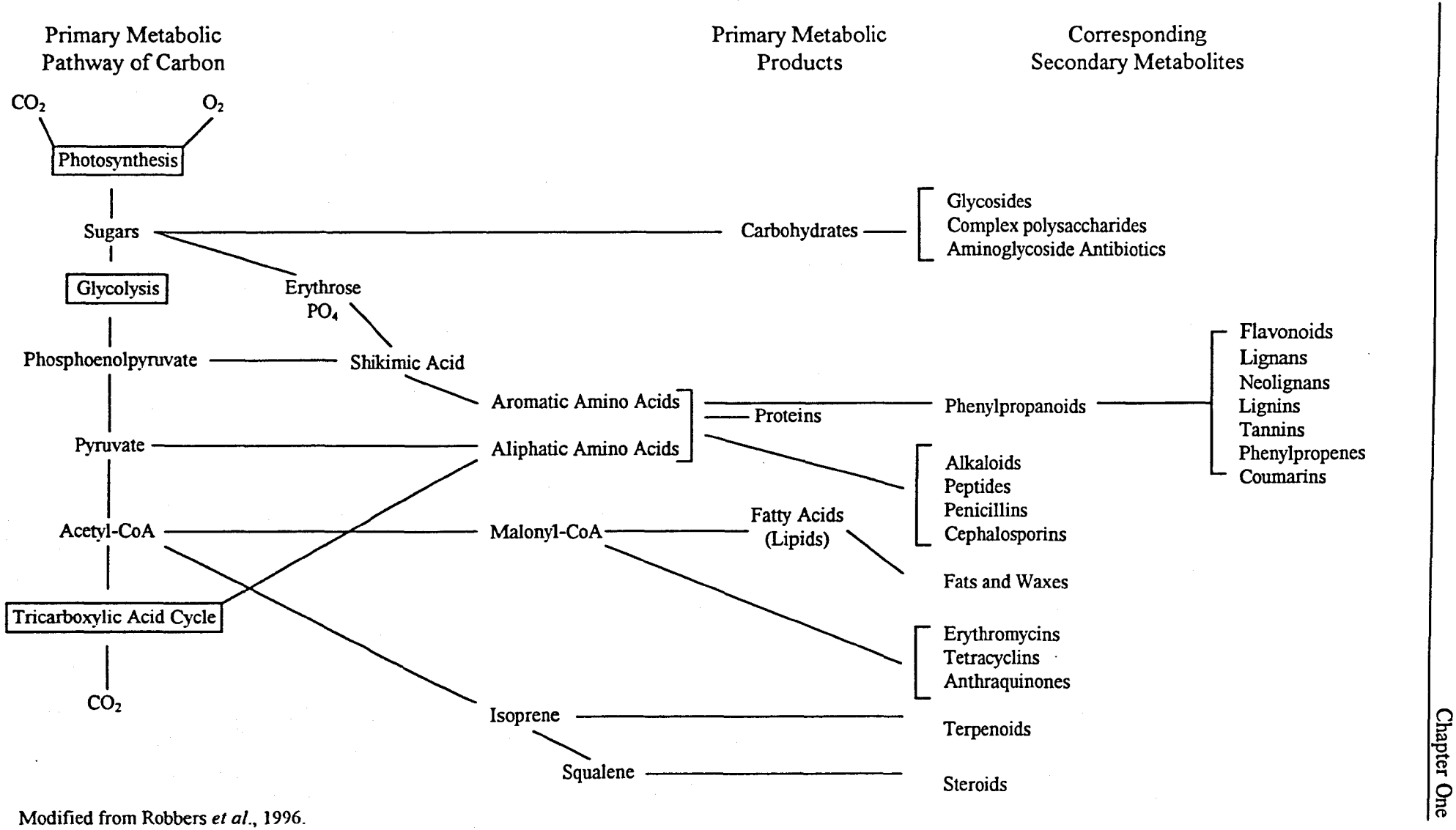
Historically, plant-derived compounds have been classified as primary or secondary metabolites (natural products). Primary metabolites are substances widely distributed in nature and occurring in higher plants in vegetative organs and seeds. These substances are necessary for growth and physiological development (Balandrin *et al.*, 1988). Secondary metabolites are biosynthetically derived from primary metabolites (Fig. 1.1), and share the same common precursors. They are often restricted to a particular taxonomic group (species, genus, related groups of families), and are not synthesized by single genes but are the result of multi-step and multi-enzyme pathways (Curtin, 1983).

Table 1.8: Traditional uses of *P. incarnata* preparations

Plant part	Preparation	Dose	Indications for use	Reference
Aerial[1]	Dried	0.25 - 1.0 g	Hysteria, insomnia, neuralgia, seizure, spasmodic asthma, nervous tachycardia	British Herbal Pharmacopoeia, 1983; Newall *et al.*, 1996
	Liquid extract[2]	0.5 - 1.0 ml, 3 times daily	Anxiety, hysteria, especially for insomnia, nervous tachycardia, spasmolytic (colitis, gastritis)	Paris and Moyse, 1967; British Herbal Pharmacopoeia, 1983; Newall *et al.*, 1996
	Tincture[3]	0.5 - 2.0 ml, 3 times daily	Anxiety, hysteria, especially for insomnia, nervous tachycardia, spasmolytic (colitis, gastritis)	Paris and Moyse, 1967; British Herbal Pharmacopoeia, 1983; Newall *et al.*, 1996
	Infusion[4] (tea)	2 - 3 cups, daily 1 - 2 cups, at night	Anxiety, gastrointestinal disorders of nervous origin, mild sleeping difficulties, nervous restlessness, sedative in neurasthenia	Tyler, 1988; Jaspersen-Schib, 1990; Bisset, 1994; Newall *et al.*, 1996
	Bath mixture	Not specified	For calming and soothing	Leung, 1980
Leaves	Tablet	1.0 - 4.0 g, 3 times daily	Helps restlessness, hypnotic to help with sleeping (anxiety, depression, light sleepers), to promote sleep (in case of insomnia), nervous irritability	Robbins, 1995

[1] Stems, flowering and fruiting tops; [2] Liquid extract [1:1 in 25% (v:v) alcohol]; [3] Tincture [1:8 in 45% (v:v) alcohol]; [4] The infusion is made pouring boiling water (*ca.* 150 ml) over *ca.* 2.0 g of the dried aerial parts (finely chopped) for 5 - 10 min.

Fig. 1.1: Interrelationships of biosynthetic pathways leading to secondary metabolites in plants



Modified from Robbers *et al.*, 1996.

Even though they are also found in animals (Luckner, 1990), most biologically active natural products have been isolated from higher plants (Howe and Wesley, 1988). Secondary metabolites are no longer regarded as waste products of metabolism (Wink, 1988) with no direct metabolic function. Instead, the biological activities of secondary metabolites, particularly their toxic effects against animals and microbial pathogens (Harborne, 1990) may indeed aid the survival of a species. Most pharmacologically active constituents of such species are products of secondary metabolism.

Secondary metabolites have no functional restriction and their importance in the interactions of plants with an environment became more clear with the advances of ecological biochemistry (Hartmann, 1995). Even though they have no apparent primary function, some such as tannins, alkaloids and terpenoids have implications ecologically. In addition to defence functions, they may serve as attractants, such as the volatile terpenes (essential oils) and pigments (flavonoids and carotenoids) (see also Section 1.3.1.2.1).

The expression of many secondary pathways is altered by genetic composition, ontogeny (stage of development) and changing environments (Robbers *et al.*, 1996), which represent biochemical adaptations of a species to different types of environmental stress such as climate, nutrient deficiency, associated flora and other factors. These biochemical adaptations may affect both primary and secondary metabolism and increase the concentration of secondary compounds in the stressed plant. For example, the increase in the concentration of the sesquiterpenoid, abscisic acid, in drought-stressed plants produces stomatal closure reducing moisture loss through transpiration (Harborne, 1993). Many secondary metabolites are allelochemicals serving as defensive chemical agents against herbivores (arthropods, vertebrates, molluscs), microorganisms (bacteria, fungi, viruses) and competing plants (allelopathy), for example, quinolizidine alkaloids in the *Leguminosae* (Wink, 1997) or insect juvenile hormones and sex pheromones (Harborne, 1997). Phytoalexins, such as the sesquiterpenoid rishitin found in the *Solanaceae*, are inducible antimicrobial compounds synthesized, *de novo*, in response to pathogenic attack (Bailey and Mansfield, 1982) [cited by Walton, 1997]. Secondary metabolites can also function as plant growth regulators, such as the gibberellins (Bramley, 1997) whilst others serve as anti-feedants, for example, lignans (see Section 1.3.1.3), sesquiterpene lactones and phenolics in chicory (Rees and Harborne, 1985).

Secondary metabolites are expensive, in terms of cellular economy, for the plant to produce requiring a balanced flow of precursors, enzymes and energy cofactors from primary metabolism. Their maintenance in high concentrations is costly for the plant because it needs synthesis from simple precursors, storage and transport.

They are accumulated in smaller quantities compared to the primary metabolites.

1.3.1.1 Secondary metabolites in *Podophyllum*, *Diphylleia* and *Passiflora*

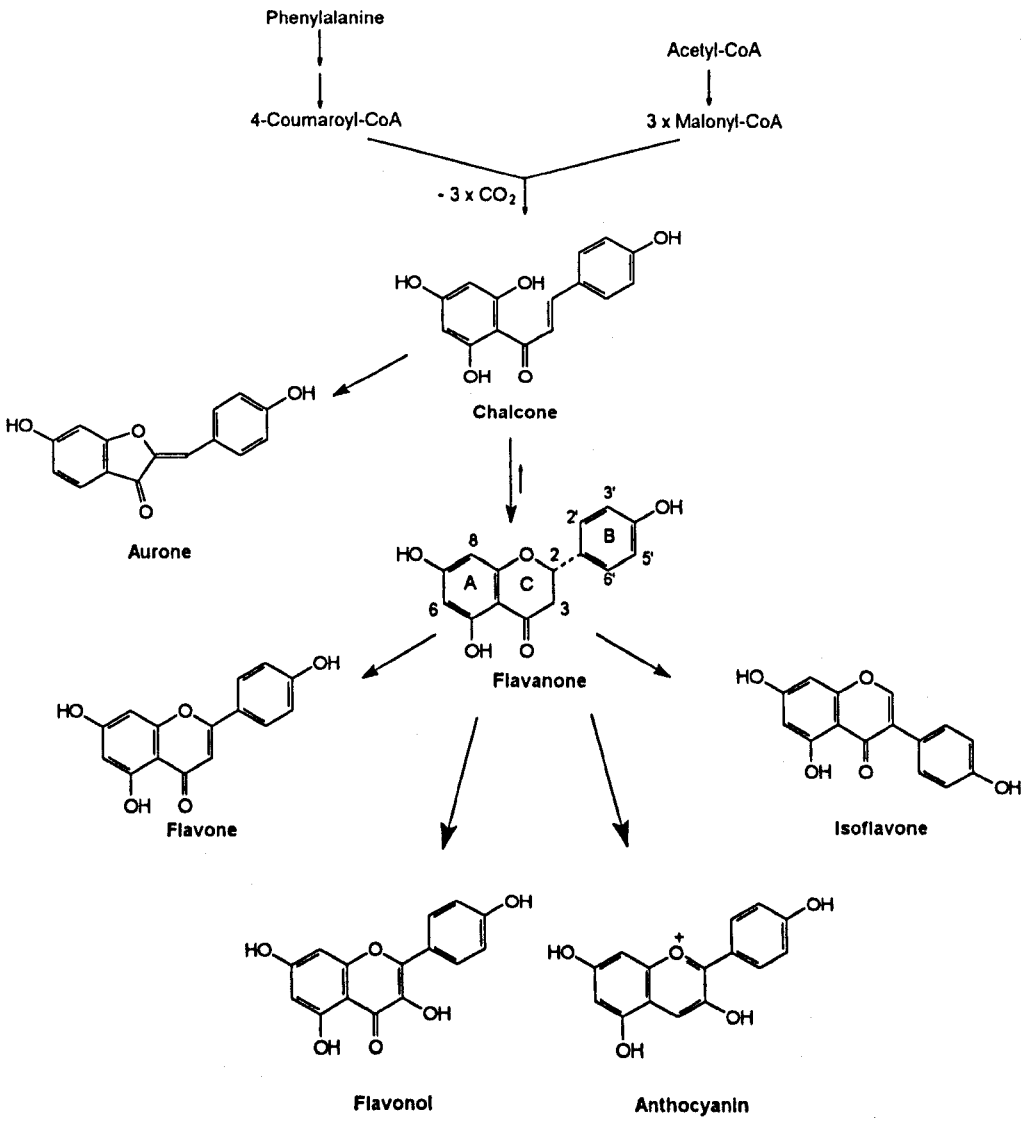
As part of phytochemical investigations of *Podophyllum* and *Diphylleia* species a number of secondary metabolites have been reported. These include the flavonoid quercetin in resin of *Podophyllum* (Farnsworth *et al.*, 1974), podoverines in *P. versipelle* cell cultures (Arens *et al.*, 1986), and kaempferol in the rhizomes of *D. sinensis* together with lignans (Ma *et al.*, 1993), water soluble polysaccharides and hemicelluloses in *P. peltatum* and *D. cymosa* (Iriki *et al.*, 1984), anthraquinones (Yin *et al.*, 1989), nitrogenated compounds and alkaloids in *Podophyllum* species (Junior, 1986). Of all the natural products found in these two genera, the lignans are of increasing significance because of their biological activity (Section 1.3.1.2.3).

Many *Passiflora* species of contain flavonoids, coumarins (Freitas, 1987; Newall *et al.*, 1996) including maltol, ethylmaltol and traces of a volatile oil (of unknown composition) in *P. incarnata* (Aoyagi *et al.*, 1974; Bradley, 1992), cyanogenic glycosides (Fischer *et al.*, 1982; Spencer and Seigler, 1983; 1984; 1985; 1987; Chassagne *et al.*, 1996), terpenoids, for example, carotenoids in the juice of *P. edulis* (Ferreira *et al.*, 1989; Winterhalter, 1990; Mercadante *et al.*, 1998), flavour constituents in the fruits of *P. edulis* fv. *flavicarpa* (Werkhoff *et al.*, 1998), tannins, resins, gums and alkaloids (Costa, 1978; Tsuchiya *et al.*, 1999). However the flavonoids play an important role in the medicinal exploitation of most *Passiflora* species (see Section 1.3.1.2.2).

1.3.1.2 Flavonoids

The term flavonoid (Lat. *flavus* = yellow) was first used to describe yellow-coloured compounds with a flavone moiety (Jovanovic *et al.*, 1998). Amongst plant phenolics, the flavonoids are the largest group and over 5000 different compounds have been structurally characterized (Strack, 1997). They are of low molecular weight, and occur either in a free state known as aglycones, when they are not in combination with a sugar, or as glycosides, in which they are bound to a sugar residue(s). Chemically, the flavonoid aglycone is formed by benzopyran-4-one (rings A and C) and a phenyl ring B as a substituent in the 2-position. They are classified according to the oxidation level of ring C which results in the major structural classes: flavones, flavanones, flavonols, anthocyanins, chalcones and isoflavones. All these classes have closely related biosynthetic pathways and chalcone acts as precursor for many of the flavonoid derivatives (Fig. 1.2).

Fig. 1.2: Scheme illustrating the position of chalcone as precursor in the biosynthesis of all classes of flavonoid



Modified from Ebel and Hahlbrook, 1982.

1.3.1.2.1 Occurrence and role of flavonoids

Flavonoids are present, characteristically, in all vascular plants (Middleton and Kandaswami, 1994) and are almost absent in algae and fungi. However, they are exceptionally produced by the green alga *Nitella* and in the fungus *Aspergillus candidans* (Markham, 1989). They are common in Bryophytes and liverworts, while in Pteridophytes they are mainly represented by bioflavonoids, proanthocyanins and chalcones. The greatest structural variety of flavonoids are recorded for the Angiosperms. Even though proanthocyanins are ubiquitous in Gymnosperms, the distribution of other groups, such as flavones, flavonol glycosides and bioflavonoids is dependent on tissue differentiation.

Flavonoids, which are water-soluble, may be present as glycosides or as free aglycones (Wollenweber, 1994). They are more widely distributed than most other secondary metabolites, occurring in all plant parts. In floral tissues, flavones, flavonols and anthocyanins are localized in the cell vacuole and function as as co-pigments, which are involved in the determination of flower colour (Harborne and Williams, 1995). They are also present in fruits and leaves, and assist with pollination and dispersal of fruits and seeds. Anthocyanins provide the most widespread natural food colours as a red range. Flavonoids have survived in vascular plants during evolution (Swain, 1975) because they are important in plant physiology and biochemistry. They may have a (speculative) physiological role, for example, light perception which is associated with their temporal accumulation during plant ontogeny in vulnerable tissues, such as young leaves or seedlings (Strack, 1997). It has been assumed that the occurrence of flavonoids in the leaf cuticle and epidermal cells serves for protection of such tissues from potential damage by ultraviolet radiation and helps in the adaptation of species to alpine, arid or semi-arid conditions.

There is an increasing interest in the important roles flavonoids play in plant survival ranging from antimicrobial compounds, such as pisatin, an isoflavonoid phytoalexin, produced by pods of *Pisum sativum* (pea) synthesized in response to pathogenic attack (Bailey and Mansfield, 1982) [cited by Walton, 1997] through to their function as growth regulators. Other flavonoids are stress metabolites. Amongst the isoflavonoids the rotenoids are insecticides, while isoflavones such as coumestrol from *Trifolium* species (clovers) show oestrogenic activity (Williams and Harborne, 1989). They can also act as signal molecules, for example, flavones, isoflavones and chalcones, and are involved in the process of nitrogen fixation in plants (Bohm, 1994; Lea, 1997). Such biological activity shows the inter relationships between flavonoids and the activation and expression of genes. In higher plants they have an ubiquitous nature and there is evidence that they are subject to a rapid turnover and degradation (Markam, 1989).

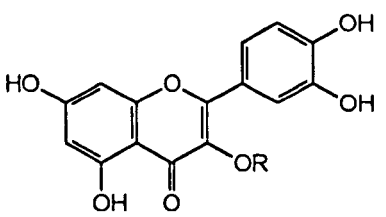
The use of flavonoids as potential taxonomic markers in plant classification and phylogeny is associated with their widespread distribution, stability and ease of identification (Bohm, 1998). The flavonoid patterns in the *Leguminosae* and *Compositae* may serve as chemotaxonomic markers for individual species (Harborne, 1989). Recently, chemotaxonomic studies have routinely included tests to identify exudate flavonoids in *Pelargonium* species where these compounds were reported for the first time (Williams *et al.*, 1997). Leaf flavonoids isolated from two *Erythroxylum* species have been suggested as chemotaxonomic markers for these taxa which are commercial sources of cocaine in their leaves (Johnson *et al.*, 1997).

1.3.1.2.2 Medicinal properties

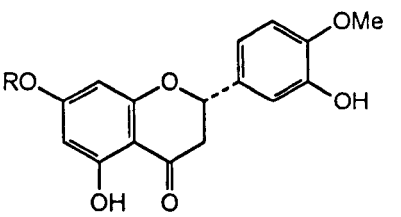
There is considerable evidence of biological activity emerging from the many *in vitro* and *in vivo* experiments and clinical reports on plant flavonoids. Their medicinal properties have been fully reviewed by Middleton and Kandaswami (1994). Much research was carried out on the physiological effects of flavonoid preparations on capillaries. The flavonol glycoside rutin (from *Ruta graveolens*) and the flavanone glycoside hesperidin (from *Citrus* peels) are included in human dietary supplements as vitamin P or permeability factor (Fig. 1.3). It has been suggested that they are useful in the treatment of ailments characterized by capillary bleeding and increased capillary fragility but more studies are required to be done to confirm their therapeutic efficacy (Bruneton, 1995). The pharmaceutical industry is interested in rutin for the treatment of capillary fragility in humans (Harborne and Baxter, 1993). Reports on the association of flavonoids with a decreased capillary fragility led to preparations containing citrus (and other fruits) together with ascorbic acid for treatment of hypertension and radiation injuries (Evans, 1996).

Flavonoids can act as protectors against cardiovascular disease, can decrease blood cholesterol, can be diuretic, hepatoprotective and can have effects on ageing (Bruneton, 1995). A flavonoid isolated from leaves of *Artemisia annua* showed significant cytotoxic activity *in vitro* against P-388, A-549, MCF-7, HT-29 and KB tumour cells (Zheng, 1994). The effect of selected flavonoids on cancer include anticarcinogenic and prodifferentiative activities. In the western diet, approximately 100 to 1000 mg of flavonoids are consumed daily. They may be health-promoting and disease-preventing compounds, and thus diets rich in flavonoids have a considerable importance (Middleton, 1996). The activities of tumour promoters in the process of carcinogenesis can be inhibited by flavonoids. For example, the isoflavone genistein found in products of soya bean consumed by Asians has been shown to protect against those types of cancers which are dependent on the hormone oestrogen such as breast, colon and prostate cancers (Messina *et al.*, 1994).

Fig. 1.3: Flavonoids known as vitamin P



Rutin; R = rhamno-glucosyl
(Flavonol)



Hesperidin; R = rhamno-glucosyl
(Flavanone)

It has been demonstrated that genistein is a potent inhibitor of expression of the mammalian stress response genes. This effect could be an important factor in the anticancer activity of genistein (Zhou and Lee, 1998). Furthermore, citrus flavonoids such as nobiletin and tangeretin have been shown, *in vitro*, to inhibit human breast cancer cells (Carrol *et al.*, 1998).

The anti-inflammatory activity of flavonoids is thought to be due to their participation in a variety of biochemical systems involved in inflammation, such as inhibition of arachidonic acid metabolism (Alcaraz and Ferrandiz, 1987). Inhibition of cAMP phosphodiesterase, which affects platelet aggregation/release, is achieved by selected flavonoids (Middleton, 1996). The flavone artemetin (from *Cordia verbenaceae* leaves) has been reported as an inhibitor, with low toxicity, using various animal models for the study of inflammation (Sertié *et al*, 1990). The anti-inflammatory effects in mice and rats of pectolinarin and linarin, flavonoids from *Cirsum subcoriaceum* and *Buddleia cordata* respectively, have been partially associated with the inhibition of the metabolic pathway of arachidonic acid (Martínez-Vázquez *et al.*, 1998). The advantage of flavonoids over the known anti-inflammatory agents may be attributed to their (1) high margin of safety, (2) ability to be totally metabolized by animal cells (thus they are not accumulated in the body) and (3) ulcer-healing activity (Duwiejua and Zeitlin, 1993). In comparison, synthetic non-steroidal anti-inflammatory drugs are frequently associated with ulceration, particularly of the gastric mucosa.

Flavonoids have also been progressively attracting attention because of their antiallergic and antiviral activities. These properties have been associated with their potent activity, *in vitro*, as inhibitors of mammalian enzyme systems such as PKC, lipoxygenase, and others (Middleton and Kandaswami, 1994). It has been recognized that inhibition of PKC offers a promising approach to the treatment of dermatoses (Tegeler *et al.*, 1995). Some flavones have been identified as better PKC inhibitors than the corresponding flavanones (Chang *et al.*, 1995). With respect to antiviral activity, they can inhibit viruses such the Polio Virus Type 1 and the parainfluenza

virus (Middleton, 1996). Flavones are of special interest because they do not induce virus resistance (Vlietinck and Vanden Berghe, 1991). In the chemotherapy of respiratory viruses, the flavone Ro-09-0179 (from *Agastache rugosa*), an antiviral agent, has been demonstrated to inhibit RNA polymerase (Challand and Young, 1997). Plant-derived flavonoids such as flavones and flavans have been reported as inhibitors of two phases in the replication cycle of the HIV virus, mainly in its adsorption and reverse transcription (Vlietinck *et al.*, 1998). Flavones and flavonols found in propolis (bee-glue) have shown antiviral activity in a test system using Type 1 Herpes Simplex Virus (Amoros *et al.*, 1992). Pinocembrin has been confirmed as the major compound responsible for the antibacterial action of propolis (Houghton *et al.*, 1995).

One of the key points about antioxidant activity of flavonoids concerns their affinity for divalent ions of heavy metals, which are involved in the production of free radicals (Younes and Siegers, 1981). Free radicals have been reported to be responsible for several disorders such as hepatic toxicity and atherosclerosis (Middleton and Kandaswami, 1994). Flavonoids, as phenolic compounds, are radical scavengers and inhibitors of lipid peroxidation and, thus function as antioxidants (Kéry *et al.*, 1996). The species *Crataegus monogyna* (hawthorn) is a promising antioxidant species and mainly contains flavones and flavonols (Bahorun *et al.*, 1994).

The pharmaceutical importance of the family *Passifloraceae* is attributed to the occurrence of flavonoids known as *C*-glycosylflavonoids (Meier, 1995a). Therefore, the presence of *C*-glycosylflavonoids was investigated by chromatography as part of this study in the medicinal species *P. edulis*, *P. incarnata* and their novel somatic hybrids (Sections 1.4.2 and 1.4.3.3.). The biological and medicinal properties of *C*-glycosylflavonoids are discussed in detail in Chapter 6.

1.3.1.3 Lignans

The widespread use of lignans in traditional medicine suggests that they are potential compounds for the development of new classes of pharmacological agents (Ayres and Loike, 1990). Therefore, investigations correlating new sources, isolation techniques, yields and chemical identification of lignans are likely to increase in number reflected, in part, by the studies presented in this thesis. Advances in analysis and identification, biological activity and biosynthesis of lignans and related compounds have recently been reviewed (Ward, 1997).

The term lignan was introduced by Haworth (in 1942) for a group of natural plant (phenolic) compounds with a carbon skeleton derived from the dimerization of two phenylpropane units coupled at the central carbon of their side-chains (Dewick, 1989). An additional term neolignan was subsequently introduced by Gottlieb (1972)

and refers to molecules containing other types of coupling. The following sections refer mainly to lignans, which is one of the classes of natural products focused on in this thesis.

1.3.1.3.1 Classification, numbering and nomenclature

No definitive classification system has yet been developed for the lignans, and this is, in part, due to the enormous diversity of structural types found within this group of compounds. According to a simplified system of classification, the lignans can be subdivided into four basic groups: "lignans" for derivatives of butane; "lignanolides" with lactone substitution for those derivatives of butanolide; "monoepoxylignans" for derivatives of tetrahydrofuran and "bisepoxylignans" for derivatives of 3,7-dioxabicyclo-(3,3,0)-octane (Freudenberg and Weinges, 1961; Weinges *et al.*, 1978). However, in the more recent literature (Bruneton, 1995) lignans have been classified into six structural subgroups:

1) Dibenzylbutanes for those with C_6C_3 units linked with an 8-8' bond (Fig. 1.4.1).

2) Monofuranoids based on cyclization resulting in three types of lignans: cyclization at 9-*O*-9', at 7-*O*-9' and 7-*O*-7' (Fig. 1.4.2a, 2b, and 2c respectively).

3) Dibenzylbutyrolactones (Fig. 1.4.3).

4) Arylnaphthalenes where the cyclization involves one aromatic carbon atom (Fig. 1.4. 4a and 4b).

5) Dibenzocyclooctanes (Fig. 1.4.5).

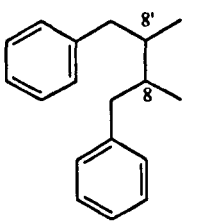
6) Furanofuranoid lignans formed by double cyclization between 7-*O*-9' and 7'-*O*-9 (Fig. 1.4.6).

Examples of the structural types mentioned above are shown in Fig. 1.4.

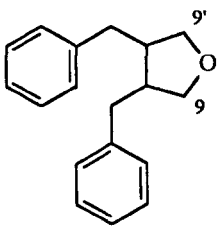
1.3.1.3.2 Distribution of lignans

Chemotaxonomically, lignans are widely distributed in vascular species (Cole and Wiedhopf, 1978) and their distribution, occurrence and evolution has been reviewed (Lewis *et al.*, 1994, 1995). They can occur in both the free state (in woody tissue) and as glycosides (in root, leaf and flower), and have been identified in some 70 plant families (Massanet *et al.*, 1989). They have been found in all tissues of angiosperms but have been isolated predominantly from dicotyledons. In gymnosperms, lignans are located mainly in woody tissues, predominantly in the *Pinaceae* and *Cupressaceae*; and are found in the tracheids where they are associated with cellular inclusions (MacRae and Towers, 1984). Even though lignans are commonly associated with plants, they are also found in man and other primates. Mammalian lignans have been identified in urine, bile, seminal fluid and serum of humans, baboons, monkeys and rats (Setchell *et al.*, 1980; Ayres and Loike, 1990).

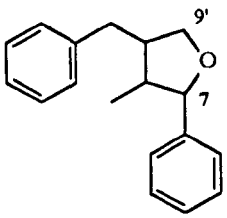
Fig. 1.4: Examples of some natural lignans



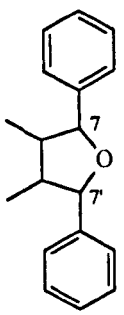
1



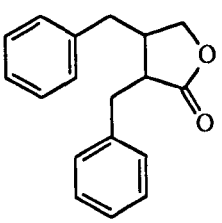
2a



2b



2c



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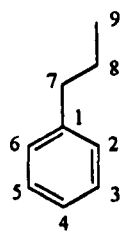

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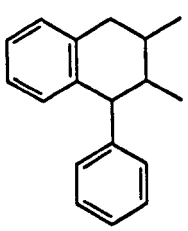
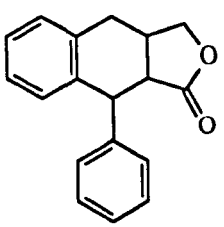
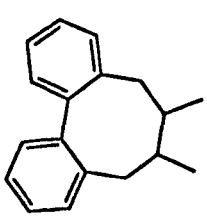


4a



4b



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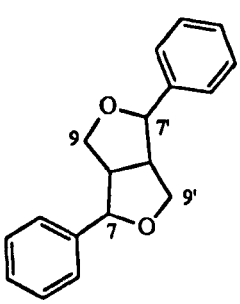


6

Relatively little is known about the biosynthesis of lignans compared to flavonoids or alkaloids (Dewick, 1989). Lignans are optically active compounds and their biosynthesis is under the control of stereospecific enzymes (Ayres and Loike, 1990). The biosynthesis of *Podophyllum*-based lignans is discussed in Chapter 4.

1.3.1.3.3 Medicinal properties

Lignans are of considerable pharmacological interest, and show a diverse spectrum of biological activities that could suggest that they could have more than one mechanism of action. The earliest recorded medicinal use of plant-based lignans dates back over 1000 years (Kelly and Hartwell, 1954). Plants, rich in lignans, have long been used in China and Japan as diuretics, analgesics, and for the treatment of diseases such as gastric and duodenal ulcers (Ayres and Loike, 1990; Pharmacopeia of P. R. China, 1977). A summary of some of the biological activities of the lignans is given in Table 1.9.

There is evidence that a diet, containing lignans, can decrease the risk of contracting certain forms of cancer (Lampe *et al.*, 1994; Mäkelä *et al.*, 1995). In this context the mammalian lignans, enterolactone and enterodiol, which are plant oestrogens, derived from cereals and vegetables, are converted by the action of intestinal microflora into substances that can act as cancer chemopreventative agents (Gang *et al.*, 1997). Their effects are similar to those produced by isoflavonoids as mentioned previously in Section 1.3.1.2.2. The addition of flaxseed, a source of plant precursors to mammalian lignans, to processed food such as bread and cereals increases the production of mammalian lignans (Nesbitt and Thompson, 1997).

Analogues of the lignan, justicidin E, have been shown to be non-redox inhibitors of 5-lipoxygenase (5-LO) activity, and inhibitors of leukotriene biosynthesis (Ducharme *et al.*, 1994). Leukotrienes have been associated with the pathology of asthma, psoriasis, and other diseases (Ford-Hutchinson, 1985). Thus, certain lignan inhibitors of 5-LO are useful as therapeutic agents for the treatment of asthma (Israel *et al.*, 1990), inflammatory bowel disease and rheumatoid arthritis (Ward, 1997).

Lignans show antiviral activity (Vlietinck *et al.*, 1998). For example, a number isolated from *Larrea tridentata* leaves demonstrated an anti-HIV activity such as inhibition of HIV replication, in blood cells from AIDS patients and protection for human lymphoblastoid cells against HIV-1 infection (Gnabre *et al.*, 1995). Other lignans such as arctigenin and trachelogenin (from *Ipomoea cairica*) and schisantherin D (from *Kadsura interior*) have been also shown, *in vitro*, to inhibit replication of the HIV virus (Schroeder *et al.*, 1991; Chen *et al.*, 1996). Gomisin D, (also from *K. interior*), exhibited the most potent anti-HIV activity among the 12 lignans obtained from an ethanolic extract of the plant (Chen *et al.*, 1997).

Table 1.9: Biological activities of lignans

Biological activity	Details of activity	Plant source	Plant part	Lignan/Extract	Reference
Active on the CNS	Mild antidepressant	*Justicia prostata*	None given	Prostalidins A, B and C	Ghosal *et al.*, 1979
Action on PAF	Inhibition of platelet aggregation	*Justicia procumbes*		Neojusticin A, justicin B and taiwanin E	Chen *et al.*, 1996
	Inhibit PAF-induced rabbit platelet aggregation	*Ocotea duckei*	Fruit	Yangambin	Castro-Faria-Neto *et al.*, 1995
Allergenicity	Causes cough, asthma and rhinitis	*Thuja plicata*	Wood (dust)	Plicatic acid	Chan-Yeung, 1973
Antihepatotoxic	Facilitate liver function	*Schisandra chinensis*	Fruit	Schisandrin B	Wagner, 1981
	Treat jaundice and other liver diseases	*Phyllanthus amarus*	Aerial parts	Phyllanthin, hypophyllanthin	Sharma *et al.*, 1993
	In vivo hepatoprotection against CCl_4	*Shisandra chinensis*	Fruit	Petroleum ether extract	Ko *et al.*, 1995
Antihypertensive		*Forsythia* species	Leaf	Pinoresinol monoglucoside	Kitagawa *et al.*, 1984
Antileukemic	Crude plant extract exhibits potent anticancer activity	*Wikstroemia viridiflora*	Powdered plant	Ethyl acetate fraction	Tandon and Rastogi, 1976
Antimicrobial	Inhibition of *Streptococcus*, *Bacillus subtilis* and *Pseudomonas aeruginosa*	*Larrea divaricata*	None given	Dihydroguaiaretic acid and norisoguaiacin	Farnsworth and Cordell, 1976
	Active against *Staphylococus aureus* and *Escherichia coli*	None given	None given	Polygamain	Sheriha *et al.*, 1987
Antioxidative	Enhances vitamin E effect and increases α-tocopherol concentration in plasma and tissue of rats	*Sesamum species*	Seed	None given	Yamashita *et al.*, 1995
	Health protector	*Sesamum indicum*	Seed	None given	Kato *et al.*, 1998

continued...

Table 1.9: continued

Biological activity	Details of activity	Plant source	Plant part	Lignan/Extract	Reference
Cathartic		*Carthamus tinctorus*	None given	2-Hydroxyarctiin	Palter *et al.*, 1972
Fungicide	Inhibition of *Fomes annosus in vitro*	*Picea abies*	Reaction zone	Hydroxymatairesinol	Shain and Hillis, 1971
Germination inhibitor	Inhibition of lettuce in the light	*Aegilops ovata*	None given	Monolactone	Gutterman *et al.*, 1980
Inhibitor of enzyme	Non-redox inhibitor of 5-LO activity	*Sesamum* species	Seed	Analogs of justicidin E	Ducharme *et al.*, 1994
Insecticidal	Inhibition of silkworm larvae	*Sesamum* species	Seed	Sesamin and kobusin	MacRae and Towers, 1984
	Synergistic action with pyrethrin/sevin	*Phryma leptostachya*	None given	Phrymarolin I	Harborne and Baxter, 1993
Interaction with human SHBG	High binding affinity to human SHBG	None given	None given	(-)-3, 4-Divanillytetrahydrofuran	Schottner *et al.*, 1998
Piscidal	Toxicity similar to rotenones	*Cleistanthus collinus* and *Justicia hayatai*	None given	Diphyllin, justicidin A and justicidin B	Anjaneyulu *et al.*, 1981; MacRae and Towers, 1984
Reduction of stress	Enhances resistance to stress	Siberian ginseng	Root	Di-O-β- diglucoside of (+)-syringaresinol	Bruneton, 1995

CNS: Central nervous system; PAF: Platelet-activating factor; SHBG: Sex hormone binding globulin; 5-LO: 5-lipoxygenase.

The potential antitumour activity of lignans is, probably, of main importance. Since 1942, lignans have been of interest in the context of cancer treatment. Several lignans, representing many structural classes, are recognised as exhibiting cytotoxic and antitumour activities. For example, the bisbenzocyclooctadiene lactones, steganacin and steganagin (from *Steganotaenia araliacea*) act as antileukemic lignans *in vivo* against P-388 leukemia in the mouse and show *in vitro* cytotoxicity against cell cultures derived from human carcinoma of the nasopharynx (Kupchan *et al.*, 1973). Substituted furofuran lignans, sesamin and episesamin, act as regulators of cholesterol and interfer in linoleate metabolism in rats (Sugano *et al.*, 1991). Novel cyclopenta[*b*]benzofuran lignans obtained by bioactivity-directed fractionation of *Aglaia elliptica* (stems/fruits) also demonstrated potent cytotoxic action against a range of human tumour cell lines such as breast cancer, human lung cancer and glioblastoma (Cui *et al.*, 1997).

The class of arylnaphthalene derivatives, particularly the aryltetralin type, contain lignans with clinical value. The arylnaphthalene lactone, koelreuterin-1, recently isolated from *Koelreuteria henryi*, showed cytotoxicity against human tumour cells (Song *et al.*, 1994). Considerable interest has centred on the *Podophyllum* lignans. In this context, podophyllotoxin reported in the genera *Podophyllum* and *Diphylleia* (Broomhead and Dewick, 1990), is one of the most important natural lignans with useful pharmacologic activity (Dewick, 1997; see also Chapter 4). Therefore, the species *P. hexandrum* and *D. cymosa* were focused on this study (see Section 1.5.1) for the establishment in tissue culture (Chapters 2 and 3) and phytochemical studies (Chapters 4 and 5).

1.4 Tissue culture

1.4.1 Tissue culture of *Podophyllum* and *Diphylleia* species

There are a number of reports concerning the tissue culture of *Podophyllum* species such as *P. versipelle*, *P. pleianthum*, *P. peltatum* and *P. hexandrum*. Cell suspension cultures of *P. versipelle* (syn. *P. mairei*) have been reported (Arens *et al.*, 1986) even though this species is poorly represented in terms of podophyllotoxin content (*ca.* 0.3%) (Broomhead and Dewick, 1990). Plant regeneration has been achieved from callus cultures of *P. pleianthum* (syn. *Dysosma pleiantha*) (Fuji *et al.*, 1986); young leaves and rhizomes proved to be the best source of explants for the production of somatic embryos which developed to plantlets (Chuang and Chang, 1987). This species has lower levels of podophyllotoxin (*ca.* 0.1%) (Jackson and Dewick, 1985) compared to *P. peltatum* and *P. hexandrum* (*ca.* 0.25% and 4.3%, respectively) (Broomhead and Dewick, 1990).

Studies of *P. hexandrum* in tissue culture are limited. Chapter 2 gives more details of tissue culture studies of this species. It has also been reported that undifferentiated callus tissues from different explants of *P. peltatum* were able to produce the aryltetralin lignan podophyllotoxin. The content quantified in callus of rhizome origin (Kadkade, 1981) was higher than that found in leaves, roots and stem cultures (Kadkade, 1982). The production of podophyllotoxin was affected by light quality and intensity and was further investigated in terms of culture medium composition. In this regard, kinetin played an important role besides NAA for callus induction and with 2,4-D for maximum podophyllotoxin production. Recently, a protocol was reported for *in vitro* propagation of *P. peltatum* (Sadowska *et al.*, 1997). According to this study, plantlets were obtained on MS medium supplemented with GA_3. In a second study, different types of buds (apical, axillary and adventitious) were induced from a terminal bud originated by rhizome tip cultures from wild plants (Moraes-Cerdeira *et al.*, 1998). The lignan contents from *in vitro* rooted buds and plantlets were assessed by HPLC and found to be at comparable concentrations to those for plants collected from wild populations (Bastos *et al.*, 1996).

To date, there are no published reports on the tissue culture of *Diphylleia* species despite its medicinal importance. Thus, the protocols developed (Chapter 3) are the first studies of the tissue culture for *D. cymosa*.

1.4.2 Current status of tissue culture of *Passiflora* species

The diversity within the genus *Passiflora* provides a great potential for germplasm storage and breeding programmes. Tissue culture techniques have helped to open up the possibility for *in vitro* propagation of *Passiflora* which would benefit the food and fruit juice industries. This biotechnological approach may allow the rapid clonal propagation of genotypes with yield potential and disease-resistance and also the multiplication of varieties that can be potentially used as rootstocks (Drew, 1997). Protocols for micropropagation of *Passiflora* species have been established for *P. edulis*, *P. edulis* fv. *flavicarpa* (Kantharajah and Dodd, 1990; Drew, 1991; Carvalho and Segura, 1994; see also references in Table 1.10), and for other species such as *P. giberti* and *P. mollissima* (Drew, 1991; Dornelas and Vieira, 1994; Drew, 1997).

Whole plants have been regenerated from isolated protoplasts, for example from leaves of *P. suberosa* (Otoni, 1995), cotyledons of *P. amethystina* and *P. cincinnata* (Dornelas and Vieira, 1993), either from embryogenic cell suspensions of *P. giberti* (Otoni, 1995) or non-embryogenic suspensions of *P. alata*, *P. amethystina*, *P. cincinnata*, *P. coccinea* (Dornelas *et al.*, 1995). Additionally, protoplasts have been successfully isolated from microspores, petals and pollen grains of other species such as *P. maliformis* (Vieira and Dornelas, 1996).

Table 1.10: Biotechnological approaches for *P. edulis*, *P. edulis* fv. *flavicarpa* and *P. incarnata*

Species	Source of explant	Result	Technique used	Reference
P. edulis	Anthers	Callus and roots[1]	Micropropagation	Tsay *et al.*, 1984
	Leaves, nodal sections	Callus, roots, shoots	Micropropagation	Kantharajah and Dodd, 1990
	Apical and nodal buds	Plant regeneration	Micropropagation	Drew, 1991
	Leaves (shoot primordia)	Plant regeneration	Micropropagation	Kawata *et al.*, 1995
P. edulis fv. *flavicarpa*	Leaves	Plant regeneration	Protoplast culture	Manders *et al.*, 1991
	Leaves	Callus, roots, shoots	Micropropagation	Amugene *et al.*, 1993
	Seedling cotyledons	Plant regeneration	Protoplast culture	Dornelas and Vieira, 1993
	Leaves	Plant regeneration	Protoplast culture	d'Utra Vaz *et al.*, 1993
	Cotyledons, hypocotyls, leaves	Regeneration, apex cultures	Micropropagation	Dornelas and Vieira, 1994
	Leaves, stems	Transgenic plants[2]	Transformation	Manders *et al.*, 1994
	Meristem tips	Establishement/enlargement of meristems	Meristem culture	Chiari *et al.*, 1994
	Leaves	Somatic hybrid plants[3]	Protoplast fusion	Otoni *et al.*, 1995
	Nodal segments	Transformed roots[4]	Transformation	Otoni, 1995
	Leaves	Somatic hybrid plants[5]	Protoplast fusion	Vieira and Dornelas, 1996
	Shoot apices	Axillary bud proliferation	Micropropagation	Faria and Segura, 1997
P. incarnata	Leaves	Somatic hybrid plants[3]	Protoplast fusion	Otoni *et al.*, 1995
	Leaves	Callus, organogenesis	Protoplast culture	Otoni, 1995

[1] Callus and roots were obtained between *P. edulis* x *P. edulis* fv. *flavicarpa* hybrids; [2] Transgenic plants were obtained by *A. tumefaciens*-mediated gene delivery; [3] Somatic hybrids between *P. edulis* fv. *flavicarpa* (+) *P. incarnata*; [4] Transformed roots were obtained by co-cultivation with *A. rhizogenes*; [5] Somatic hybrids between *P. edulis* fv. *flavicarpa* (+) [*P. alata*, *P. amethystina*, *P. cincinnata*, *P. coccinea*, *P. giberti*].

As mentionated previously (Section 1.2.2.5) the worldwide juice industry based on passion fruit is dependent on the varieties *P. edulis* and *P. edulis* fv. *flavicarpa*. The former has been reported to be highly resistant to the fungal disease caused by *Fusarium oxysporum* f. *passiflorae* (Oliveira, 1987), but susceptible to the bacterium *Xanthomonas campestris* f. *passiflorae* (Neto *et al.*, 1984). *P. edulis* fv. *flavicarpa* has showed some resistance to the nematode *Meloidogyne javanica* but is vulnerable to *Fusarium* (Oliveira, 1987). The existing genetic variability, variation in the phenotypic characters and the possibility to transfer, into cultivated species, useful traits, such as resistance to fusariosis, bacteriosis, root nematodes and cold tolerance found in wild species are useful to *Passiflora* breeding programmes (Drew, 1997). Therefore, somatic hybridization is one of the options for the introduction of agronomically desirable genes, and thus to overcome sexual incompatibilities (Anthony *et al.*, 1999a). This has been reported for *P. edulis* fv. *flavicarpa* and *P. incarnata* (Otoni, 1995) and between *P. edulis* fv. *flavicarpa* and five wild-type species (Vieira and Dornelas, 1996).

Genetic manipulation studies on the susceptibility of *P. edulis* fv. *flavicarpa* to *Agrobacterium tumefaciens* have demonstrated the possibility of obtaining transgenic plants (Manders *et al.*, 1994) and also to introduce resistance to soybean mosaic virus and passion fruit woodiness virus (d'Eeckenbrugge *et al.*, 1998). Transformation studies using biolistics-mediated transient gene expression of β-glucuronidase has been reported in embryogenic calli from suspension cells of *P. giberti* (Otoni, 1995). Biotechnological techniques used for *P. edulis*, *P. edulis* fv. *flavicarpa* and *P. incarnata*, which are the species focused on in this study are summarised in Table 1.10.

1.5 Thesis objectives

1.5.1 Choice of *Podophyllum hexandrum* and *Diphylleia cymosa*

These two species were chosen for study because they are important medicinal species for sources of lignans with antitumour activity. *Podophyllum* roots yield podophyllotoxin which is used as commercial precursor for the semi-synthesis of etoposide and teniposide, known anticancer drugs. The successful introduction of these drugs has created a demand for podophyllotoxin.

Commercially there are few plant sources for podophyllotoxin, a relatively rare natural product, and thus the availability of this lignan is limited. The synthesis of this molecule is uneconomic and its production still comes from extraction from plant material, but the process gives non-optimal levels of podophyllotoxin. The conversion of podophyllotoxin to etoposide and teniposide is multistage, and some of the steps resulted in low yields of intermediate compounds, leading to a high market price for

these drugs. There is thus an urgent need for maintainable supplies of podophyllotoxin, which potentially could be achieved through tissue culture propagation of *Podophyllum*.

The high concentration of *Podophyllum* lignans in leaves of *D. cymosa* has clear commercial value, thus the present study was directed towards the establishment of a methodology for its tissue culture and an assessment of lignan content in undifferentiated callus and cell suspensions.

The novel tissue culture methodologies for both *P. hexandrum* and *D. cymosa* were to be developed to determine whether such *in vitro* systems could, in practice, be used as an alternative source of podophyllotoxin and the related lignans.

1.5.2 Choice of *Passiflora* species

Passiflora incarnata and *P. edulis* have a widespread use as sedatives and tranquillisers in popular medicine. Flavonoids are considered to be biologically active compounds and could be responsible for some of the known pharmacological effects of *Passiflora* species. For the analysis of polyphenolic compounds such as flavonoids, reverse-phase HPLC and TLC methods are generally used. In this study a focus was to develop a sensitive assay for the qualitative and quantitative analysis of flavonoids in *P. edulis* fv. *flavicarpa*, *P. incarnata*, and their somatic hybrid.

1.5.3 Specific research objectives

1.5.3.1 *Podophyllum hexandrum*

I) To improve existing tissue culture protocols, especially in terms of reducing the time required for callus initiation from root explants and also to improve upon published micropropagation systems. This approach will also be used to evaluate the optimisation of culture conditions, leading to an efficient regeneration system as a prerequisite to *Agrobacterium*-mediated transformation experiments.

II) To isolate aryltetralin lactone lignans from rhizomes/roots of *P. hexandrum* and *P. peltatum* using PLC, in order to obtain authentic standards. To identify lignans in these extracts by comparison with standards, using TLC, HPLC, ^{1}H NMR, MS and determination of melting points. To use some of the authentic standards obtained from rhizomes/roots of *P. hexandrum* and *P. peltatum* in phytochemical studies of whole plants and tissue cultures of *D. cymosa*.

1.5.3.2 *Diphylleia cymosa*

I) To establish tissue cultures of *D. cymosa* from different explant sources, including the establishment and optimisation of growth conditions for callus and cell suspension cultures. Additionally, to investigate the effects of media combinations and growth regulators, photoperiod, on biomass production and retention of totipotency.

II) To assess, by HPLC, the aryltetralin lactone lignan content in a range of plant parts from individuals of *D. cymosa*, to determine a representative lignan profile for this species. Particular emphasis was to be placed on leaf and petiole tissues, which were also to be used as source materials for tissue culture. The lignan content of such tissue-cultured plant material would be determined, by comparison with *Podophyllum* standards (Section 1.5.3.1).

III) To evaluate cryopreservation for storage of cell suspensions of *D. cymosa* and to provide a constant supply of viable cells, free from seasonal effects, for use in future studies of the biosynthesis and metabolism of aryltetralin lignans.

IV) To study the possible effects of Pluronic F-68 (a non-ionic, co-polymer surfactant) on callus grown from leaf explants.

1.5.3.3 *Passiflora* species

I) To determine, on a comparative basis, the presence of flavonoids, of medicinal value, in four independent somatic hybrids [*P. incarnata* (+) *P. edulis*] and their parents. To evaluate the range and type of flavonoids in their leaf tissues by chromatographic approaches.

Chapter Two

Tissue Culture Studies of *Podophyllum hexandrum*

2.1 Introduction

Studies of *P. hexandrum* in tissue culture are limited despite its economic value as a source of natural lignans with antitumour activity (Chapter 4, Section 4.1.2). This could be partially attributed to the long juvenile phase displayed by plants coupled with a poor fruit setting (Van Uden *et al.*, 1990) and the fact that it has never been extensively cultivated and is collected from the wild (see Section 2.1.1). The other *Podophyllum* species (*P. peltatum*, *P. pleianthum* and *P. versipelle*) have received considerable attention in terms of tissue culture (Chapter 1, Section 1.4.1). However, these species contain only low yields of lignans such as podophyllotoxin when compared to *P. hexandrum* (Jackson and Dewick, 1985; Broomhead and Dewick, 1990).

In *P. hexandrum*, only a few studies of root culture have been published. Embryogenic callus and cell suspensions derived from roots of plant germplasms, now maintained at Nottingham, from *in vitro* cultured seedlings have been reported (Rahman, 1988). However, such embryogenic calli did not develop when cultured on agar-solidified media. Lignans and their glycosides were not found in these calli. In a second study, callus and cell suspensions were evaluated in relation to podophyllotoxin (Van Uden *et al.*, 1989). Cultures of dark-grown callus had higher contents of podophyllotoxin (*ca.* 0.3% d. wt.) compared with light-grown cultures. Root-derived callus from *in vitro* cultured seedlings showed positive results in terms of lignan production (Heyenga *et al.*, 1990). This study has also shown that for some cell lines, high levels of podophyllotoxin are related to the tissue differentiation status elicited by BAP. Moreover, the presence of auxins, in particular 2,4-D, was necessary for callus induction. According to these studies, B5 medium showed better results in terms of callus growth than MS medium.

Plant regeneration has been achieved via somatic embryogenesis (Arumugan and Bhojwani, 1990). Somatic embryos were formed on callus derived from zygotic embryos cultured on MS medium supplemented with BAP and IAA. Globular somatic embryos were formed with 2,4-D and BAP. NAA (or a high concentration of sucrose) was required in order to promote development of somatic embryos.

In addition, cell suspension cultures of *P. hexandrum* have been studied in order to improve their podophyllotoxin production by feeding biosynthetic precursors (Van Uden *et al.*, 1990b) including coniferin, an intermediate from the phenylpropanoid pathway, which was converted to podophyllotoxin. This lignan was

released into the medium when the substrate (coniferin) was supplied with 0.5% (v:v) isopropanol as a permeabilizing agent. However, coniferin is not commercially available (Woerdenbag *et al.*, 1990), therefore, coniferyl alcohol was the substrate of choice and when complexed with β-cyclodextrin, was shown to enhance podophyllotoxin content in *P. hexandrum* cell suspensions (Woerdenbag *et al.*, 1990). Furthermore, in bioconversion studies, cell cultures accumulated podophyllotoxin and its β-D-glucoside after feeding with desoxypodophyllotoxin as a cyclodextrin complex (Van Uden *et al.*, 1995).

2.1.1 The importance of conservation of *P. hexandrum* germplasm

In the early 1900's, the exportation of wild collected *P. hexandrum* rhizomes from India as a source of income resulted in over-exploitation (Chatterjee, 1952). Unregulated collection of the rhizomes and roots by untrained persons in the past has reduced plant populations in easily accessible areas, making the species vulnerable. Additionally, the costs of collection, processing and transport of the material are considered high (Badhwar and Sharma, 1963).

These factors along with the plant's medicinal importance have encouraged efforts to preserve and cultivate the species on a commercial scale. As a result, several experiments on its cultivation were conducted in India (Krishnamurthy *et al.*, 1965; Nautiyal *et al.*, 1987). Concern has been expressed about the shortage of rhizomes. The situation has been aggravated by the fact that an adulteration of Indian *Podophyllum* has been discovered in Banjar (one of the Himalayan States) where, for some time, roots of *Ainsliaea latifolia* have been collected in large amounts and used to adulterate *P. hexandrum* (Puri and Jain, 1988). This problem has been exacerbated by the fact that the plant, once common in the Himalayas, is now considered as a rare species and collection of rhizomes from the wild is prohibited (Gupta and Sethi, 1983). Fortunately, *P. hexandrum* and other Himalayan medicinal plants are under protection from the Government of India. Currently, supplies of wild rhizomes come from China (Konuklugil and Shaw, 1996).

Like other species considered rare, *Podophyllum* needs to be protected in its natural environment (Schumacher, 1991). The National Parks and sanctuaries in the Himalayan region retain the basic ecological conditions for *in situ* conservation of *Podophyllum* (Gupta and Sethi, 1983). *Podophyllum* has been highlighted as a genus also in need of garden conservation (Brickell and Sharman, 1986). It is one of the few genera of medicinal species now conserved in India as living collections using *ex situ* conservation through botanical gardens (Jain, 1994). In 1996, three national collections of *Podophyllum* species were set up by the National Council for the Conservation of Plants and Gardens (NCCPG) in order to preserve cultivated stocks

in Britain (Shaw, 1999). In addition, a few studies on tissue culture have been undertaken to preserve and multiply germplasm of *P. hexandrum* (Heyenga, 1989; Arumugan and Bhojwani, 1990; Nadeem *et al.*, 2000).

2.1.2 Root cultures as a source of secondary metabolites

In the biosynthesis of secondary metabolites, root cultures were initially targeted in order to confirm that nicotine synthesis in *Nicotiana tabacum* occurs in the roots and then the alkaloid is transported to the leaves (Dawson, 1942). Root cultures, in general, show a slow growth, and were thus replaced by callus and cell suspension cultures. Although callus and cell suspensions are characterized by more rapid growth, problems associated with low product yields and culture instabilities have limited their industrial application (Toivonen, 1993). Moreover, genetic instability (somaclonal variation) occurs frequently when cells are maintained in a dedifferentiated state (Ronchi, 1995). Therefore, the interest in root and shoot cultures has been renewed as an alternative approach to the production of plant secondary metabolites. A wide range of secondary metabolites such as flavourings (from *Glycyrrhiza glabra*); insecticides (rotenoids and thiophenes); dyes, for example sanguinarine from *Sanguinaria canadensis* which also has anti-bacterial activity (Signs and Flores, 1990) and others has been isolated from roots (Loyola-Vargas and Miranda-Ham, 1995). Furthermore, root cultures have the following advantages over callus and cell suspensions: the differentiated root is required to enhance biosynthesis expression; genetic and productive stability is maintained for up to one year; and such a system is compatible with immobilization (Collin and Edwards, 1998). Roots have been suggested as an engineering strategy to improve overall secondary metabolite production (Payne *et al.*, 1991). Root cultures can include transformed or hairy root cultures, the latter obtained after transformation of plant tissues by *A. rhizogenes*.

2.1.3 *Agrobacterium*-mediated transformation

The primary objective of *Agrobacterium*-mediated transformation has been to improve methods of inserting foreign and novel genes into the plants. The pathogenic soil bacteria *A. rhizogenes* and *A. tumefaciens* infect a wide range of species via wound sites, inducing root formation or development of tumours, characteristics of hairy root and crown gall diseases (Davey *et al.*, 1994). This process involves the transfer and integration of a segment of T-DNA region (transfer DNA) from the bacterial Ri (root-inducing) or Ti (tumour-inducing) plasmids into the plant genome (Davey *et al.*, 1994). Studies have shown that over 120 different plant species (dicotyledons, monocotyledons and gymnosperms) are susceptible to *Agrobacterium*-mediated gene delivery (Birch, 1997).

Transformation is triggered by activation of virulent genes by wound substances. These are phenolic compounds, for example acetosyringone (4-acetyl-2,6-dimethoxyphenol), synthesized in the plant (Toivonen, 1993). T-DNA contains a variety of genes and most of them are involved in the formation of the tumour or in the synthesis of opines. The tumour cells result from the cell divisions triggered by the production of auxins and cytokinins by the enzymes encoded in the T-DNA (Hiei *et al.*, 1997). The T-DNA can carry genes encoding the synthesis of sugar and aminoacid conjugates termed opines, for example agropine (*ags*), nopaline (*nos*) and others, which can be used by the invading bacteria as a source of energy, carbon and nitrogen (Saito *et al.*, 1992). The opines are synthesized by enzymes and are not found in untransformed tissues.

2.1.3.1 Applications of *Agrobacterium* to medicinal plants

In the last few years, several advances have been made concerning the production (and over-production) of secondary metabolites; production of transgenic plants and biotransformation of products (Saito *et al.*, 1992; Wysokinska and Chmiel, 1997). Transformed roots of *Linum flavum* produce 1.5 to 3.5% (d. wt.) of the lignan 5-methoxypodophyllotoxin (Oostdam *et al.*, 1993) which has strong cytotoxic activities similar to podophyllotoxin (Van Uden *et al.*, 1990a). Production of secondary metabolites and transgenic plants using a strain of Japanese *A. rhizogenes* has been reported for medicinal species within the genera *Papaver*, *Duboisia* and *Angelica* (Shimomura and Yoshimatsu, 1994). Solasodine, an alternative to diosgenin for the synthesis of steroid drugs, was obtained from hairy root cultures of *Solanum aviculare* in high levels compared to callus and cell suspensions (Kittipongpatana *et al.*, 1998). Furthemore, hairy root cultures of *D. lanata* have been shown to accumulate higher contents of anthraquinones and flavonoids compared to untransformed roots (Prade *et al.*, 1997). Accumulation of arteannuin B and artemisinic acid has been reported in regenerants of *Artemisia annua* following transformation with *A. rhizogenes* (Banerjee *et al.*, 1998). These compounds are considered precursors of the antimalarial artemisinin (Akhila *et al.*, 1990) which also is active against multidrug-resistant *Plasmodium fasciparum* strains (Vergauve *et al.*, 1998).

Although transformed root cultures produce the same metabolites found in the intact root cultures, new compounds have been identified in hairy root cultures which were not present in their untransformed counterparts (Table 2.1).

Table 2.1: Novel compounds identified in transformed root cultures

Plant species	Secondary products	Reference
Catharanthus trichophyllus	anthraserpine and its derivatives	Davioud *et al.*, 1989a, b
Hyoscyamus albus	N-methylpyrrolidinyl-cuscohygrine, 4-hydroxylittorine	Doerk-Schmitz *et al.*, 1994
Swertia japonica	8-*O*-primevero-sylbellidifolin	Ishimaru *et al.*, 1990
Valeriana officinalis	valdiate	Gränicher *et al.*, 1995
Sesamum indicum	anthraquinones	Ogasawara *et al.*, 1993

Shooty teratoma cultures of *A. annua* transformed by wild type nopaline strains of *A. tumefaciens* have been reported to produce slightly higher levels of artemisinin than those of non-transformed shoots or plantlets (Ghosh *et al.*, 1997). Transformed shoot cultures of *Pimpinella anisum* (anise) using *A. tumefaciens* strain T37 have low yields of essential oils compared to untransformed shoots (Salem and Charlwood, 1995). Although an increase of secondary metabolites was not achieved, shoot cultures could be used as a model system for studying the effects of insertion of genes on the production of secondary metabolites (Salem and Charlwood, 1995). Furthermore, callus and root cultures of *Coleus forskohlii* transformed by *A. tumefaciens* strain C58 have been reported to accumulated forskolin (Mukherjee *et al.*, 1996).

In the present work, three bacterial strains of *A. rhizogenes* and *A. tumefaciens* were used to infect different organs and tissues of *P. hexandrum* in order to establish whether *Agrobacterium*-mediated transformation *per se* could be used for this species.

2.2 Materials and methods

2.2.1 Plant material and seed production

The identity of the *P. hexandrum* cv. 'Majus' plants maintained at the University of Nottingham was confirmed by JMH Shaw (Department of Pharmaceutical Sciences, University of Nottingham). The cv.'Majus' represents an inbred line albeit of unknown origin, commonly cultivated as a garden ornamental plant in the UK. Plants were originally obtained from Bressingham Gardens (Bressingham Plant Centre, Bressingham, Norfolk, UK). In the first year, plants were

cultivated individually in 10.0 cm diam. pots containing a mixture of Levington M3 soil-less (Fisons Plc., Ipswich, UK) and John Innes No. 2 composts (Joseph Bentley Ltd., Barrow-on-Humber, UK) (1:1, v:v), maintained in a cold frame and then planted out. Seeds of self pollinations were produced and used to increase stocks. Ripe fruits of *P. hexandrum* cv. 'Majus' (Plate 2.1.A) were harvested (in August) from a population of approx. 20 self-pollinated individuals, grown from seed.

2.2.2 Preparation of explants for tissue culture

2.2.2.1 Surface sterilisation of seeds

Seeds were sterilised by one of two methods:

Fresh fruits were washed briefly in running water prior to disinfection of seeds. After harvesting, ripe fruits were immediately surface sterilised by immersion in 70% (v:v) methcol (Appendix 1) for 5 min and dissected aseptically (Plate 2.1.B). Seeds (n = 50) with their entire placental column were washed by stirring (15 min) with sterile, reverse-osmosis water. Seeds were also removed from fruits, left for 20 - 30 min in running water, and washed three times with sterile, reverse-osmosis water. Seeds, free of the placental column, were surface sterilised in [5, 10, 15 and 20% (v:v)] Domestos bleach solution (Appendix 1) with 0.2% (v:v) Tween 20 (polyoxyethylenesorbitan monolaurate; Sigma, Chemical Co., St. Louis, USA) for 5, 10, 15 or 20 min, followed by three washes in sterile, reverse-osmosis water. The whole sterilisation process was repeated once, the last two washes of which were of 10 min duration. Seeds were blotted dry onto sterile filter paper disks (90 mm diam.) (Whatman International Ltd., Maidstone, Kent, UK) followed by germination.

2.2.2.2 Seed germination

The effect of storage as a pre-treatment for the germination of seeds was investigated using sterilised seeds (n = 140). Seeds were kept in 14.0 cm diam. Petri dishes (Bibby Sterilin Ltd., Stone, UK) and maintained in the dark at 22 ± 1°C for 30 d prior to use under dessicating conditions. Stored seeds were then scarified (n = 70) or left without scarification (n = 70). Germination of seeds (scarified and non-scarified) was carried out in 9.0 cm diam. Petri dishes (Sterilin Ltd., Middlesex, UK), containing a moist (sterile, reverse-osmosis water) sterile filter paper disk (70 mm diam., No.1; Whatman, UK). Ten seeds were cultured per dish which was sealed with Nescofilm (Nippon, Shoji Kaisha Ltd., Osaka, Japan). Cultures were incubated in the dark at 22 ± 1°C until the emergence of the radicle. The experiment consisted of seven replicates and was repeated three times. In order to facilitate germination, freshly sterilised seeds (n = 30) without any storage pre-treatment were scarified under aseptic conditions by removing a section of seed coat through an incision at the

opposite side of the hilum area. Controls without scarification (n = 150) were also set up. Cultures (10 seeds per dish) were left in the dark at 28°C until the emergence of the radicle. The experiment consisted of three replicates for scarified seeds and 15 replicates for control. The experiment was repeated five times. In all treatments, germination frequencies were scored based on the emergence of the radicle.

Statiscal analyses of the results included one-way ANOVAs incorporating a post-Hoc Tukey-Honestly Significant Difference (Tukey-HSD) test, and a probability of $P < 0.05$ was considered significant.

2.2.3 Axenic culture of plants

After the radicle emerged, (approx. 35 d after plating seeds) (Plate 2.1.C), the germinated seeds were cultured on a semi-solid basal medium in order to produce roots for tissue culture experiments. Germinated seeds (n = 60) were placed on the surface of 20 ml per dish of 0.2 normal strength B5 medium (Appendix 2), pH 6.0, solidified with 0.6% (w:v) agar (Sigma Chemical Co., St. Louis, USA) (Heyenga *et al.*, 1990). This medium, designated MRG medium, was dispensed into 9.0 cm diam. Petri dishes. The experiment consisted of five seeds per dish and 12 replicates and was repeated three times. Half of the Petri dishes were kept under diffuse light (3.8 μmol m^{-2} s^{-1}) at 22 ± 1°C whilst the other dishes were maintained in the dark at 28°C. All cultures were assessed after 3 weeks.

Full-strength BGS and MS media supplemented with or without growth regulators were also assessed in order to grow *in vitro* seedlings as a source of plant material for tissue culture experiments (Sections 2.2.5 and 2.2.6) and transformation (Section 2.2.11). Germinated seeds (scarified and non-scarified, Section 2.2.2.2) were individually transferred onto 50 ml full-strength BGS medium (n = 30 seeds; Appendix 2) with growth regulators and 90 ml full-strength MS medium (n = 30 seeds; Appendix 2) lacking growth regulators. BGS medium was dispensed in 175 ml screw-capped jars (Beatson Clarke and Co. Ltd., Rotherham, UK) and MS medium into Magenta culture vessels (Sigma Chemical Co., Dorset, UK). Cultures were maintained under diffuse light (3.8 μmol m^{-2} s^{-1}) at 22 ± 1°C for 3 weeks and then transferred to a 16 h photoperiod under fluorescent light (42 μmol m^{-2} s^{-1}, provided by "Daylight" fluorescent tubes; Philips Lighting Ltd., Croydon, UK) at 22 ± 1°C.

2.2.4 Vernalization of dormant plants

In an attempt to break the pre-determined dormancy period in *P. hexandrum* plants harvested during the autumn/winter, a total of eight pots containing dormant plants (with buds, rhizomes and roots) (Plate 2.1.E) were maintained in an illuminated vernalization chamber (Hoover, UK) with a 16 h photoperiod (3.0 - 14.5 μmol m^{-2} s^{-1})

at 4°C. In another attempt to break dormancy, four cultivated plants (5 - 6 years-old) (Plate 2.1.F) from the stock of plant germplasm held at the University of Nottingham were also maintained in glasshouse conditions at 20 ± 2°C (night) and 22 ± 2°C (day) with natural daylight supplemented by a 16 h photoperiod (151 μmol m^{-2} s^{-1}, provided by TLD 58W 33 "Daylight" fluorescent tubes; Philips Lighting Ltd., Croydon, UK). After 8 weeks, the first pot from the vernalization chamber was transferred to the glasshouse under the same growth conditions as described above. The remaining pots were individually taken out, at 7 d intervals from the vernalization chamber to the glasshouse to determine the optimum vernalization period.

2.2.5 Establishment of callus cultures

Roots (8.0 - 10.0 cm in length) (Plate 2.1.D) were harvested from *in vitro* grown seedlings in MRG medium (Section 2.2.3) and approx. 0.5 cm was removed from each end of the root explant (hypocotyl and root tip). Sections (0.6 - 0.7 cm in length) were placed horizontally onto the callus induction medium. This medium consisted of 0.5 normal strength B5 medium supplemented with 1.0 mg l^{-1} 2,4-D, 0.1 mg l^{-1} BAP, 1.0 mg l^{-1} GA_3, 2% (w:v) sucrose and solidified with 0.7% (w:v) agar, pH 5.8 (Heyenga *et al.*, 1990), designated MCG medium. MCG medium (20 ml) was dispensed into 9.0 cm diam. Petri dishes with ten explants per dish. The experiment consisted of ten replicates and was repeated five times. Cultures were maintained in the dark at 22 ± 1°C and assessed for callus induction every for 4 weeks.

2.2.6 Establishment of root-derived cell suspension cultures

Cell suspension cultures were initiated from cream, friable root-derived callus (4 months old) on MCG medium although some portions of the calli exhibited an embryogenic appearance (Section 2.3.4). Calli (1.0 cm diam., approx. 1.5 g f. wt.) were transferred to a 100 ml Erlenmeyer flask with 40 ml UM liquid medium (Appendix 2). Flasks were placed on a rotary shaker (120 rpm) in the dark at 25 ± 2°C. Flasks were left with continuous agitation for 14 d without subculturing and thereafter 5 ml of fresh MCG medium was added to each flask. After a further 7 d culture, the spent medium was partially replaced (volume for volume) with 30 ml fresh UM medium. After a further 7 d culture, the contents of individual flasks were transferred to 50 ml fresh UM (250 ml flasks) to produce stock cultures. The flasks were maintained in the dark throughout the experiment.

Plate 2.1: Plants, fruits and germination *in vitro* of seeds of *Podophyllum hexandrum*

A. *P. hexandrum* cv. 'Majus' plants (approx. 8 years old) from a population of self-pollinated individuals, grown from seeds of the same inbred line, and cultivated at the University of Nottingham. Plants were photographed in the late summer and show ripe fruits. (x 0.10).

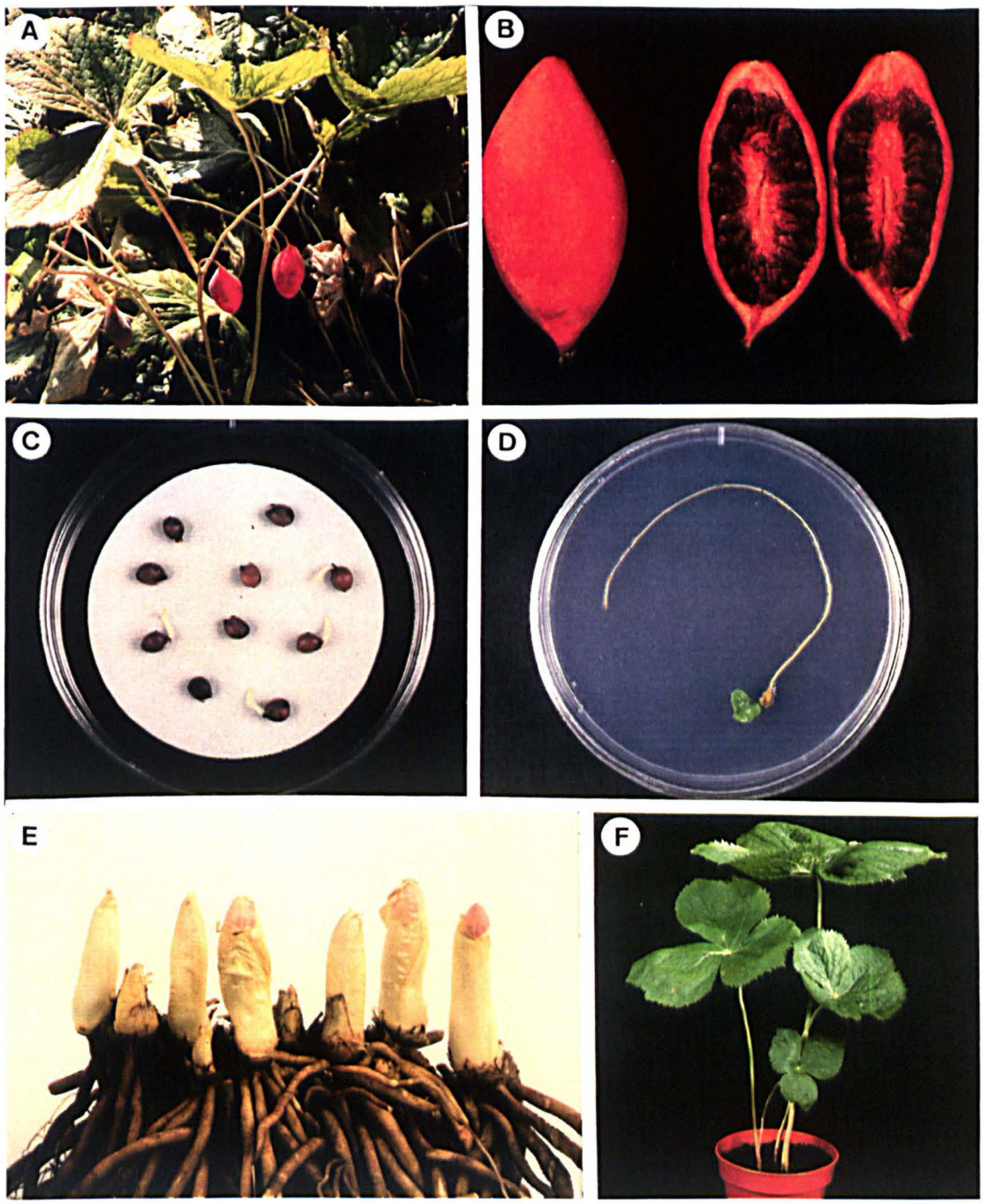
B. Ripe fruits, each containing approx. 50 seeds, harvested from a population of self-pollinated seed-derived plants. (x 0.75).

C. Seeds germinating on moist filter paper in a 9.0 cm diam. Petri dish after 35 d in the dark. (x 0.93).

D. An axenic seedling cultured for 21 d under diffuse light on 0.2 strength B5 medium lacking growth regulators and semi-solidified with 0.6% (w:v) agar. (x 0.93).

E. Dormant buds, rhizomes and roots of *P. hexandrum* cv. 'Majus' used as material for vernalization experiments. (x 1.0).

F. A plant of *P. hexandrum* cv. 'Majus' (approx. 5 - 6 years old) maintained in the glasshouse at 20 ± 2°C (night) and 22 ± 2°C (day) with natural daylight supplemented by a 16 h photoperiod (151 μmol m^{-2} s^{-1}) provided by "Daylight" fluorescent tubes. (x 0.40).


A
B
C
D
E
F

2.2.7 Determination of cell suspension viability

The viability of root-derived cell suspensions was assessed 4 d after subculture using the FDA method (Widholm, 1972). Cells were stained with FDA (stock solution: 10 µl of 5 mg l^{-1} FDA in acetone). Viable cells, labelled with FDA, were examined in a UV microscope (Nikon Diaphot TMD inverted microscope, model XF-EF with fluorescence attachment) at 495 nm and showed green-yellow fluorescence. A minimum of 70 cells was scored for each Erlemeyer flask and viability was expressed as the percentage of fluorescing cells (Appendix 3).

2.2.8 Plant regeneration

2.2.8.1 Somatic embryogenesis of root-derived callus (from cell cultures)

Embryogenic cell suspension cultures obtained in UM medium (see Results, Section 2.3.5) were evaluated for their morphogenic responses on agar-solidified media with a range of growth regulators. Small cell aggregates (0.40 g f. wt.) containing embryos were sieved (nylon sieve, 45 µm pore size) (see Results, Section 2.3.5) and transferred to 5.5 cm diam. Petri dishes (Bibby-Sterlin, Stone, UK) with either 10 ml of full-strength MS medium lacking growth regulators or MS medium supplemented with: BAP (0, 0,23 and 0.45 mg l^{-1}) in combination with GA_3 (0 and 0.97 mg l^{-1}) designated MSB media (see Results, Section 2.3.6, Table 2.4); GA_3 (0.25, 0.50 and 1.0 mg l^{-1}) designated MSG media; BAP (0 and 1.0 mg l^{-1}) with zeatin (0.5 and 1.0 mg l^{-1}) designated BZ media (see Results, Section 2.3.6, Table 2.5). Cell aggregates with embryos were also transferred to half-strength MS medium (Appendix 2) either alone or with the same combinations of BAP and GA_3 mentioned earlier (designated MB media; see Results, Section 2.3.6, Table 2.6) or with only GA_3 (designated MG media). Three cell aggregates (each cell aggregate approx. 7.0 mm diam.) containing embryos were cultured per dish and each treatment consisted of three replicates. Cultures were incubated either in the dark or in a 16 h photoperiod under fluorescent light (30 µmol m^{-2} s^{-1}). The experiments were repeated twice.

Embryogenic root-derived calli were separated from non-embryogenic cells prior to culture on different (proliferation/differentiation) media (see Results, Section 2.3.6, Table 2.7). Embryogenic calli (n = 14) transferred from BZ2 medium to BZ1 medium and similarly from BZ2 to BZ1 medium in combination with 0.23 mg l^{-1} 2,4-D (n = 16); and embryogenic calli (n = 8) from MSB3 medium were either placed on MB3 medium or MB3 medium in combination with 0.23 mg l^{-1} 2,4-D (n = 12). Media (10 ml) were dispensed in 5.5 cm diam. Petri dishes and cultures were maintained in the dark at 22 ± 1°C for 12 weeks. Calli were subcultured every 4 weeks onto fresh media and kept under the same conditions. After 4 weeks, all calli were examined and the number of embryos produced was assessed (see Results, Section 2.3.6.1.1).

2.2.8.2 Rooting of somatic embryo-derived plantlets

A sub-study was undertaken to promote rooting since not all somatic embryo-derived plantlets developed roots (see Results, Section 2.3.6.1). Experiments were carried out using IBA and a lipo-oligosaccharide NGR234, since it has been speculated that the latter compound is capable of inducing rooting of recalcitrant species [Cocking cited in Babaoglu (1996)].

2.2.8.2.1 Effects of IBA on rooting of somatic embryos

Somatic embryo-derived plantlets (n = 24) were individually transferred from MSB3, MSB5, BZ4 and MB5 media to 20 ml half-strength MS medium with IBA (0.1 and 0.3 mg l^{-1}) contained in 60 ml capacity screw-capped glass jars (Beatson Clarke and Co. Ltd., Rotherham, UK). Plantlets were pre-cultured (7 or 11 d) in the dark at 22 ± 1°C and thereafter transferred separately to half-strength MS medium lacking growth regulators. Cultures were maintained under a 16 h photoperiod (42 µmol m^{-2} s^{-1}) at 22 ± 1°C for 4 weeks.

2.2.8.2.2 Effects of nod-factor on rooting of somatic embryos

The effects of the Rhizobial lipo-oligosaccharide NGR234 [nodulation (Nod) factor] on root stimulation of plantlets obtained from somatic embryos (see Results, Section 2.3.6.1.1) were studied. Somatic embryo-derived plantlets (n = 20) from MSB5, BZ4 and MB5 media were individually pre-cultured on 20 ml full-strength MS medium with NGR234 (10^{-5} M) contained in 60 ml glass jars. Cultures were maintained in the dark for 7 d at 22 ± 1°C and thereafter transferred to half-strength MS medium lacking growth regulators. Cultures were maintained under a 16 h photoperiod (42 µmol m^{-2} s^{-1}) at 22 ± 1°C for 4 weeks.

2.2.8.3 Initiation of organogenesis

Roots (8.0 - 10.0 cm in length) were harvested from *in vitro* grown seedlings (Section 2.2.3) (Plate 2.4.A) and were studied for organogenic responses (Silva *et al.*, 1998). Explants excised below the cotyledons (hypocotyl through to root tips) (1.5 - 2.0 cm length) were sectioned and cultured in 250 ml Erlenmeyer flasks with 50 ml MS liquid medium containing NAA (0; 0.5; 1.0; 1.5; 2.0; 2.5; 3.0 mg l^{-1}) or IBA (0; 0.5; 1.0; 1.5; 2.0; 3.0 mg l^{-1}). Eleven root explants were cultured per treatment and four replicates were established for experiments which were repeated three times. Cultures were incubated on a rotary shaker (90 rpm) in the dark at 25 ± 1°C. After 45 d culture, regenerated plants (see Results, Section 2.3.6.2) were transferred to a new 250 ml Erlenmeyer flask with 50 ml of fresh liquid MS medium lacking NAA and

IBA. Cultures were maintained under similar conditions, but with a 16 h photoperiod (25 μmol m^2 s^{-1}).

2.2.9 Transfer and acclimatisation of regenerated plants

Regenerants (approx. 10.0 cm in height) were removed from the Erlenmeyer flasks and their roots were gently washed with sterile, reverse-osmosis water. Individual plants were transferred to 9.0 cm diam. pots containing a mixture of Levington M3 soil-less compost, John Innes No. 3 compost (Joseph Bentley Ltd.) and Perlite (Silvaperl Ltd., Gainsborough, UK) (6:6:1, v:v:v). The surfaces of the pots were covered with Perlite and, after watering, polyethylene bags were placed over each plant for the first 20 d to allow a waxy cuticle to form, thus minimising wilting. The covers were gradually perforated and completely removed after a further 41 d. Pots were watered weekly with Bentley's liquid nutrient solution (Joseph Bentley Ltd., Barrow-on-Humber, UK).

2.2.10 Histology of regeneration

Buds (structures similar to nodules) (1.2 - 2.4 mm diam.) that formed on the root explants (Results, Section 2.3.6.2) were excised after initiation in liquid MS medium with growth regulators and embedded for light microscopy as described by Davey *et al.* (1993), except that osmium tetroxide and uranyl acetate were not used. Explants were fixed overnight at 4°C in 2% (v:v) glutaraldehyde with 2% (w:v) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2). The material was dehydrated through a series of washes with graded ethanol [10, 30, 50, 60, 70, 80, 90, 100% (v:v)]. Dehydrated explants were embedded in LR White acrylic resin (Agar Scientific, UK). Semi-thin sections (2 μm) were cut with glass knives and collected on glass slides. They were dried on a hot plate at 50°C and stained with 0.5% (w:v) toluidine blue in aqueous 0.1% (w:v) sodium tetraborate, prior to microscopic examination.

2.2.11 Cytological studies of cultivated and regenerated plants

Root tip squashing was performed in order to determine the number and structure of *P. hexandrum* chromosomes and indicate the degree of possible somaclonal variation. However, only one regenerant survived acclimation and was available for these studies and consequently, the degree of somaclonal variation could not be assessed. Root tips of seven seed-derived cultivated plants were used as a control for the chromosome counts.

2.2.11.1 Selection and treatment of root tips

Root tips (5.0 - 10.0 mm), white in colour (which indicated active growth) were selected and excised from the regenerated plant (6 months-old) (see Results, Section 2.3.7). This material was collected in a universal bottle filled with 10 ml of reverse-osmosis water. Root tips were blotted dry and a number of them were immediately pre-treated using the procedure described by Jong (1997) with 2.0 mM aqueous solution of 8-hydroxyquinoline (Merck Ltd., Lutterworth, UK) whilst some root tips were left untreated. Treated and untreated root tips were kept at room temperature (20 ± 4°C) in the light for 2.30 h and then fixed in an alcoholic mixture of ethanol:acetic acid (3:1, v:v) and stored in the dark at 4°C.

2.2.11.2 Preparation of root tip squashes

Fixed root tips (treated and untreated) were squashed by two methods:

2.2.11.2.1 Feulgen squash technique

Hot hydrolysis was carried out by transferring fixed root tips into 30 ml screw-topped glass bottles with 10 ml of 1 M HCl pre-heated and equilibrated at 60°C (for 10 min). Hydrolysed root tips were stained in 5 ml Feulgen's reagent (Schiff's reagent; Fox, 1969) (Merck Ltd., Lutterworth, UK) for 1 h at 20 ± 4°C (ambient temperature). Stained root tips were transferred to a microscope slide (Blue Star MicroSlides, Chance Propper Ltd., Warley, UK) containing a drop of 45% (v:v) glacial acetic acid. Root caps (approx. 3.0 mm) containing the dividing cells were excised from the roots, macerated using hypodermic needles and gently warmed.

Slides of *P. hexandrum* root tips from the cultivated plants and from the tissue culture regenerant were examined using phase contrast microscopy (Leica Mikroskopie and System GmbH, Wetzlar DM/LS, Germany) to determine somatic chromosome numbers.

2.2.11.2.2 Aceto-orcein squash technique

Root tips were squashed in one drop of 1% (w:v) orcein in 45% (v:v) acetic acid (for 1 - 2 min) before applying a clean coverslip. Root tissues were dispersed by tapping the coverslip and squashed. Slides of root tips from the tissue culture regenerant were examined using the same phase contrast microscopy procedure as used to determine somatic chromosome counts. This procedure was followed for chromosome counts of root tips from seven cultivated plants (controls). Squashes were permanently fixed by immersing the slides in liquid nitrogen. Slides were then immersed in methanol, air-dried and stored at 4°C.

2.2.12 *Agrobacterium*-mediated transformation of *P. hexandrum*

2.2.12.1 Preparation of explants

Aerial parts (leaf laminae and petioles) were harvested from approx. 200 cultivated seed-derived plants (Section 2.2.1). The leaf lamina were excised from their petioles and both washed under tap water. Leaves were surface-sterilised in 5.0% (v:v) Domestos bleach solution (Appendix 1) with 0.2% (v:v) Tween 20 for 10 min, followed by five rinses with sterile, reverse-osmosis water. Petioles were immersed in 10% (v:v) Domestos bleach solution with 0.2% (v:v) Tween 20 for 20 min and washed as described previously. Explants were blotted dry onto sterile filter paper disks (90.0 mm diam.). After removing the lateral margins, leaf laminae were sectioned into squares (1.0 cm^2 approx.) whilst petiole explants were cut into 1.0 cm lengths.

Explants were also excised from 30 d old axenic seedlings (Section 2.2.3) cultured on full-strength MS medium lacking growth regulators (Appendix 2). The explants were cut into sections of different sizes: cotyledon (8.0 mm in length), hypocotyl (6.0 mm), true leaf (1.0 cm^2) and petiole (6.0 - 7.0 mm). Explants (from cultivated plants and *in vitro* seedlings) were used in transformation with different strains of *Agrobacterium* (Sections 2.2.12.3 and 2.2.12.4).

2.2.12.2 Bacterial strains and plasmids

The strains and plasmids of *A. rhizogenes* and *A. tumefaciens* used in these experiments are listed in Table 2.2. Supervirulence in the *A. rhizogenes* strain R1601 has been attributed to the presence of the plasmid TVK291 (Pythoud *et al.*, 1987) whilst in the *A. tumefaciens* strain 1065 (Curtis *et al.*, 1994) this is due to the presence of the hypervirulent plasmid pTOK47, which contains the virulence genes *virB*, *virC* and *virG* (Jin *et al.*, 1987).

2.2.12.3 Culture conditions and maintenance of *Agrobacterium* strains

Suspension cultures of strain LBA 9402 were maintained on YBM medium (Appendix 2.2) whilst strain 1065 was maintained on LB medium (Appendix 2.2). The other bacterial strains were maintained on APM medium (Appendix 2.2). The antibiotics utilised in the maintenance of the bacteria are listed in Table 2.2. YBM, APM and LB media were made semi-solidified with 1.5% (w:v) agar. The bacterial stock solutions were kept at 4°C (dark).

Overnight cultures of *A. tumefaciens* strain 1065 in LB medium were grown from cultures initiated on semi-solidified LB medium with 1.5% (w:v) agar, 50 mg l^{-1} kanamycin sulphate and 5 mg l^{-1} tetracycline-HCl from glycerol stocks which were maintained in 20% (v:v) glycerol stock at -70°C.

2.2.12.4 Inoculation of explants from cultivated plants with *A. rhizogenes*

Leaf and petiole explants were surface-sterilised (Section 2.2.12.1) and cut into portions (1.0 cm^2 areas and 7.0 mm lengths for leaf and petiole respectively). Leaf explants were wounded with a scapel on their abaxial surface in order to facilitate contact between tissue and *Agrobacterium*. The wounded tissues were incubated, abaxial surface down, (10 explants per dish for both leaf and petiole) in Petri dishes each containing 20 ml full-strength MS medium (Appendix 2). A total of 100 explants from both leaves and petioles were incubated in the dark at 28°C for 2 d (pre-culture period).

Pre-cultured explants were immersed in 20 ml full-strength MS liquid medium (Appendix 2) lacking growth regulators containing two dilutions [1:10 and 1:20 (v:v)] of an overnight suspension culture of *A. rhizogenes* strain R1601 for 20 min. Alternatively, leaf and petiole explants were also inoculated with the same bacterial cell culture with 100 µM acetosyringone for 10 min. Control explants were immersed in MS liquid medium. All explants were blotted dry on sterile filter paper disks (70.0 mm diam.) and transferred to 9.0 cm diam. Petri dishes containing 20 ml full-strength MS medium (Appendix 2) lacking growth regulators. Explants were co-cultivated in the dark at 28°C for 3 d. The explants were washed in MS liquid medium with 500 mg l^{-1} cefotaxime (Claforan, Roussel Laboratories, Uxbridge, UK) and were blotted dry. Post-inoculated explants were transferred to 20 ml full-strength MS medium lacking growth regulators and containing 500 mg l^{-1} cefotaxime. Half of the explants were incubated in the dark and the remainder in the light at 28°C. The inoculated explants were subcultured to full-strength MS medium with cefotaxime every 3 weeks. Ten replicates were carried out for each treatment and experiments were repeated seven times.

2.2.12.5 Inoculation of *in vitro* explants with *A. rhizogenes* or *A. tumefaciens*

Three strains of *A. rhizogenes* (LBA 9402, R1601 and 8196) and three strains of *A. tumefaciens* (C58, T37 and 1065) were used in experiments (Table 2.2). Cotyledon, hypocotyl, true leaf and petiole explants (Section 2.2.10.1) were immersed with each *Agrobacterium* strain in a 1:1 (v:v) mixture of an overnight liquid culture of *Agrobacterium* diluted with liquid MS medium [1:5; 1:10 and 1:20 (v:v)] for a period of 10 and 20 min. Seven explants of either cotyledons or leaves, nine explants of hypocotyls or ten explants of petioles were inoculated per dish. The inoculated explants were blotted dry on sterile filter paper disks (70.0 mm diam.) and transferred to 9.0 cm diam. Petri dishes containing 20 ml MS medium. The dishes were sealed with Nescofilm. For each treatment, three replicate dishes were prepared and the experiments were repeated twice. Cultures were incubated in the dark at 22 ± 1°C for

Table 2.2: Background to the *Agrobacterium* strains used for transformation of *P. hexandrum*

Agrobacterium strain	Plasmid, gene, opine and comments	Maintenance of bacteria	Reference
LBA 9402[a]	pRi1855; Rif derivative of NCPPB 1855; agropine	YBM	Ooms *et al.*, 1985
R1601[a*]	pRiA4b pTVK291 in trans; chimaeric *npt* II cointegrated into TL-DNA; *nos-npt*II-*nos*; agropine; kanamycin-resistant	APM + ampicillin and kanamycin sulphate (50 μ mg l^{-1})	Pythoud *et al.*, 1987
8196[a]	pRi8196; mannopine	APM	Vervliet *et al.*, 1975
C58[b]	pTiC58; nopaline	APM	Holsters *et al.*, 1980
T37[b]	pTiT37; nopaline	APM	Sciaky *et al.*, 1978
1065[b*]	pTOK47; 35S-*gus*-intron; *nos-npt*II-*nos*; *vir*B; *vir*C; *vir*G; nopaline; tetracycline-resistant	LB + 50 mg l^{-1} kanamycin sulphate + 5 mg l^{-1} tetracylin-HCl	Curtis *et al.*, 1994

[a] *A. rhizogenes*; [b] *A. tumefaciens*.
* Supervirulent strain.

2 d. The same number of explants were used for the controls (uninoculated explants). After co-cultivation, all explants (inoculated and uninoculated) were transferred to 9.0 cm diam. Petri dishes containing 20 ml MS medium supplemented with the appropriate antibiotics (Table 2.2).

2.2.12.6 Histochemical assay for the detection of GUS expression

GUS expression in inoculated explants was assayed using a buffer solution containing X-gluc (5-bromo-4-3-chloro-3-indolyl-β-D-glucuronic acid, Gold Biotechnology, St. Louis, USA) (Jefferson *et al.*, 1987). The assay solution used was a modification from Jefferson *et al.* (1987), which consisted of 0.2 M Na_2HPO_4 with 0.2 M NaH_2PO_4 buffer (pH 7.0), 0.5 mg l^{-1} Na_2EDTA, 0.1% (v:v) Triton X-100, 0.1 M $K_4Fe(CN)_6.3HO$, 0.1 M $K_3Fe(CN)_6$ and 0.5 mg l^{-1} X-Gluc which was dissolved in dimethyl formamide. Seven days after co-cultivation with *A. tumefaciens* strain 1065, explants were randomly selected and tested histochemically for GUS activity. The explants were immersed in a freshly prepared X-gluc buffer dispensed in 1.5 ml microfuge tubes. Explants were incubated overnight at 37°C in the dark. After the incubation period, the assay buffer was removed and replaced with 90% (v:v) ethanol. For both cotyledons and leaf explants, the ethanol was replaced every 5 h in order to remove chlorophyll and to improve visualisation of the blue colour. All explants were viewed under a light microscope (Vickers Instruments, Japan) to identify the localisation of the blue colour resulting from expression of the *gus* reporter gene.

2.3 Results

2.3.1 Seed sterilisation

The effectiveness of the two methods of sterilisation of *P. hexadrum* seeds was investigated. After 4 weeks, fungal contamination was observed in most seeds from fruits which had been sterilised with methcol. Seed contamination was also observed with Domestos at the lower concentrations [5, 10 and 15% (v:v)] and for any treatment of less than 20 min. Domestos [20% (v:v)] and 0.2% (v:v) Tween 20 for 20 min was effective for sterilisation.

2.3.2 Axenic culture of plants

2.3.2.1 Seed germination

In all treatments, the radicle emerged after approx. 35 - 40 d, for both stored and fresh seeds with or without pre-treatments. Although seed germination occurred at all temperatures, the extent of the response varied as described in Table 2.3. There was no significant difference between the treatments B, C and D. Seed germination was significantly ($P < 0.05$) lower in treatment A compared to all other treatments. A

normal root system was obtained for seeds (both scarified and non-scarified) stored at 28°C. Treatment C was chosen as the routine procedure for seed germination since the seeds were non-scarified and less prone to contamination.

Table 2.3: Comparison of germination between fresh and stored seeds of *P. hexandrum* subjected to different pre-treatments

Treatment	Germination condition	Pre-treatment	Number of seeds cultured	% germinating seeds (mean ± s.d.)[c]
A	22 ± 1°C[a]	Scarification[A]	70	8.57 ± 0.54
B	28°C[b]	Scarification[B]	30	96.67 ± 5.77
C	22 ± 1°C[a]	Non-scarification[C]	70	94.71 ± 0.77
D	28°C[b]	Non-scarification[D]	150	95.00 ± 0.76

[a] Seeds were sterilised and stored for 30 d (dark) immediately after harvesting from the ripe fruits; [b] Fresh seeds were sterilised and used after harvesting from the ripe fruits; [c] Values are based on the number of replicates per treatment: pre-treatments A and C (n = 7), pre-treatments B and D (n = 3 and n = 15 respectively).

2.3.2.2 Establishment of *in vitro* cultured seedlings

Plant growth on all the media (MRG, BGS and MS) was assessed by visual observation. Axenic seedlings were established on the three media tested. Initially, the two green, enlarged cotyledons were fused along their base. On MRG medium, seedlings exposed to the diffuse light at 22 ± 1°C showed a long tap root and seedling growth was better than those in the dark at 28°C. Axenic seedlings were obtained on both full-strength BGS and MS media, however, growth was better on the latter. The cotyledons were elliptic in appearance with entire margins and displayed fused terminal lobes forming a long cotyledonary tube; the hypocotyl was short and the plumule emmerged as a small subtrifoliate leaf (Plate 2.4.A).

2.3.3 Vernalization

There was no growth response from the eight (dormant) adult plants maintained in the glasshouse following vernalization for 8 weeks. Their underground parts (buds, rhizomes and a highly developed root system) remained dormant. The maintenance of four plants in the glasshouse during winter did not break dormancy.

2.3.4 Establishment and maintenance of callus cultures

Callus induction was assessed visually. Although, the response of root explants from seedlings obtained on MRG medium was slow, 100% of the explants gave rise to callus (Plate 2.2.A). Callusing of root explants was initiated only after 60 d culture on MCG medium. It was not possible to record increases of callus fresh weight since the growth rate was negligible. After three subcultures onto fresh MCG medium, they turned cream coloured or whitish. Some parts of the calli developed a nodular surface indicating embryogenic potential (Plate 2.2.B) even though root explants were still attached. Accumulation of phenolic compounds was observed at the region of contact between callus and medium and in the root explants, though these were left attached to support embryogenesis. Subculturing was undertaken every 4 weeks onto fresh MCG medium. Since the nodular appearance of some calli could be an indication of somatic embryogenesis, they were subcultured in their entirety.

2.3.5 Establishment and maintenance of embryogenic cell suspensions

Friable, embryogenic root-derived callus cultured on MCG medium was dispersed in liquid UM medium. The embryogenic cell suspensions were either cream or brown in colour and the culture medium became slightly cream-coloured. These differences in colour have been already reported (Van Uden *et al.*, 1989) indicating the production of lignans and thus both cell lines were subcultured. Differentiation of the cells started after 90 d. After several subcultures, the differentiated cell cultures were composed of small cell aggregates (approx. 5.0 mm diam.), single, spherical and elongated cells (cytoplasmically dense) and also contained differentiated embryos. The cell cultures retained embryogenic potential for 6 months. When the established cell suspension was filtered through a nylon sieve, single and fused somatic embryos and small aggregates were harvested. Although globular embryos predominated, embryos at different stages of development (late globular, heart- and torpedo-shapes) were also observed. Histological studies (see Results, Section 2.3.8) and attempts at regeneration (see Results, Section 2.3.6) were undertaken with the nodular embryogenic cell aggregates and the somatic embryos produced in UM liquid medium.

The percentage cell viability of embryogenic suspensions, measured 4 d after subculture on UM medium, was 94%.

2.3.6 Plant regeneration

2.3.6.1 Somatic embryogenesis on root-derived callus from cell cultures

2.3.6.1.1 Development and germination of somatic embryos

Somatic embryos obtained from embryogenic root derived-calli in UM liquid medium were cream and light-brown in colour. The surface of 90% calli became smooth, shiny and nodular in appearance after 14 d on calli cultured on MSB3, MSB4 and MSB5 media. Differentiation of somatic embryos started after 30 d on MSB3 or MSB5 media and only in the dark. In contrast, no further development was observed in calli cultured on MSB4 medium although roots were produced. After 30 d, the results observed with calli cultured on MSB media are described in Table 2.4.

Table 2.4: Response of embryogenic calli from root explants cultured on MSB media assessed after 30 days

	GA_3 (mg l^{-1})	
BAP (mg l^{-1})	0	0.97
0	MSB1[5]	MSB2[0]
0.23	MSB3[1,2,4ab]	MSB4[1,3,4a]
0.45	MSB5[1,2,4]	MSB6[5]

[0] = no response; [1] = callus growth; [2] = shoot formation; [3] = root production; [4] = development of somatic embryos ; [5] = callus became dark-brown.
[a] = green; [b] = cream.

There was no response from calli on the MSG media. However, development and germination of somatic embryos was observed when calli were cultured on the BZ media (Table 2.5). Growth of calli was observed after 14 d in all media except in BZ3 medium since some calli became dark-brown. The calli responses after 30 d are described in Table 2.5. At this stage, the percentage of calli giving rise to green shoots was 35% and 20% for calli cultured on BZ2 and BZ4 respectively. This contrasted with the higher germination frequency of somatic embryos that occurred in BZ1 medium (Plate 2.2.D). However, on BZ1 medium, shoot development and growth of embryogenic calli was visually better compared to those cultured on BZ2, BZ3 and BZ4 media. Somatic embryos also occurred on the surface of callus cultured on BZ3 medium but these did not develop further.

After 14 d, calli cultured on the MB media showed distinct colouration differences (green, cream, whitish, pale yellow and light-brown calli). After a further 10 d, even before first subculture somatic embryos at different stages of development were observed on calli cultured on MB5 medium in the dark. They were pale yellow in colour and appeared on the surface of light-brown calli (Plate 2.2.E). After 30 d,

Table 2.5: Response of embryogenic calli from root explants cultured on BZ media assessed after 30 days

	BAP (mg l^{-1})	
Zeatin (mg l^{-1})	0	0.97
0.5	BZ1[1,2,3,4bc]	BZ2[1,2,3,5ac]
1.0	BZ3[1,2,4,5]	BZ4[1,2]

[0] = no response; [1] = callus growth; [2] = shoot formation; [3] = root production; [4] = development of somatic embryos ; [5] = callus became dark-brown.
[a] = green; [b] = cream; [c] = whitish.

greening of somatic embryos was observed on several media (Table 2.6). Production of somatic embryos and subsequent shoot formation only occurred on 42% of calli on MB5 medium in the dark (Plate 2.2.F).

Table 2.6: Response of embryogenic calli from root explants cultured on MB media assessed after 30 days

	GA_3 (mg l^{-1})	
BAP (mg l^{-1})	0	0.97
0	MB1[1,4be]	MB2[0]
0.23	MB3[1,2,3ac]	MB4[1d]
0.45	MB5[1,2,3,4acd]	MB6[5]

[0] = no response; [1] = callus growth; [2] = shoot formation; [3] = root production; [4] = development of somatic embryos ; [5] = callus became dark-brown.
[a] = green; [b] = cream; [c] = whitish; [d] = pale yellow; [e] = light-brown.

Proliferation of somatic embryos on embryogenic root-derived calli was observed, although there was no significant difference between treatments. After 90 d, the responses of the embryogenic calli after transfer from BZ2 and MSB3 media to different media for proliferation/differentiation are summarised in Table 2.7. Rooting (2%) of developed embryos maintained in the dark was obtained in treatment A (Table 2.7) (Plate 2.3.A). Differentiation was observed on BZ1 medium (Plate 2.3.B) although globular somatic embryos predominated. Secondary embryogenesis was only observed in treatment B (Table 2.7). Proliferation of somatic embryos followed by their differentiation was observed in treatment D (Table 2.7) (Plate 2.3.C, D). However, such proliferation only progressed to the globular stage of development.

2.3.6.1.2 Effects of IBA and nod-factor on stimulation of root development

Somatic embryo-derived plantlets from MSB3, MSB5, BZ4 and MB5 media pre-cultured on half-strength MS medium with IBA (0.1 and 0.3 mg l^{-1}) failed to develop roots. Plantlets from MSB5 medium and pre-cultured on full-strength MS medium containing 10^{-5} M nod-factor produced roots (50%) within 35 d after transfer to half-strength MS medium, lacking growth regulators and maintained in the light at 22° ± 1°C. This experiment could not be repeated due to the fact that insufficient numbers of plantlets were available for replication. In contrast, rooting was inhibited for plantlets from BZ4 and MB5 media and cultured on full-strength MS medium lacking growth regulators but supplemented with nod-factor.

2.3.6.2 Organogenesis from root cultures in liquid medium

Root explants cultured on full-strength MS liquid medium with NAA gave rise to adventitious buds after 30 d; root formation was also observed (Table 2.8). These pale yellow, compact structures (4 - 5 per explant) were formed along the entire whole root length (Plate 2.4.B, C). They grew slowly and later developed into plants. Plant regeneration was achieved after 45 d on MS liquid medium with 1.0 mg l^{-1} NAA or no NAA (Plate 2.4.E). Plant regeneration was not obtained with IBA. Optimum *in vitro* regeneration was in MS liquid medium lacking NAA, with 48% of root explants giving rise to at least one plant; 14% regeneration of explants was obtained with 1.0 mg l^{-1} NAA. Root development of these plants was better in medium lacking NAA compared to that in medium with NAA, whereby roots were thinner and shorter. Root cultures failed to undergo regeneration when the NAA concentration was increased to above 2.0 mg l^{-1}.

Table 2.8: The effect of NAA on adventitious bud formation on root explants cultured on MS liquid medium assessed after 30 days

NAA (mg l^{-1})	No. of root explants producing adventitious buds (mean ± s.d.)[1]
0	11 ± 0[a]
0.5	10 ± 0[a]
1.0	11 ± 0[a]
1.5	11 ± 0[a]
2.0	11 ± 0[b]
2.5	No response
3.0	No response

[1] Values are based on 4 replicates per treatment (n = 11 root explants throughout). The experiment was repeated 3 times. [a] = new root formation; [b] = phenolic oxidation.

Plate 2.2: Somatic embryogenesis on root-derived callus of *Podophyllum hexandrum*

A. Pale yellow, friable calli from root explants cultured on MCG medium after 60 d in the dark. (x 0.93).

B. Whitish, nodular embryogenic callus cultured on MCG medium after 90 d in the dark. (x 2.0).

C. First cell division of an embryogenic cell giving rise to a two-celled proembryo in liquid UM medium. (x 1528).

D. Germination of somatic embryos on calli cultured on BZ1 medium after 60 d in the dark and then transferred to the light. (x 1.0).

E. Somatic embryos on light brown calli cultured on MB5 medium after 24 d in the dark. (x 1.9).

F. Shoot formation from somatic embryos on callus cultured on MB5 medium after 60 d in the dark. (x 0.66).

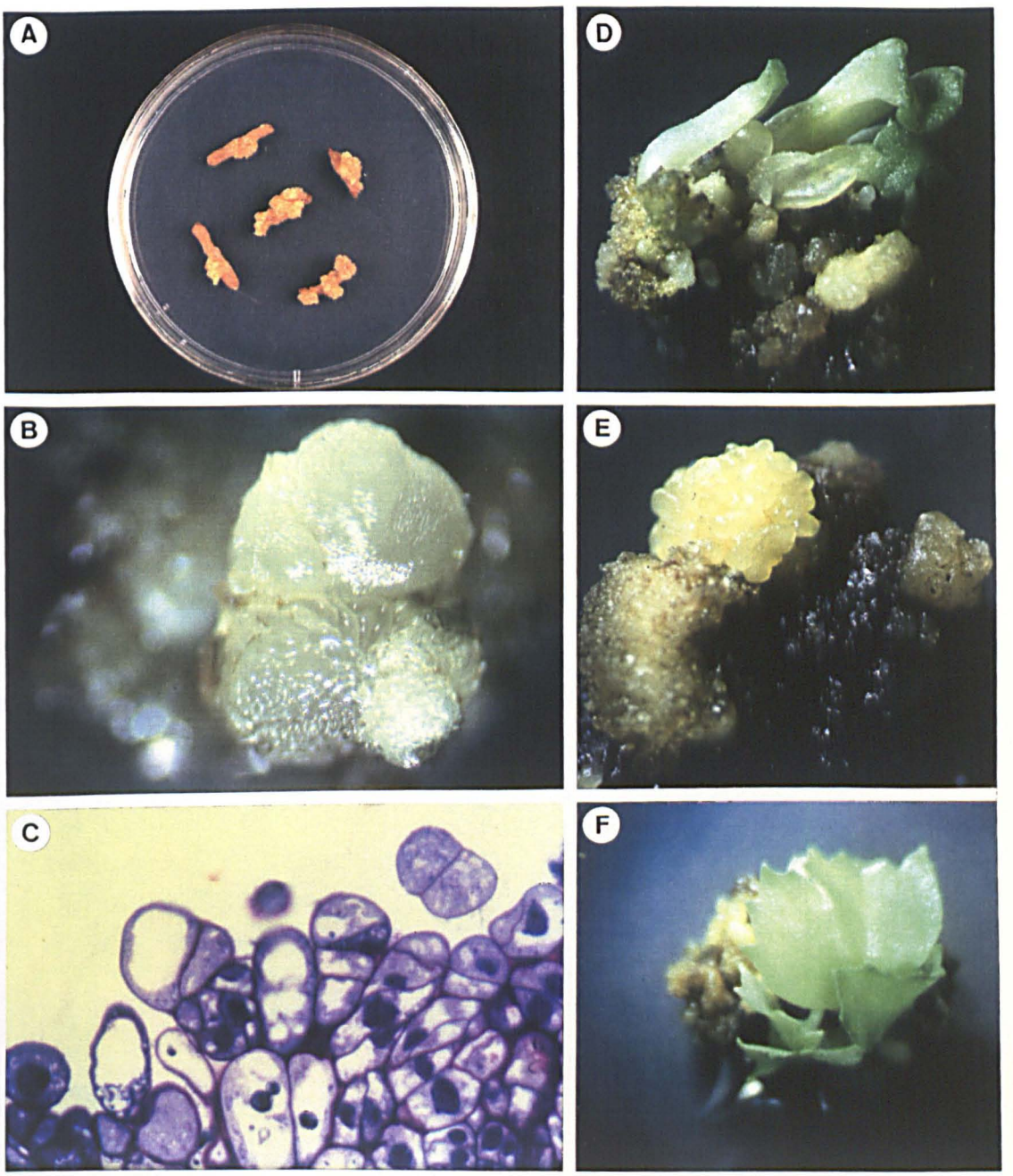

A
D
B
E
C
F

Table 2.7: Effects of exogenous growth regulators on somatic embryo proliferation and differentiation from root-derived calli of *P. hexandrum*

Treatment	Medium designation	Mean of somatic embryos per callus (mean ± s.d.)	Development stage of somatic embryos reached following different treatments			Number of germinated somatic embryos	Number of germinated somatic embryos with roots
			Globular	Heart	Cotyledonar		
A	BZ[1]	17.21 ± 11.43[a]	+ +	+	+ +	12	2
B	BZ1 + 2,4-D[2]	21.56 ± 28.87[b*]	+ +	-	+	-	-
C	MB3[3]	7.62 ± 3.74[c]	+	-	-	-	-
D	MB3 + 2,4-D[4]	16.67 ± 16.60[d]	+ +	-	+	+	-

[1] Addition of 0.5 mg l^{-1} zeatin to full-strength MS medium; [2] Additions of 0.5 mg l^{-1} zeatin and 0.2 mg l^{-1} 2,4-D to full-strength MS medium; [3] Addition of 0.25 mg l^{-1} BAP to half-strength MS medium; [4] Additions of 0.25 mg l^{-1} BAP and 0.2 mg l^{-1} 2,4-D to half-strength MS medium.

[a] (n = 14 embryogenic aggregates); [b] (n = 16); [c] (n = 8); [d] (n = 12).

* Secondary embryogenesis was observed. The number of (+) present and (-) absent signs indicates the response.

Plate 2.3: Effects of growth regulators on differentiation and germination of somatic embryos on root-derived callus of *Podophyllum hexandrum*

A. Germinated somatic embryo cultured initially on BZ2 medium and transferred to BZ1 medium (treatment A) after 90 d. (x 0.8).

B. Differentiation of somatic embryos [(heart, open arrow) and (torpedo, thick arrow)] after culture on BZ1 medium (treatment A) for 90 d. (x 1.8).

C. Embryogenic calli cultured for 90 d on MB3 medium with 2,4-D (treatment D). (x 2.0).

D. A germinating somatic embryo at the cotyledonary stage after 90 d on MB3 medium with 2,4-D (treatment D). (x 2.4).

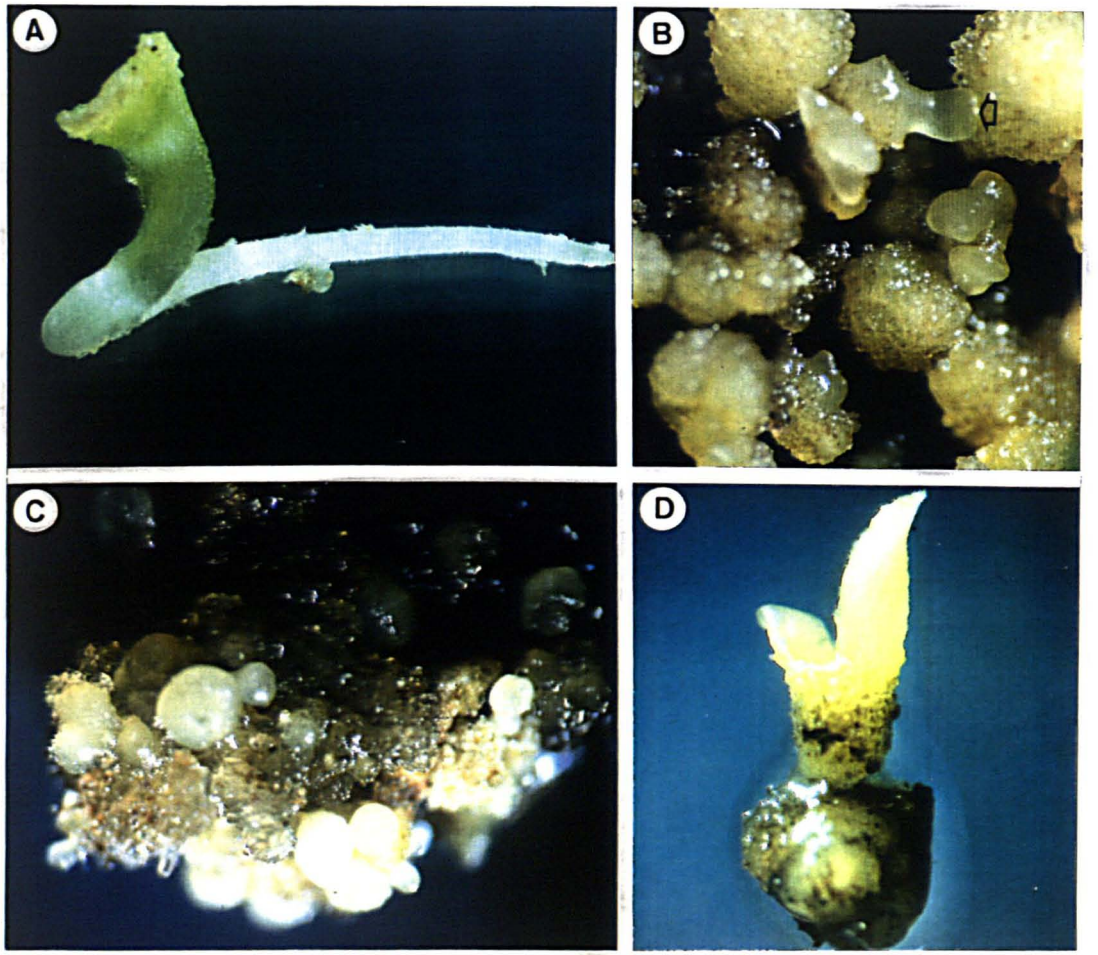

A
B
C
D

Plate 2.4: Plant regeneration via organogenesis from roots of *Podophyllum hexandrum* cultured in MS liquid medium

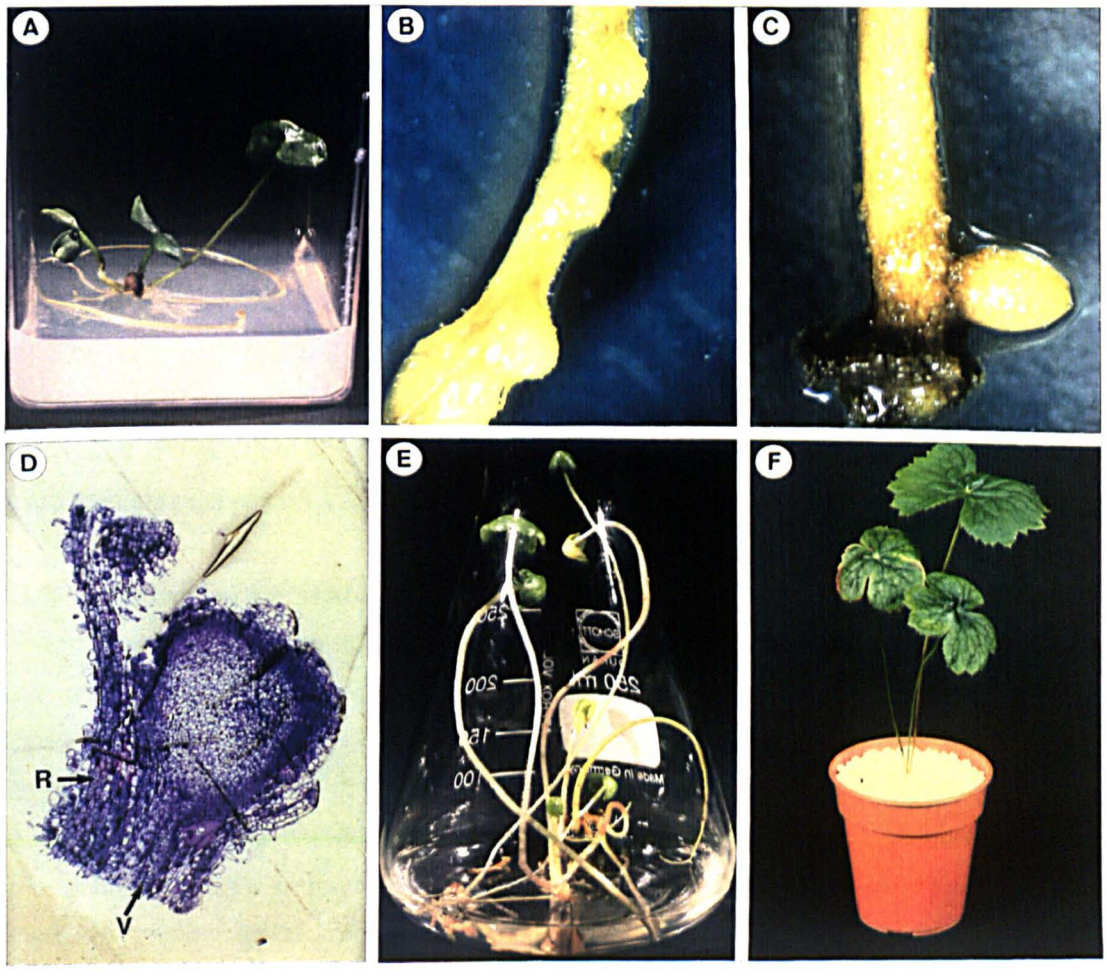
A. An axenic seedling cultured on full-strength MS semi-solidified agar medium lacking growth regulators after 35 d in the light. (x 1.25).

B,C. Adventitious bud formation on root explants cultured on full-strength MS liquid medium lacking growth regulators. (x 8.5).

D. Light micrograph of adventitious bud formation in a root explant (R) cultured on MS liquid medium lacking growth regulators, showing the vascular connection (V) of the bud with the parental tissue. (x 153).

E. Regenerated plants cultured in MS liquid medium lacking growth regulators and maintained under a 16 h photoperiod (25 μmol m^{-2} s^{-1}) provided by "Daylight" fluorescent tubes. (x 1.06).

F. A root explant derived plant 6 months-old after transfer to 9.0 cm diam. pot containing a mixture of Levington M3 soil-less compost, John Innes No. 3 compost and Perlite (6:6:1, v:v:v). (x 0.28).


A
B
C
D
R
V
E
F

2.3.7 *Ex vitro* establishment of regenerated *Podophyllum* plants

After 4 weeks culture on full-strength MS liquid medium without growth regulators and under light conditions, regenerated plants (n = 14) were transferred *ex vitro*. Six months-old plants were normal in terms of height and morphology (Plate 2.4.F) when compared to their seed-derived counterparts. However, only one regenerated plant from the original 14 transferred *ex vitro*, survived in the glasshouse for more than six months.

2.3.8 Histology

Histological studies revealed that the two alternative pathways of regeneration, somatic embryogenesis and organogenesis, were observed in the present work with *P. hexandrum*. Somatic embryogenesis occurred via a callus phase. Light microscopic examination revealed the first division of an embryogenic cell giving rise to proembryos in UM liquid medium (Plate 2.2.C). Organogenesis of roots was observed via bud formation in MS liquid medium. Sectioning of spherical and oval-shaped structures showed the formation of adventitious shoot buds in the root explants cultured on full-strength MS liquid medium. These adventitious bud structures appeared as organ primordia showing initial provascular cells. The occurrence of vascular connections between meristematic centres and the parental tissue was also revealed by histological examination (Plate 2.4.D).

2.3.9 Cytology of cultivated and regenerant *P. hexandrum* plants

The somatic chromosome numbers were determined from actively dividing root tip cells obtained from the cultivated plants and the one regenerated *P. hexandrum* plant. Somatic chromosome numbers for cultivated plants at early anaphase indicated that the *P. hexandrum* germplasm was diploid ($2n = 2x = 12$), confirming published chromosome data (Siddique *et al.*, 1990). The chromosomes were relatively large in length ranging from 9 - 17 μm (Plate 2.5.A). The nucleolar organizer region (NOR) was characterised by four easily identifiable secondary constrictions; one of them appeared to be located in a metacentric (m) chromosome. The cytology of the regenerant did not show any obvious difference compared to the cultivated control seed-derived plants. Exceptions were the incidence of a few abnormalites such as the presence of an extra decondensed chromatin body which was observed at later anaphase in the regenerant *Podophyllum* plant (Plate 2.5.B). The somatic chromosome number analysed was the same for control plants. The NORs were located in two pairs of chromosomes and were observed as two secondary constrictions. In both cultivated and regenerated *P. hexandrum*, chromosomes were easily stained with low background stain (the cytoplasm did not take up stain) and

cells showed a high mitotic index (about 8% for untreated tissues). Root tips with no pre-treatment were used to observe every stage of mitotic division.

It was not possible to obtain cytological observations from other regenerant *P. hexandrum* plants since only one plant remained viable after transfer to compost.

2.3.10 *A. rhizogenes and A. tumefaciens*-mediated transformation

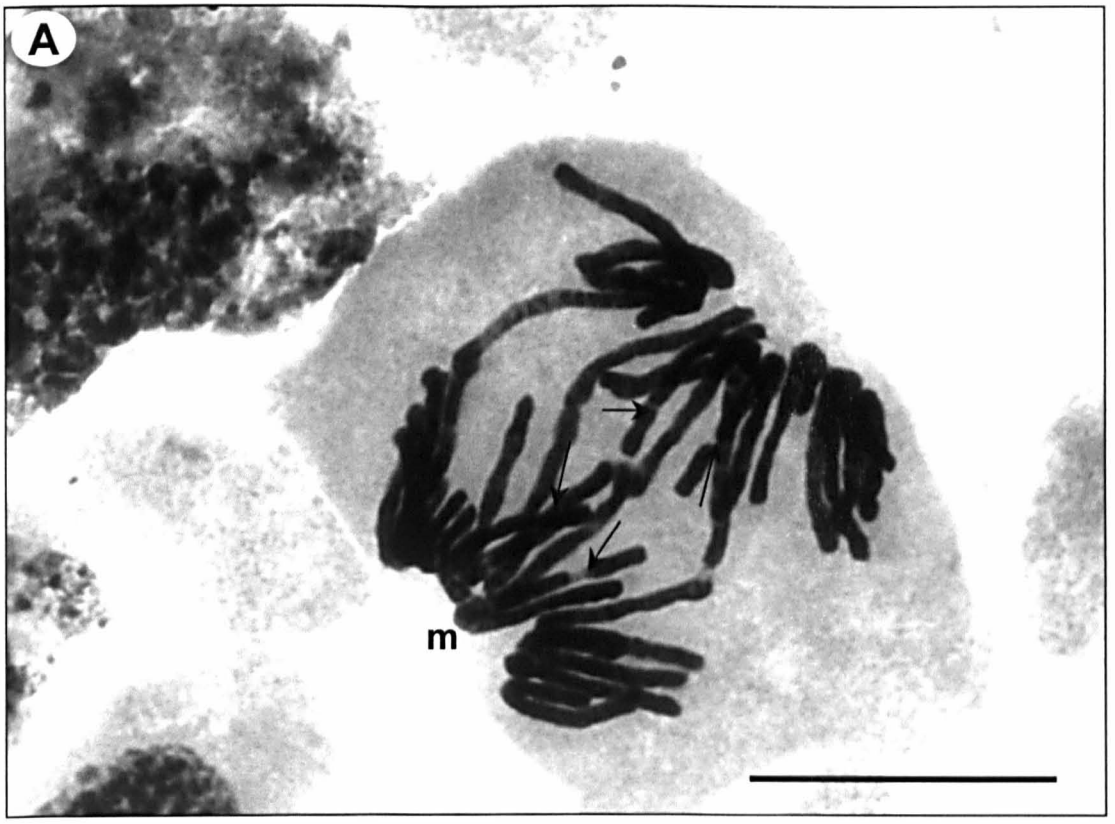
Initial studies, involving the transformation of leaf and petiole explants from cultivated plants, using *A. rhizogenes* strain R1601 alone or in combination with acetosyringone (100 μM) showed that these explants failed to exhibit a response. All inoculated explants produced an abundance of phenolic compounds in culture. Uninoculated leaf explants (controls) remained green whilst uninoculated petiole explants (controls) became brown at their cut surfaces.

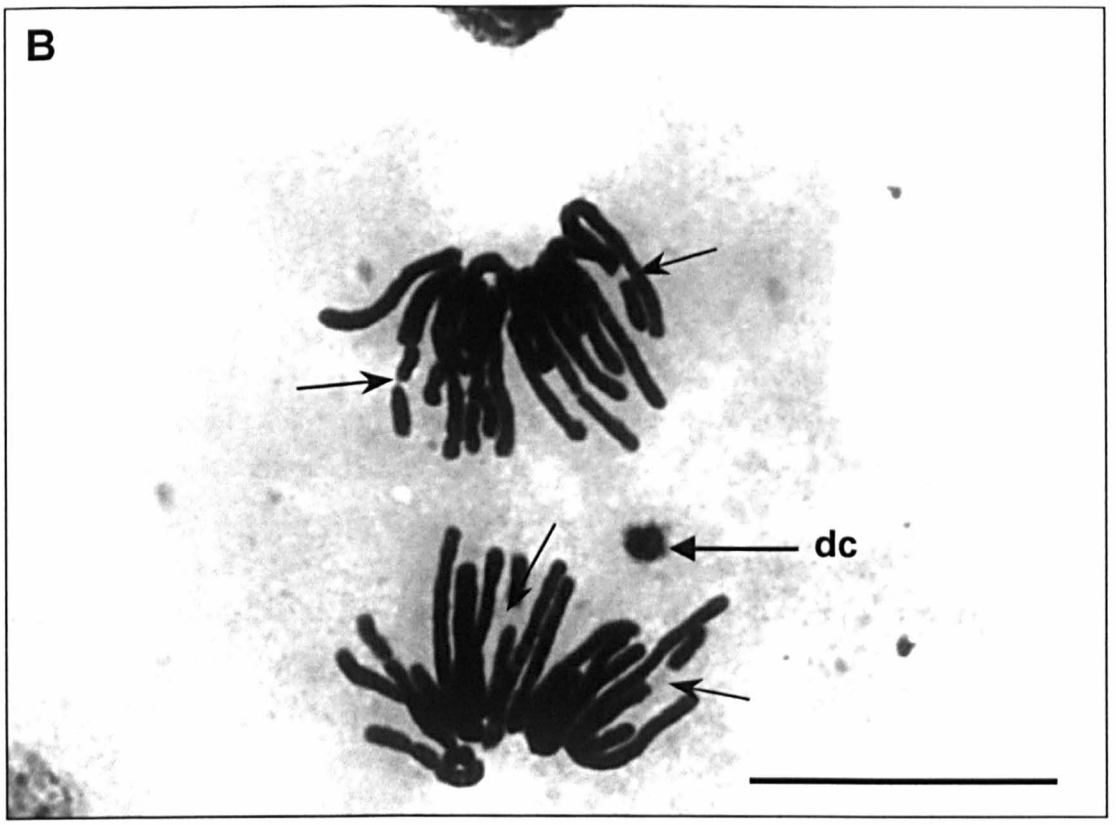
There was no response from the cotyledon, hypocotyl, true leaf and petiole explants excised from *in vitro* seedlings and inoculated with strains LBA 9402, R1601 and 8196 of *A. rhizogenes*. Furthermore, these explants failed to be transformed when inoculated with *A. tumefaciens* strains C58 or T37. Expression (GUS histochemical staining) of the *gus* reporter gene was thus not detected in leaf explants. Although contamination was not observed in any of the explants, the incidence of tissue necrosis was much higher for explants inoculated with the strains of *A. tumefaciens* (up to 90% for hypocotyls, true leaves and petioles and 48% for cotyledons) compared with uninoculated explants (control cultures).

Plate 2.5: Cytogenetic comparison of cultivated and regenerated plants of *Podophyllum hexandrum*

A. Early anaphase in a control seed-derived plant showing a complement of $2n$ = 2x = 12 somatic chromosomes comprising relatively large chromosomes. The preparation was stained with aceto-orcein reagent. Note the metacentric (m) chromosome and the nucleolar organizer regions (NORs, arrows). (Bar = 10 μm).

B. Late anaphase in a regenerated tissue culture-derived plant of *Podophyllum* showing the NORs (arrows) as secondary constrictions in two pairs of chromosomes. The preparation was stained with Feulgen and aceto-orcein reagents. Note the extra decondensed chromatin (dc). (Bar = 10 μm).


A
m


B
dc

2.4 Discussion

The protocol for surface sterilisation of the fruits with methcol (Heyenga *et al.*, 1990) was ineffective. Seed sterilisation was best achieved with 20% (v:v) Domestos and 0.2% (v:v) Tween 20 for 20 min.

P. hexandrum (and *Diphylleia*) species have been reported to show pseudomonocotyledonous seed germination (Shaw, 1996a). Studies focusing on seed germination of high altitude plants have reported that the fruit pulp of *P. hexandrum* contains germination inhibitors and a post-harvesting ripening period is thus required (Nautiyal, 1982). The presence of inhibitors in the seed coat and in the embryo itself has also been reported (Rahman, 1988). During the present study, although there were no significant differences between the treatments tested, a post-harvesting ripening period of 30 d was required for *in vitro* seed germination. Research (Dewick and Shaw, 1988) has shown that germination of seeds extracted from fresh fruits (if they were sown immediately) occurs in early spring after a cold winter. Seeds are highly sensitive to desiccation. Seeds purchased dry have shown a lower germination rate, however, cleaned seeds stored in moist conditions at 4°C retain full viability for at least two years. Several studies have reported a variation in the germination rates between accessions of this species (Nautiyal, 1982; Deno, 1993; Shaw, 1996a). In the present study, scarification was considered unnecessary, in contrast with other reports (Heyenga *et al.*, 1990). In addition and in the context of tissue culture, during scarification the seeds need to be handled carefully in order to avoid contamination. If seeds are stored in moist and dark conditions, spontaneous germination occurs within 35 to 40 d.

P. hexandrum, like other species of the genus *Podophyllum* such as *P. peltatum* and *P. pleianthum*, showed a seasonal availability. To overcome the limited supply of plant material for experiments, axenic cultures were successfully established. In the present studies, axenic seedlings grew either on full-strength BGS medium with growth regulators or full-strength MS medium lacking growth regulators. However, the overall growth of plants showing a normal morphology was superior when germinated seeds were transferred to the latter medium.

Plants maintained in the glasshouse remained dormant. Attempts to break dormancy by transferring a few plants from the glasshouse to a vernalization chamber and then returning them to glasshouse conditions were unsuccessful. This species may require a longer period of vernalization.

In *P. hexandrum*, an initial callus response from root explants on MCG medium has been reported within three weeks of culture (Heyenga *et al.*, 1990). These workers demonstrated that friable callus was associated with the presence of BAP, GA_3 and 2,4-D in the culture medium. In the present study, callus initiation in

MCG medium was very slow and did not occur before two months. This could be due to the fact that the present study utilised a different germplasm. Moreover, callus was friable in texture and did not become embryogenic in appearance until a further 14 d of successive subculture. In *P. hexandrum*, somatic embryogenesis only occurred under dark conditions. This result was consistent with an earlier study of *P. pleianthum* (*Dysosma pleiantha*) (Chuang and Chang, 1987) another medicinal species with lignans within the family *Berberidaceae*. According to this study, one month old embryogenic callus became brown and lost its totipotency when it was transferred to the light. This may be attributed to biochemical changes leading to the production of phenolic compounds. These compounds have been shown to interact with intra- and intercellular metabolism, particularly auxin metabolism. Such alteration of hormonal balances would affect the ability to express cellular totipontecy. These observations would suggest that somatic embryogenesis production within the subfamily *Podophylloideae* occurs only under dark conditions. Somatic embryogenesis *per se* was not affected by browning of the callus tissues. Such browning may be attributed to the presence of quinones derived by the oxidation of phenolic compounds and to several other factors such as the presence of tannins, endogenous compounds which are produced or result from existing biosynthetic pathways, wounding of the tissues, and medium composition deficiencies (Preece and Compton, 1991). It was interesting to note that root-derived callus of *P. hexandrum* tended to become brown (and embryogenic) after several subcultures onto fresh medium. Thus, during subculture régimes, whole brown calli were transferred to fresh medium in order to promote somatic embryogenesis.

In *P. hexandrum*, somatic embryos were successfully produced by embryogenic cell suspensions cultures initiated from root-derived callus, cultured in liquid UM medium under dark conditions. These cell cultures could thus be an important alternative source of lignans for phytochemical studies focusing on the development of high yielding clonal lines for lignan production.

This is the first report of the recovery of somatic embryo-derived plantlets of *P. hexandrum* being successfully rooted in response to the lipo-oligosaccharide NGR234 even though this result was not statistically significant. This compound which is isolated from *Rhizobium* species has been reported to nodulate more than 70 genera of legumes (Price *et al.*, 1992). The lipo-oligosaccharides exhibit the ability to induce nodule organogenesis in roots of legume host plants (Spaink *et al.*, 1991). Several effects, for example initiation of meristematic cell divisions, elicitation of root hair deformation, induction of mitosis and expression of early plant genes have been attributed to these signal compounds (De Jong *et al.*, 1993; Spaink *et al.*, 1998;). Moreover, oligosaccharides can act as elicitors to podophyllotoxin production in

callus cultures of *Juniperus chinensis* (Muranaka *et al.*, 1998). According to the latter, podophyllotoxin increased fifteen-fold by treatment with oligosaccharide. In addition, rhizogenesis of recalcitrant species could be stimulated by the inclusion of the Rhizobial lipo-oligosaccharides in the culture medium [Cocking cited in Babaoglu (1996)]. In the current study, the difficulties of rooting of somatic embryo-derived plantlets is in agreement with a previous report (Arumugan and Bhojwani, 1990). In this latter study, rooting of micropropagated shoots was not achieved. Therefore, the inclusion of lipo-oligosaccharide NGR234 in the culture medium opens a new approach for rooting of somatic embryo-derived plantlets of *P. hexandrum*, a species which shows some recalcitrance in tissue culture.

These studies with *P. hexandrum* confirm that plant regeneration via organogenesis was partly successful. Adventitious bud regeneration was obtained from *in vitro* cultured root explants using full-strength MS liquid medium containing either no or low concentrations of NAA but no IBA. Plant regeneration via root-derived buds of this species was obtained in accordance with field observations of cultivated plants [Selivanova-Gorodkova; cited in Frye (1977)]. Root cuttings *per se* may provide a basis for increased reproduction rates in *P. hexandrum* according to an ecological study carried out with other *Podophyllum* species (*P. delavayi* and *P. mairei*) growing in Omei Shan, China (Zhuang *et al.*, 1993). However, it was not clear from these studies if the authors were referring to root-derived buds from wild species or artificially maintained root cuttings from cultivated rhizomes (Shaw, 1996a). It is important to emphasise that the results described in this chapter represent the first report of direct organogenesis for *P. hexandrum* based on root cultures in liquid medium. This strategy may be used for the *in vitro* conservation of other species in the genus *Podophyllum*.

The cytological studies showed that the somatic chromome number was $2n = 2x = 12$ for the cultivated (control) plants. These studies revealed the presence of large chromosomes in this species in accordance with other reports (Siddique *et al.*, 1990). The occurrence of the NORs, represented by secondary constrictions, was also consistent with other studies of *P. hexandrum* (Kosenko, 1979; Siddique *et al.*, 1990). In the sole regenerant of *Podophyllum*, the mitotic index (from untreated tissues) indicated a high percentage of cells in a population undergoing mitosis. Cytologically, the regenerant showed that only a few chromosomes were damaged; however, only a single plant was analysed. The occurrence of structural and numerical chromosome variations in a regenerant of this species has been already reported (Arumugan and Bhojwani, 1994) and was attributed to the growth regulators used in particular 2,4-D, which was the growth regulator of choice in both studies.

The present studies demonstrate that *P. hexandrum* could not be transformed using the *Agrobacterium* strains evaluated. This result highlights the recalcitrance of *Podophyllum* species towards transformation, a conclusion supported by the fact that to date there has been no published reports either of successful transformation or production of transgenic plants within this genus. In the present studies, the addition of 100 μM acetosyringone in order to theoretical enhance virulence of *A. rhizogenes* strain R1601 failed to produce hairy roots or perhaps this strain was not sufficiently virulent. This could be also attributed to the state of tissue that was to be infected, genotype, conditions of tissue culture, inoculation, co-cultivation time and vectors used (Hiei *et al.*, 1997; Vergauve *et al.*, 1998). The non-responsiveness of *P. hexandrum* to Agrobacteria infection may also be caused, in part, by the presence of the aryltetralin lignans in this species leading to an inactivation of the Agrobacteria (Alfermann, 1999).

Although important results focusing on the tissue culture of *P. hexandrum* have been described in this chapter, more studies are needed in order to overcome several problems encountered, such as rooting of shoots and improved maintenance of plantlet development post transfer to the soil.

Chapter Three

Establishment of *Diphylleia cymosa* Tissue Cultures

3.1 Introduction

Leaves and rhizomes of *Diphylleia* species provide an alternative source of natural lignans and some have clinical value (see Chapter 4, Section 4.1.2). Despite the medicinal importance of *Diphylleia* species, they are still collected in the wild although *D. cymosa* has already been suggested for commercial cultivation (Broomhead and Dewick, 1990).

Within the subfamily *Podophylloideae* containing *Diphylleia* and *Podophyllum* species, studies here have focussed only on the response of the latter in tissue culture (Chapters 1, Section 1.4.1 and 2, Section 2.1). No work concerning the tissue culture of the three species of *Diphylleia* (*D. cymosa*, *D. grayi* and *D. sinensis*) has been published to date. This chapter is directed towards the development and establishment of a tissue culture system for *D. cymosa*. Outcomes of these studies provided the starting material used for the assessment of lignans (Chapter 5, Section 5.2.1).

It is noteworthy that the novel protocols described in this chapter are the first studies carried out on the tissue culture of *D. cymosa*.

3.1.1 *In situ* and *ex situ* conservation of *Diphylleia* germplasm

Diphylleia species grow in areas under protection such as the national forests and national parks in North America and Japan (Ying *et al.*, 1984). In the wild, the North American species, *D. cymosa*, is restricted to the Appalachian mountains but is not considered to be an endangered species. The Asian species *D. grayi* and *D. sinensis* grow on the mountains in Japan and China (Foster, 1989). *D. sinensis* is becoming scarce owing to destruction of its natural habitat and over-collection for medicinal use by the local population (Ying *et al.*, 1984). Since there are no published reports, to date, referring to cell and tissue culture of *Diphylleia*, it is increasingly important to collect and evaluate the wild genotypes, which are traditional sources of genetic variability, in order to preserve these gene pools for future research. *Ex situ* conservation for *Diphylleia* species has been carried out on a small scale at botanic gardens, such as the Royal Botanic Gardens of Edinburgh (Scotland), where one accession of *D. cymosa* and two of *D. grayi* are grown (Walter *et al.*, 1995). The Gardens of the Blue Ridge (North Carolina, USA), a commercial horticultural nursery, has its own stock of *D. cymosa*. In an effort to conserve cultivated stocks of species of *Diphylleia* in Britain, the NCCPG (Chapter 2, Section 2.1.1) has set up

three national collections in 1996 (Shaw, 1999). One important *ex situ* method of conservation which has not yet been exploited for *Diphylleia* species is via tissue culture. In this study, the first attempts to establish tissue culture of *D. cymosa* were thus undertaken.

3.1.2 Applications of non-ionic surfactants in plant cell cultures

Over the past 30 years, several studies have focused on the role of non-ionic, co-polymer surfactants as protecting and stimulating-agents of cultured animal cells (Handa-Corrigan *et al.*, 1992; Wu, 1995). Recently, they have been exploited, at low concentrations, in order to improve the growth of cells, protoplasts, organs and tissues in plant cell cultures of trees and species of medicinal, ornamental and agronomic importance (Lowe *et al.*, 1994). Among them, Tween 20 and *Pluronic*® F-68 have been reported to stimulate shoot production from seedling cotyledons resulting in phenotypically normal plants of *Corchorus capsularis* (jute), (Khatun *et al.*, 1993a). Tween 20 is a permeabilisation agent for tropane alkaloids derived from *Datura innoxia* hairy roots (Boitel-Conti *et al.*, 1995). After treatment with this surfactant, transformed roots released more hyoscyamine and scopolamine in the culture medium compared to control cultures. *Pluronic*® F-68 (Poloxamer 188) is formed by a central, hydrophobic poly(oxypropylene) group in which hydrophilic poly(oxyethylene) groups are linked in the two laterals (Lowe *et al.*, 1993). *Pluronic*® F-68 is the most widely used non-ionic surfactant. Several properties have been attributed to this surfactant such as the stimulation of shoot regeneration in leaves of *Chrysanthemum morifolium* (Khehra *et al.*, 1995) and hypocotyls of *Eucalyptus urophylla* (Tibok, 1995). Furthermore, *Pluronic*® F-68 has been shown to enhance pigmentation production such as anthocyanin in leaf-derived callus of *Hypericum perforatum* (St. John's Wort), (Brutovská *et al.*, 1994) and also to release intracellular anthraquinones from cell suspensions of *Morinda citrifolia* (Bassetti *et al.*, 1995). These findings emphasize the potential use of *Pluronic*® F-68 in plant biotechnology in particular the production, release and/or biotransformation of secondary metabolites. In addition, enhancement of T-DNA delivery for genetic transformation of *Triticum aestivum* (wheat) has been, in part, attributed to the presence of *Pluronic*® F-68 in the inoculation medium, leaving cells free of surface-tension which promotes conditions for *A. tumefaciens* attachement (Cheng *et al.*, 1997). The cryoprotectant properties of *Pluronic*® F-68 reported for embryogenic cells cultures of *Oryza sativa* (Japonica rice), non-embryogenic cells of *Lolium multiflorum* and *Moricandia arvensis* involving the increase of their post-thaw cell viability and biomass (Anthony *et al.*, 1996; 1999b; Craig *et al.*, 1997) could provide an alternative strategy for increasing

cell recovery from cryostorage and for culturing cells which are unresponsive to post-thaw protocols.

Various mechanisms have been proposed for the interaction of *Pluronic*® F-68 with plant cells although more research is necessary. One explanation is that the surfactant may increase membrane permeability and therefore increase the uptake of nutrients and/or growth regulators and oxygen into cultured cells, tissues and organs. It may also alter carbon dioxide release from cells (Lowe *et al.*, 1993). Moreover, biochemical changes such as the alteration of metabolic enzymes would allow biochemical pathways to operate more efficiently thereby conferring the growth-stimulating effects of *Pluronic*® F-68.

Experiments with *Pluronic*® F-68 and Tween 20 were undertaken in order to investigate whether, the stimulation of callus growth of *Solanum dulcamara* cultured with surfactant (Kumar *et al.*, 1991), could be extended to leaf-derived callus of the species *D. cymosa*.

3.1.3 Cryopreservation of plant genetic resources

The last decade has seen an ever-growing international awareness of the value of germplasm conservation (Chapter 1, Section 1.1.4). There is an urgent need for the *in vitro* conservation of germplasms from rare, elite and endangered species, in particular those of recalcitrant types whose seed cannot be preserved due to sensitivity to desiccation (Bajaj, 1995). Furthermore, *in vitro* conservation can be applied to vegetatively propagated species and material modified through molecular biology technologies (Grout, 1990a). The options for *in vitro* conservation may be categorized into slow-growth systems and cryopreservation (Monette, 1995). In the former category, conservation is long-term but with the risk of an high incidence of somaclonal variation (Monette, 1995) whilst cryopreservation offers long-term preservation of valuable germplasm and prevents genetic variability (Kartha, 1987).

Cryopreservation is a relatively new technique and involves the non-lethal storage of biological materials at ultra-low temperatures (Benson, 1994) in liquid nitrogen. The low temperature (-196°C) of liquid nitrogen, at least in theory, suppresses chemical reactions in the cells and provides genetic stability (Grout, 1990b). However, macromolecular damage and formation of free radicals can occur at -196°C which influence stability and cell survival (Grout, 1995). Techniques of plant cryopreservation involve either slow cooling or vitrification procedures (Grout, 1995). The protocols for slow cooling comprise the following stages: pre-growth, pre-treatment with chemical cryoprotectants, freezing, storage, thawing (or warming), post-thawing and recovery (Withers, 1985; Seitz, 1987). Chemical cryoprotectants are classified as penetrating or non-penetrating (Benson, 1994) and are added to the

freeze mixture to prevent cell damage during the freezing process. There is a wide range of cryoprotectants; the most common of which are dimethyl sulphoxide (DMSO), glycerol, sucrose and methanol, all classified as penetratives (Grout, 1995; Dawson, 1992). Cryoprotectants need to have high solubility in water and low toxicity to cells (Dawson, 1992) and are more effective as combined mixtures at low concentrations than as single cryoprotectants (Bajaj, 1991). Preservation of viability depends on the ability to reduce the stresses of cryopreservation (Grout, 1995) and thus, viability assays are needed, after thawing, to assess the effectiveness of the protocol employed. These include analysis of growth and dye assays such as reduction of triphenyl tetrazolium chloride (TTC) and FDA. These are useful assays for assessing viability and stability in callus and cell suspensions (Towill, 1991), while the post-thawed regrowth is the best indicator of recovery (Benson, 1994). Furthermore, biochemical and molecular analyses have been employed to assess genetic stability of cryopreserved cultures (Harding and Benson, 1995).

Cryopreservation technology has been applied to all types of plant cell systems such as protoplasts, callus, cell suspensions, tissues, organs, embryos and plantlets (see review by Withers, 1985; Razdan and Cocking, 1997) from herbaceous and woody species. Cryopreserved callus of *Lavandula vera* (lavender) has retained its capacity for biotin production and differentiation potential (Watanabe *et al.*, 1983) and while cell cultures have been successfully cryopreserved (Kuriyama *et al.*, 1990), some lines have shown sensitivity to ammonium ions (Kuriyama *et al.*, 1996). Cryopreservation of callus of *Chamomilla recutita* has also been reported (Cellárová *et al.*, 1992). Earlier studies using *Digitalis lanata* have already demonstrated that 70% of shoot tips survived cryopreservation and 30% regenerated shoots (Diettrich *et al.*, 1986). After cryopreservation, hairy root cultures have been shown to retain secondary metabolite production such as artemisinin in *Artemisia annua* (Teoh *et al.*, 1996) and ginsenoside (saponins) in *Panax ginseng* (Yoshimatsu *et al.*, 1996). Furthermore, post cryopreservation studies have shown that a diverse spectrum of species such as *Atropa belladona*, *Datura innoxia* and *Nicotiana tabacum* (Bajaj, 1988) and other species have been successfully regenerated (for references see Anthony *et al.*, 1998).

3.1.3.1 Cryopreservation of cell cultures from medicinal plants

Plant cell cultures of medicinal plant species are an unlimited source of primary and secondary metabolites, enzymes and novel products (Alfermann and Petersen, 1995). Therefore, their long-term storage is important for the conservation of germplasm and for the extraction of target compounds. Although cell suspensions are an excellent source of natural products of commercial interest, the problem of

genetic and biosynthetic instability may arise after continuous subculture and their long-term utilisation (Mannonen *et al.*, 1990). In order to overcome these problems, cryopreservation has been used to establish a safe repository of cell cultures which can be re-introduced into culture after thawing and recovery (Benson and Hamill, 1991). Additionally, through this method it may be possible to reduce the costs of storage and maintenance of cultures under standard growth conditions and to provide insurance against the risk of contamination during the periodical subcultures (or loss by equipment failure).

Suspension cultures that produce secondary metabolites have been cryopreserved following standard protocols (Towill, 1991) (Section 3.1.3); some results are summarised in Table 3.1. After cryopreservation, many cell lines retain their biosynthetic potential with or without the same patterns of genetic integrity (Benson and Hamill, 1991; see also Table 3.1). Cryopreserved embryogenic cell suspensions of *D. lanata*, after three years, retain their ability to undergo biotransformation whilst stability of DNA content, growth rate and embryogenic capacity are unaltered (Diettrich *et al.*, 1985).

Table 3.1: Cryopreservation of cell suspension cultures from medicinal plants

Species	Biosynthetic potential	Reference
Anisodus acutangulus	Synthesis of hyoscyamine and scopolamine	Zheng *et al.*, 1983
Catharanthus roseus	Stable synthesis and accumulation of alkaloids	Chen *et al.*, 1984
	Recovery of indole alkaloids	Mannonen *et al.*, 1990
Chrysanthemum cinerariaefolium	High pyrethrin biosynthesis	Hitmi *et al.*, 1997
Digitalis lanata	Preservation of biotransformation (cardenolides) capacity	Seitz *et al.*, 1983; Seitz, 1995
Dioscorea deltoidea	Retained composition and quantity of steroids	Popov *et al.*, 1995
Lavandula vera	Retained the biosynthetic ability to synthesize biotin	Watanabe *et al.*, 1985; Tanaka and Takahashi, 1995
Panax ginseng	Ginsenosides production	Mannonen *et al.*, 1990
Papaver somniferum	Increased sanguinarine production	Friesen *et al.*, 1991

It is particularly important to determine optimum conditions to preserve high producing cell lines (Friesen *et al.*, 1991) therefore this aspect of cryopreservation requires ongoing investigation.

3.2 Materials and methods

3.2.1 Plant material

This study utilised plant germplasm maintained at the University of Nottingham (Plate 3.1.A). The identity of *D. cymosa* plants was confirmed by JMH Shaw (Department of Pharmaceutical Sciences, University of Nottingham). Plants were originally collected from the wild and propagated clonally by division of rhizomes at the Gardens of the Blue Ridge (North Carolina, USA) and sent to the University of Nottingham. Rhizomes (approx. 3 to 4 years old) were planted individually in 10.0 cm diam. pots containing a mixture of Levington M3 soil-less and John Innes No. 3 composts (2:1, v:v) and maintained in a cold frame to protect from frost and rain since rhizomes were susceptible to rotting if kept too wet and when dormant. It was decided, for all experiments, that the explants would be harvested for the tissue culture experiments and analysis of lignans at the same time in the morning in order to encourage comparable results, as in other systems it is known (Evans, 1996) that there can be changes in amount and/or composition of natural products in the different organs of the plant.

3.2.2 Preparation of explants

3.2.2.1 Surface sterilisation of leaves and petioles

The entire aerial part of the petiole was excised together with the leaf laminae from about 20 plants. Two different leaf and petiole ages were used: 10 d old and 28 - 35 d old (measured from shoot emergence above ground) respectively. The leaf lamina was excised from their petioles and both explants were washed briefly under tap water. Leaves were surface-sterilised in 5.0% (v:v) Domestos bleach solution (Appendix 1) with 0.2% (v:v) Tween 20 for 10 min, followed by five rinses with sterile, reverse-osmosis water, the last two washes of which were of 10 min duration, to ensure that no Domestos remained. Petioles were immersed in 10% (v:v) Domestos bleach solution with 0.2% (v:v) Tween 20 for 20 min, and rinsed as described above. All surface-sterilised explants were blotted dry onto sterile filter paper disks (90.0 mm diam.). Some leaves and petioles were stored into 14.0 cm diam. Petri dishes for 6 months at -70°C for later analysis of lignan content (Chapter 4).

Leaf laminae were cut transversely into strips removing the lateral margins. The strips showing the central vein were cut into squares (1.0 cm^2) and placed with their abaxial surfaces on to the medium surface. Approximately 1.0 cm of tissue was

removed from each end of the petiole explants and sections (1.0 cm in length) were then placed horizontally onto the medium (Section 3.2.3.1).

3.2.3 Assessment of growth regulators and basal media

3.2.3.1 Callus cultures

Studies were undertaken to assess media requirements and growth conditions for callus induction and culture. These experiments focused on the initiation of callus from different explants and the subsequent maintenance of callus *in vitro*. Such defined conditions could then be applied to the development of an effective and reproducible protocol for the tissue culture of *D. cymosa*. Leaf and petiole explants were tested for their responses to different growth regulator régimes.

Leaf and petiole explants were initially tested for callus initiation on full-strength B5 medium (Appendix 2) with various concentrations of growth regulators: 2,4-D (0, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 mg l^{-1}) designated B5D media.; 2,4-D (0, 0.25, 0.5, 1.0 and 2.0 mg l^{-1}) in combination with BAP (0, 1.0, 2.0, 3.0 and 4.0 mg l^{-1}) designated BD media; NAA (0, 0.25, 0.5, 1.0 and 2.0 mg l^{-1}) with BAP (1.0, 2.0, 3.0 and 4.0 mg l^{-1}) designated BN media.

A second series of experiments was also carried out to attempt callus induction from leaf and petiole explants cultured on full-strength MS medium (Appendix 2) with various concentrations of growth regulators: 2,4-D (0, 0.1, 0.5, 1.0 and 2.0 mg l^{-1}); designated MSD media; BAP (0, 1.0, 2.0, 3.0, 4.0 mg l^{-1}) designated MSB media; BAP (0, 0.5, 2.0 and 3.0 mg l^{-1}) in combination with NAA (0, 1.0, 2.0 and 3.0 mg l^{-1}) designated MSBN media. The auxin picloram was assessed at five different concentrations (4.8, 7.2, 9.6, 12.1 and 14.5 mg l^{-1}) and was included in the MS media and designated MSP media (Appendix 2). MS medium supplemented with 2.0 mg l^{-1} 2,4-D (UM medium; Appendix 2) was also tested for callus initiation.

Media were supplemented with 3.0% (w:v) sucrose and solidified with 0.8% (w:v) agar, pH 5.7. Media (20 ml) were dispensed into 9.0 cm diam. Petri dishes. Ten and five explants were cultured per dish for leaves and petioles respectively. Each treatment consisted of seven replicates and the experiments were repeated at least three times. Petri dishes were sealed with Nescofilm and cultures were maintained in the dark for 4 weeks (22 ± 1°C).

3.2.3.2 Effects of CPPU (forchlorfenuron) and 2,4-D on embryogenesis

The effects of CPPU as the sole growth regulator and CPPU in combination with 2,4-D in the induction of direct embryogenesis in leaf and petiole explants was also investigated. Leaves (10 d old) and petioles were surface-sterilised (Section 3.2.2.1) and explants were transferred to 9.0 cm Petri dishes containing 20 ml of MS

medium with CPPU only (0.24 and 0.5 mg l^{-1}) or 0.24 mg l^{-1} CPPU in combination with 2,4-D (1.0 and 2.0 mg l^{-1}). Ten and five explants were cultured per dish for leaves and petioles respectively and each treatment was replicated twice. Cultures were maintained under the same grown conditions as described in Section 3.2.3.1.

3.2.3.3 Effects of TDZ (thidiazuron) on organogenesis

Leaf and petiole explants were prepared and cultured as described in Section 3.2.3.2 on MS media with TDZ (0.1, 0.01 and 0.001 mg l^{-1}).

3.2.4 Regeneration of somatic embryos

Experiments involving partial desiccation, replacement of agar with agarose, dark and light régimes and variations in auxin and cytokinin concentrations were undertaken in order to promote development and germination of somatic embryos formed on leaf and petiole-derived calli in turn produced on different media (see Results, Section 3.3.5.1).

3.2.4.1 Somatic embryogenesis on leaf-derived callus

3.2.4.1.1 Development of somatic embryos originated on UM medium

Callus exhibiting somatic embryogenesis and originally initiated and cultured on UM medium was transferred to 5.5 cm diam. Petri dishes containing 10 ml of MS medium lacking growth regulators. Three Petri dishes were used as replicates (two calli per dish). Cultures were incubated in the dark (22 ± 1°C) for 6 d. Browning of embryos prompted their transfer to half-strength MS medium without growth regulators (see Results, Section 3.3.5.2.1). Cultures were maintained with a 16 h photoperiod under fluorescent light (30 µmol m^{-2} s^{-1}) at 22 ± 1°C for 8 weeks.

Somatic embryos appeared on leaf-derived calli after 10 - 11 subcultures on UM medium (see Results, Section 3.3.5.1). Regeneration was attempted by transferring these calli, with embryos, to MS medium with half-strength nitrate (Appendix 2). The medium (10 ml) was dispensed into 5.5 cm Petri dishes and two calli were placed in each dish. Cultures were maintained in the dark for 4 weeks and then transferred to the light under the same growth conditions as described above.

3.2.4.1.2 Development of somatic embryos originated on MSP media

These experiments were conducted in order to study the effects of BAP with NAA, zeatin, a high sucrose level and GA_3 on the differentiation and germination of somatic embryos formed on leaf-derived calli on MSP2 and MSP3 media (see Results, Section 3.3.5.1). Calli bearing globular embryos were transferred to half-strength MS medium containing 1.0 mg l^{-1} BAP in combination with 0.001 mg l^{-1} NAA; MS

medium with 1.0 mg l^{-1} zeatin and to half-strength MS medium lacking growth regulators. Ten ml aliquots of media were dispensed into 5.5 cm Petri dishes with two calli per dish. Cultures were kept in the dark for 15 d and then transferred to a 16 h photoperiod under fluorescent light (30 μmol m^{-2} s^{-1}) for 7 d.

Germination of these embryos was not obtained on half-strength MS medium (see Results, Section 3.3.5.2.2). These calli with embryos were thus transferred to different media with ABA and agarose (Sections 3.2.4.1.3 and 3.2.4.1.4). Cultures on half-strength medium supplemented with BAP and NAA gave enlargement of somatic embryos, however, no fully differentiated embryos were obtained (see Results, Section 3.3.5.2.1). Embryo differentiation was attempted by transfer to 10 ml of high-sucrose N6 medium (Appendix 2) dispensed into 5.5 cm Petri dishes. After the medium had solidified, a small aliquot (100 μl of a 0.5 mg l^{-1} GA_3 stock solution) was spread at the centre of the medium surface and one callus, with embryos (from a previous treatment with BAP and NAA), was placed per dish. A sterile cotton bud was used to spread the GA_3 solution on to the top of the embryos. In addition, controls (callus bearing embryos formed on MSP3 medium which were not pre-cultured in the presence of BAP and NAA) were prepared. The experiment consisted of three replicates and was repeated twice. All cultures were maintained in the dark for 7 d and then transferred to 16 h photoperiod under fluorescent light (30 μmol m^{-2} s^{-1}) for a further 7 d. Subsequently, callus was transferred to Petri dishes containing high-sucrose N6 medium but lacking GA_3. Cultures were kept as described above at 22 ± 1°C for 28 d.

3.2.4.1.3 Effects of ABA and partial desiccation on embryogenesis

Embryogenic calli and somatic embryos cultured on half-strength MS medium were subjected to partial desiccation in order to study such effects on embryo germination. The presence of ABA combined with a high sucrose level was also evaluated. Embryogenic calli (3 pieces; 8.0 mm diam.) and somatic embryos (n = 6), were transferred directly on to a sterile filter paper disk (70.0 mm diam.) placed in an empty 9.0 cm diam. Petri dish. Cultures were left for 10 h under light at room temperature. After treatment, the partially desiccated calli and embryos were gently placed on MS6 medium (Appendix 2) supplemented with ABA (0.1 or 0.2 mg l^{-1}). During the first 15 d, cultures were kept in the dark and then moved to the light after subculture on the same medium but without ABA. Controls were also set up that lacked ABA. Two replicates were used for each treatment.

3.2.4.1.4 Effects of ABA and agarose on embryogenesis

Embryogenic calli cultured on half-strength MS medium or pre-cultured on the same medium supplemented with BAP and NAA were used to study the effects of agarose combined with ABA on embryo germination. A two-step protocol was carried out by transferring the calli (3 pieces of 8.0 mm diam.) to 5.5 cm Petri dishes containing MS medium supplemented with ABA (0.1 or 0.2 mg l^{-1}) and semi-solidified with 1% (w:v) Sea Kem Le agarose (FMC Corporation, USA). Cultures were incubated in the dark for 7 d and transferred to the light for another 7 d. Calli were transferred to MS medium, semi-solidified with 0.4% (w:v) agarose and incubated in a 16 h photoperiod under fluorescent light (30 µmol m^{-2} s^{-1}) at 22 ± 1°C for 4 weeks. Two replicates were used for each treatment. Three subcultures (every 3 - 4 weeks) on MS medium lacking ABA, semi-solidified with 0.4% (w:v) agarose were performed over a period of 11 weeks.

3.2.4.1.5 Effects of GA_3 on somatic embryo development

Germinated embryos and embryo-like structures cultured on half-strength MS medium semi-solidified with 0.4% (w:v) agarose (see Results, Section 3.3.5.2.3) were transferred to 5.5 cm Petri dishes containing 10 ml half-strength MS medium supplemented with 0.5 mg l^{-1} GA_3 and kept in the light. Embryos were individually transferred onto 40 ml half-strength MS medium lacking growth regulators and with 0.2% (w:v) sucrose and solidified with 0.2% (w:v) Phytagel (Sigma, UK) in 175 ml screw-capped jars (Beatson Clarke and Co. Ltd., Rotherham, UK).

3.2.4.2 Somatic embryogenesis on petiolar callus

3.2.4.2.1 Effects of zeatin

Calli, with embryogenic structures, formed on petiolar explants cultured on BN medium (see Results, Section 3.3.1.3), were utilised to study the induction of somatic embryogenesis. Compact or frosted calli cultured on BN15, BN16, BN17, BN18 and BN20 media were individually transferred to 40 ml MSZ medium in 175 ml screw-capped jars. The petioles were left attached to the individual callus portions so as to possibly promote embyrogenesis. Petioles were placed horizontally on the surface of the medium in such way that the calli were in direct contact with the medium. Cultures were maintained in the dark (22 ± 1°C) for 3 weeks and then cultured in a 16 h photoperiod under fluorescent light (30 µmol m^{-2} s^{-1}) at 22 ± 1°C.

3.2.4.2.2 Effects of zeatin and BAP

Calli with the same embryogenic appearance and explant and induction media origins (Section 3.2.4.2.1) (except callus from BN20 medium and including those

from BN14 medium) were transferred to MS medium supplemented with BAP (0 and 1.0 mg l^{-1}) in combination with zeatin (0.5 and 1.0 mg l^{-1}) (designated BZ media). Calli with attached petioles were placed onto media in 175 ml glass jars (2 calli per jar) and cultures were maintained in the dark (22 ± 1°C). To investigate whether light affected embryogenic response, calli from BN18 were also kept continuously in a 16 h photoperiod under fluorescent light (30 μmol m^{-2} s^{-1}) at 22 ± 1°C.

3.2.4.3 Rooting of somatic embryos

The effects of IBA and NAA as pre-culture treatments on the stimulation of root development were investigated using germinated embryos cultured on half-strength MS medium and N6 medium (see Results, Section 3.3.5.2.1). Individual, embryos from half-strength MS medium were transferred to 40 ml half-strength MS medium supplemented with sucrose [0.3% (w:v)] and with or without IBA (0.1, 0.5 mg l^{-1}) and/or 0.1 mg l^{-1} IBA in combination with 0.1 mg l^{-1} NAA in 175 ml screw-capped glass jars. After 8 d (in the dark) cultures were transferred to half-strength MS medium supplemented with sucrose but lacking growth regulators and cultured under a 16 h photoperiod in the light (30 μmol m^{-2} s^{-1}) at 22 ± 1°C. Cultures were transferred to half-strength MS medium supplemented with sucrose [0.2 or 0.3% (w:v)] and maintained under the same conditions. Germinated embryos from N6 medium were transferred to N3 medium (Appendix 2) with or without auxins and cultured, in the light, as described earlier in this Section. Root development responses were assessed after 21 d culture for all treatments.

3.2.5 Histology

Embryogenic leaf-derived calli with differentiated somatic embryos cultured on UM medium were embedded for light microscopy as described in Chapter 2, Section 2.2.10. Histochemical studies were carried out in order to detect starch using the Pizzolato method (Pizzolato, 1978). This consisted of the identification of starch using tannic acid, ferric chloride and toluidine blue. Semi-thin sections were treated with 5% (v:v) aqueous tannic, 3% (v:v) aqueous ferric chloride and stained with 0.05% (w:v) toluidine blue in 1% (v:v) aqueous sodium borate.

3.2.6 Cytological analysis of cultivated and regenerated plants of *D. cymosa*

Root tip squashing was performed in order to determine the number and structure of *D. cymosa* chromosomes. It was important to note that only one regenerant was used as a source of root tips for the cytological analysis. The other plantlets did not survive in culture, therefore, the available material for these experiments was very limited.

3.2.6.1 Selection, treatment and preparation of root tips

Root tips (5 - 10 mm) were selected and excised from the regenerated plantlet and treated as described previously (Chapter 2, Section 2.2.11.1). To prepare root tip squashes, fixed root tips were squashed in Feulgen or aceto-orcein (Section 2.2.11.2). Mitotic root cells were examined and chromosomes counted. Only the aceto-orcein squash technique was followed for the chromosome counts of root tips of five glasshouse cultivated plants (controls).

3.2.7 Effects of surfactants on biomass growth of leaf-derived callus

These experiments were undertaken to assess the effects of non-ionic surfactants on the biomass and growth of leaf-derived calli cultured on UM medium. This medium was selected on the basis that earlier positive responses were obtained in terms of callus proliferation (see Results, Section 3.3.1.7).

A 10% (w:v) stock solution of commercial grade *Pluronic* ® F-68 (Sigma, Poole, UK) and 10% (v:v) solution of Tween 20 were freshly prepared, filter-sterilised (0.2 μm pore size filter Minisart; Sartorius, UK) and added to molten UM medium at a range of concentrations. Friable, leaf-derived callus (0.2 g f. wt.) was transferred on to 20 ml UM medium supplemented with *Pluronic* ® F-68 [0.001, 0.01 and 0.1% (w:v)]. Two calli were cultured per 9.0 cm Petri dish and each treatment was replicated five times. Cultures were kept in the dark, under the same conditions as described in Section 3.2.3.1. Similarly, leaf-derived callus was also transferred to UM medium supplemented with Tween 20 [0.001, 0.01 and 0.1% (v:v)] and cultured as for the *Pluronic* ® F-68 assessments. Controls lacking *Pluronic* ® F-68 and Tween 20 were set up and the experiment was repeated three times.

The growth of the callus was determined by recording fresh and dry weights every 15 d, during a 60 d period without subculture. The calli (n = 10, from each treatment) used for fresh weight determination were oven-dried at 60°C overnight prior to reweighing for dry weight determination. Means and standard deviations (s.d.) were used for results throughout. Statistical analyses included one-way ANOVAs incorporating a post-Hoc Tukey-Honestly Significant Difference (Tukey-HSD) test; a probability of $P < 0.05$ was considered significant.

3.2.8 Establishment and maintenance of cell suspensions

3.2.8.1 Initiation of leaf-derived cell suspension cultures

Cell cultures were initiated from fast-growing portions of pale yellow, friable leaf-derived callus (approx. 2.0 g f. wt.) by transfer to a 100 ml Erlenmeyer flask with 13 ml UM liquid medium (callus initiation medium). Callus derived from leaf explants on MSP2 medium (Appendix 2) was also chosen to initiate cell suspensions. Friable

callus (approx. 0.5 g f. wt.) was transferred to a 100 ml flask containing 13 ml MSP2 liquid medium. Callus showing signs of browning was discarded. Flasks were placed on a rotary shaker (120 rpm) in the dark at 25 ± 2°C. After 11 d culture, the contents of one flask were transferred to a 250 ml Erlenmeyer flask containing 10 ml fresh UM medium. The culture medium was replaced with 13 ml of fresh medium within 9 d and these cells thereafter became stock cultures. After 9 - 10 d culture, 5.5 ml PCV of suspension, with 20 ml of conditioned (spent) medium, were transferred to a 250 ml flask containing 15 ml of fresh medium. After 3 d culture, 2 ml fresh medium was added to the flask. After a further 3 d culture, 5 ml PCV and 18 ml of conditioned medium were transferred to a new 250 ml flask containing 19 ml fresh UM medium. The culture medium was replaced with fresh medium every 8 d. The subculture régime consisted of the addition of 2 ml fresh medium after each subculture at 4 d intervals (without removing conditioned medium) and after a further 4 d of culture, the replacement of 3 ml of conditioned medium with 3 ml of fresh medium. The amount of fresh medium per subculture was gradually increased up to 49 ml in total.

3.2.8.2 Initiation of petiole-derived cell suspension cultures

Petiole-derived suspensions were initiated from the same form of callus (approx. 2.0 g f. wt.) as used for leaf-derived cell cultures. Callus was transferred to a 100 ml Erlenmeyer flask containing 20 ml UM liquid medium. Flasks were placed on a rotary shaker (120 rpm) under the same growth conditions (Section 3.2.8.1). After 7 d culture, 5 ml of conditioned medium was replaced with 5 ml of fresh medium. After a further 5 d, 5 ml of conditioned medium was replaced with 7 ml of fresh medium. This was repeated every 7 d over a period of 14 d. After 4 weeks, the contents of one flask were transferred to a 250 ml Erlenmeyer flask but fresh medium was not added. In addition, stock cell cultures were kept in a 100 ml flask with 2 ml of fresh medium and 20 ml of culture medium containing cell suspensions. After 7 d, the culture medium was gradually replaced with fresh medium up to 30 ml in total.

3.2.8.3 Maintenance of cell suspensions

Friable, fast growing cell suspensions derived from leaves were maintained by transferring 5.0 ml PCV to a 250 ml flask with 50 ml of fresh UM medium. These cells were subcultured weekly and four lines were established as stock cultures. All cultures were maintained under the same conditions as described in Section 3.2.8.1.

Petiole-derived cell suspensions were maintained in a 100 ml flask and were routinely subcultured every 7 d. Cells from mother cultures (1.5 ml PCV), with or without 9.0 ml of conditioned medium, were resuspended in 20 ml of fresh medium. Cell suspensions were cultured under the same conditions as described in Section

3.2.8.1. Two lines were maintained by weekly subculture of 1 ml PCV with 10 ml of conditioned medium and 20 ml of fresh medium. Petiole-derived cell suspensions that were kept in a 250 ml flask became brown quickly (see Results, Section 3.3.5.8.1) therefore, a few flasks were maintained for the determination of growth curves (Section 3.2.8.5). These cells (5.0 ml PCV) were harvested weekly from the spent medium and resuspended with 50 ml of fresh medium. Leaf and petiole-derived cell suspensions were maintained in order to assess the growth of cells (Section 3.2.8.5) and their lignan content (Chapter 5).

3.2.8.4 Determination of cell suspension viability

The viability of cell suspensions of leaf and petiole was assessed 3 d after subculturing using the same FDA method as described previously (Chapter 2, Section 2.2.7). At least 50 cells per sample were counted and viability was expressed as the percentage of fluorescing cells (Appendix 3).

3.2.8.5 Growth curves of cell suspensions

The growth (biomass) of cells was measured by determination of increases in PCV (Hall, 1991). Aliquots [5 ml (PCV)] of cells from established suspension cultures (6 and 13 months old for leaf and petiole respectively) were transferred to a 250 ml Erlenmeyer flask with graduated (10 ml) side-arms and containing 50 ml fresh UM medium. Cultures were placed on a rotary shaker under the conditions described in Sections 3.2.8.1 and 3.2.8.2 but without weekly subculture. Growth of the cells was monitored from day 0 (day of initiation) until a stationary growth phase was reached (23 - 25 d). Five readings for each flask (total of four flasks) were recorded daily over a period of 25 d by dispensing 10 ml of the cell suspension into the side-arm of the flask to give a SCV. Growth curves of leaf and petiole cell suspension cultures were plotted as a mean value ± s.e. of SCV against time. This experiment was repeated twice.

3.2.9 Cryopreservation of petiole-derived cell cultures

3.2.9.1 Pre-growth treatment

Prior to cryopreservation, cell suspensions (13 months old) taken 7 d post-subculture were partially dehydrated by replacing the culture medium with 35 ml of fresh UM liquid medium supplemented with 6% (w:v) mannitol (BDH Laboratory Supplies, Poole, UK). The cell suspensions were cultured for 4 d under the same conditions as given in Section 3.2.8.2. Two experiments were undertaken and each consisted of three treatments: cells with cryoprotectant mixture were cryopreserved and then stored in liquid nitrogen (treatment 1, with cryoprotectant A or B; Section

3.2.9.2); cells without cryoprotectant mixture were cryopreserved by transferring to liquid nitrogen (treatment 2); partially dehydrated cells without cryoprotectant and non-cryopreserved (treatment 3, control).

3.2.9.2 Chemical cryoprotection and controlled freezing

After 4 d culture, cells were harvested from mannitol-supplemented UM medium and sieved (64 μm pore size) prior to transfer to 2.0 cm^3 polypropylene vials (Sarstedt, Leicester, UK). To each vial (approx. 0.2 g f. wt. of cells) was added 0.75 ml of a freshly prepared cryoprotectant mixture. The components of cryoprotectant mixtures tested were: 0.5 M DMSO, 0.5 M glycerol and 1.0 M sucrose (cryoprotectant mixture A); and 1.0 M DMSO, 1.0 M glycerol and 1.0 M sucrose (cryoprotectant mixture B). Cells were chilled for 1 h on iced water and frozen from 0°C to -35°C, at -1°C min^{-1} and held at this temperature for 35 min in a programmable freezer (Planer Biomed, Middlesex, UK) before storage at -196°C in liquid nitrogen for 7 d.

3.2.9.3 Post-thaw recovery of cryopreserved cells

Cells (all treatments) were thawed by immersion of vials in sterile, reverse-osmosis water (45°C) for 2 - 3 min followed by removal of excess cryoprotectant. The cells from each vial were transferred to two superimposed 5.5 cm sterile filter paper disks overlaying 20 ml aliquots of UM medium in a 9.0 cm Petri dish. Cells were cultured for 3 d in the dark (22 ± 1°C). Then, the upper filter paper disk supporting the cells was transferred to fresh UM medium and cultured for 1 d under the same conditions. Each treatment consisted of 20 replicates and was repeated twice. The cell viability and biomass were assessed after thawing (Section 3.2.9.4).

3.2.9.4 Determination of post-thaw viability and biomass of cells

The post-thaw viabilities of cryopreserved and non-cryopreserved cells were assessed 4 d after thawing using the TTC assay, according to a modified method of Steponkus and Lamphear (1967). This assay was based on the enzymatic reduction of TTC to produce red formazan, which was measured in a Perkin Elmer UV/VIS spectrometer, Lambda Bio (Perkin Elmer, Beaconsfield, UK), for viable cells; data was recorded using an ultraviolet spectrometer. TTC buffer (3 ml) consisting of 0.6% (w:v) TTC in 0.05 M Na_2HPO_4 - KH_2PO_4 buffer and 0.05% (v:v) Tween 80 (Sigma, UK), pH 7.4, was added into a 16 ml screw-capped centrifuge tube (Corning, NY) containing cells (50 mg). The cell suspensions were incubated in the dark (28 ± 1°C) for 16 - 18 h followed by one washing with sterile, reverse-osmosis water. The tetrazolium salt was extracted with 7 ml 95% (v:v) ethanol in a boiling water bath for

5 min. The volume in each tube was adjusted to 10 ml with 95% (v:v) ethanol. The absorbance of cells (20 samples from each treatment) was measured spectrophotometrically at 490 nm. The fresh weight of thawed cells was also recorded after 90 d culture for biomass determination.

3.2.9.5 Re-initiation of cryopreserved and non-cryopreserved cells

Calli (approx. 2.0 g f. wt.) from thawed cryopreserved cells lacking cryoprotection (Treatment 2) and partially dehydrated unfrozen cells (Treatment 3) cultured for 90 d post-thawing were used to re-initiate cell suspensions in liquid UM medium, following a previous protocol (Section 3.2.8.2). This experiment was conducted in order to verify if cell suspensions could be re-initiated thus was undertaken on a small-scale.

3.3 Results

3.3.1 Assessment of callus initiation on different media

3.3.1.1 B5D media

There was no response from leaf and petiole explants cultured on these media. After 35 d in culture, an intense phenolic oxidation was observed in all the explants.

3.3.1.2 BD media

The leaf and petiole explants did not respond to these media.

3.3.1.3 BN media

No response from leaf explants was observed. However, after 38 d on BN media, petiole explants had swollen and 27 - 33% of explants showed callus initiation around the cut end of the petiole. Following a further 22 d culture on B5 medium supplemented with NAA and BAP, these explants gave distinct types of calli (in colour and appearance) described in Table 3.2. Some calli appeared to be potentially embryogenic (Plate 3.1.B) and later experiments confirmed this (see Results, Sections 3.3.5.3.1 and 3.3.5.3.2). None of the explants kept in the light exhibited callus formation.

3.3.1.4 MSD media

Callus initiation was observed after 90 d from leaf explants cultured on media containing 0.5, 1.0 or 2.0 mg l^{-1} 2,4-D, however, there was no continued growth. Explants (50 to 60%) became necrotic on MSD media with 0.1 - 1.0 mg l^{-1} 2,4-D. Petiole explants cultured on this media gave no response.

Table 3.2: Callusing response of petiole explants cultured on BN media assessed after 60 days

	BAP (mg l^{-1})			
NAA (mg l^{-1})	1.0	2.0	3.0	4.0
0	BN1^{0}	BN2^{0}	BN3^{0}	BN4^{0}
0.25	BN5^{0}	BN6^{1}	BN7^{0}	BN8^{1}
0.5	BN9^{1}	BN10^{0}	BN11^{1}	BN12^{0}
1.0	BN13^{0}	BN14^{2}	BN15^{2d}	BN16^{2b}
2.0	BN17^{2}	BN18^{3ac}	BN19^{2}	BN20^{3a}

^{0} = no response; ^{1} = callus; ^{2} = compact callus; ^{3} = frosted callus.
^{a} = pale green; ^{b} = pale yellow; ^{c} = yellowish-cream; ^{d} = yellowish-green.
Shading indicates response of interest.

3.3.1.5 MSB media

No response from leaf and petiole explants was observed.

3.3.1.6 MSP media

Callusing of leaf explants was initiated after 30 d culture on MS medium supplemented with 4.8, 7.2 or 9.6 mg l^{-1} picloram (MSP2, MSP3 and MSP4 media respectively), although the percentage of leaf tissue explants that gave rise to callus was very low (6% on MSP2, 9% on MSP3 and 3% on MSP4 media). Calli cultured for up to 3 months on MSP2 and MSP3 media were slow growing. The callus showed different colours and textures with different concentrations of picloram. The callus was pale green and friable with 4.8 and 9.6 mg l^{-1} picloram whilst at a concentration of 7.2 mg l^{-1}, callus was pale yellow (compact or frosted) or pale green and friable. At this stage, the number of calli obtained was different for the three treatments (n = 8 calli per dish on MSP2 medium, n = 4 calli on MSP3 or MSP4 media). Even though picloram was included in MS medium for callus induction, growth was generally slow (Fig. 3.1). All calli became brown and died after 7 months culture on MSP2, MSP3 and MSP4 media.

Initially, intense browning and staining of the media was observed for all treatments. Interestingly, this was characterised by the presence of a rusty-red coloured pigment mainly in the immediate area around the callus cultures (Plate 3.1.C). To overcome this problem and to preserve cultures, calli (all treatments) were subcultured routinely, every 21 d, to fresh MSP2, MSP3 and MSP4 media as appropriate and maintained in the dark (22 ± 1°C).

Callusing responses from petioles were obtained after 30 d culture on MSP2, MSP3 and MSP4 media. Petiole sections cultured on these media produced dark

brown exudates at the cut surfaces but maintained a green colour. Although petiole explants cultured on MSP medium gave rise to callus, the extent of the response was varied even after 120 d culture: 38% of explants on MSP2, 20% on MSP3, 30% on MSP4, 15% on MSP5 and 5% on MSP6 medium. Calli were pale green, friable or pale yellow and frosted in all treatments, except on MSP3 medium where they were whitish and compact.

3.3.1.7 UM medium

Callus derived from leaf explants maintained on UM medium developed in the vicinity of the cut ends and along the central veins (54% of explants gave callus; n = 49) after 60 d. Due to the incidence of browning and necrosis, 28% of explants were discarded. Therefore, subculturing of callus was undertaken every 21 d during the first 4 months. The combination of 2.0 mg l^{-1} 2,4-D and 0.25 mg l^{-1} kinetin induced a pale yellow, watery, friable callus (Plate 3.1.D). Callus initiation whitish-yellow, frosted callus was occasionally observed. It was found that some calli had embryogenic potential (Section 3.3.5.1). The same type of callus (whitish-yellow, watery, friable) was obtained from petioles (33% of explants gave rise to callus) after 60 d of culture. Although, leaf explants cultured on UM medium gave rise to callus, the growth rate was slow based on the fresh weight increase of callus (Fig. 3.2) and dry weight. For example, the mean dry weight of leaf-derived callus was 0.008 ± 0.006 g (n = 10) after 3 months of culture and had increased to 0.031 ± 0.016g (n = 10) after a further 30 d of culture. In the case of petiole-derived callus, the growth rate was even slower than leaf-derived callus and no detectable increases in fresh weight were found over the culture period.

3.3.2 Maintenance of callus cultures

After the first 4 months of culture on induction medium, the level of phenolic oxidation decreased and leaf and petiole-derived calli were regularly subcultured onto fresh UM, MSP2, MSP3 and MSP4 media. The fastest growing portion in the callus was always selected for subculturing every 4 weeks. The maintenance of friable calli from leaf and petiole explants cultured on different media was required for the initiation of cell suspensions (Section 3.3.5.8.1) and analysis of lignan content (Chapter 5, Section 5.2.1).

Fig. 3.1: Fresh weight gain profile of callus from leaf explants of *D. cymosa* cultured on MSP medium

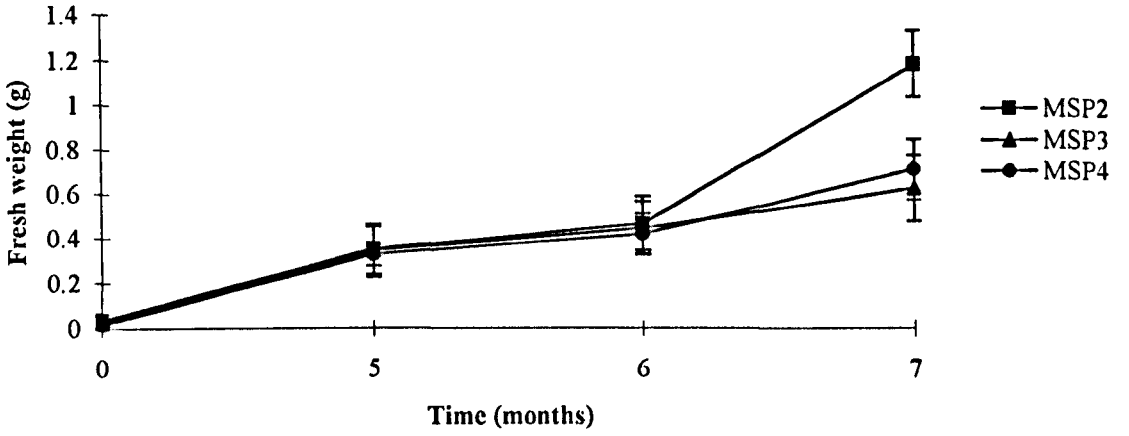
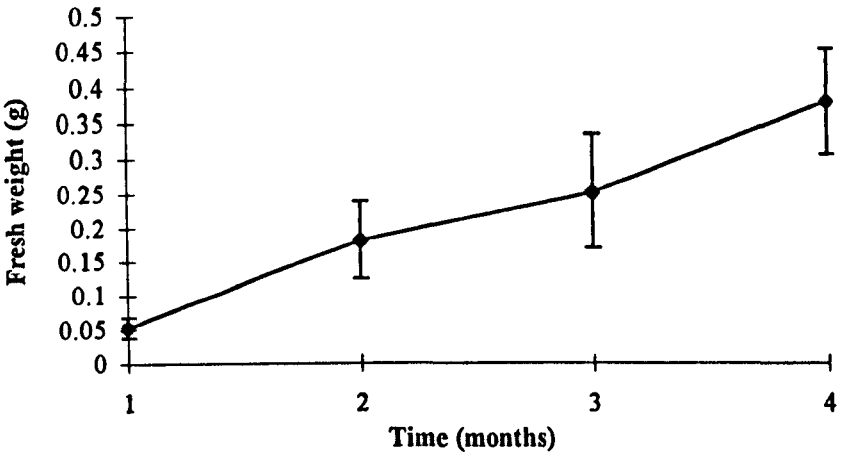


Fig. 3.2: Fresh weight gain profile of callus from leaf explants of *D. cymosa* cultured on UM medium



Values are mean ± s.d. of 5 replicates from experiments repeated 3 times.
n = 10 throughout.

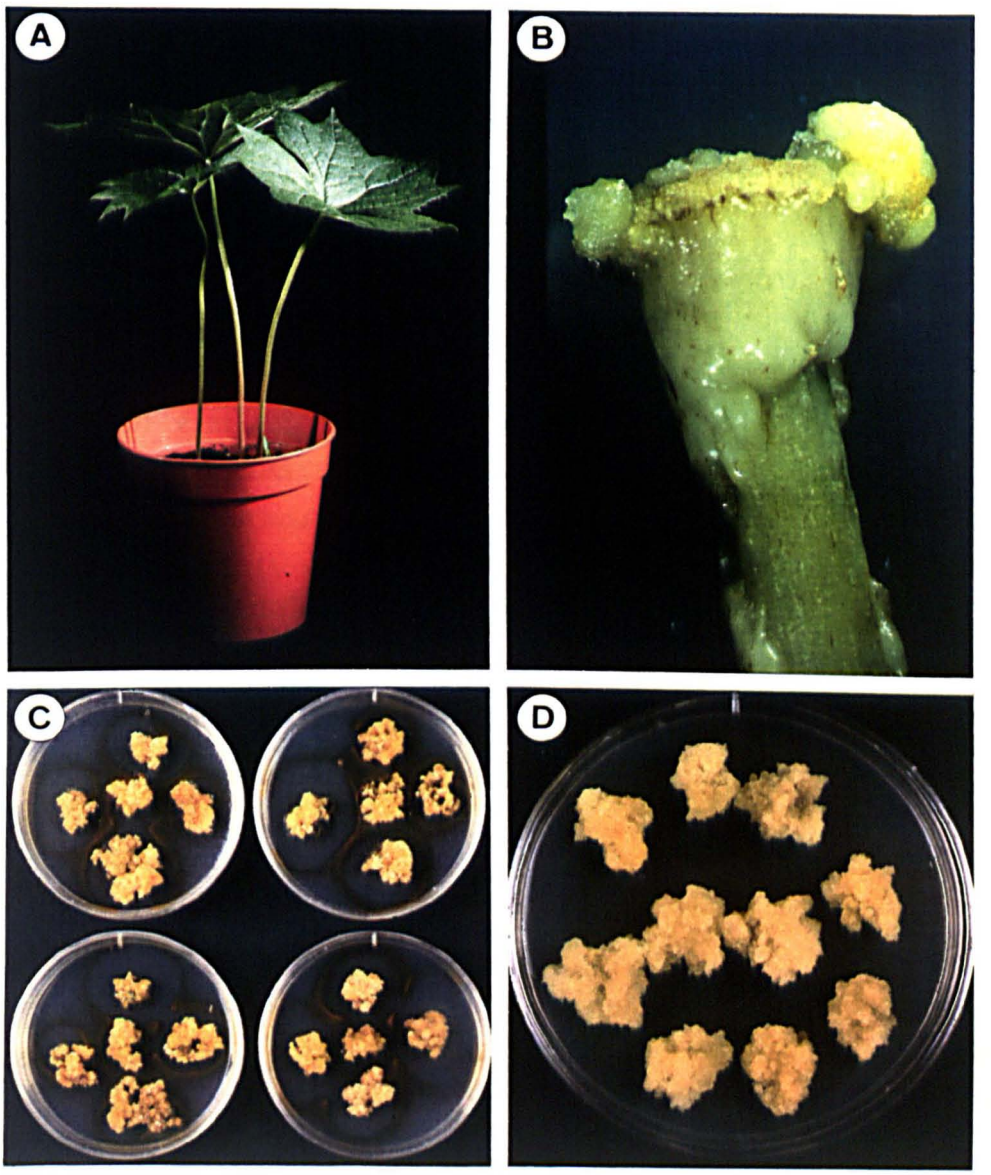
Plate 3.1: Callus formation in leaf and petiole explants of *Diphylleia cymosa*

A. *D. cymosa* plant (5 years old; from a propagated clone) cultivated at the University of Nottingham and used as starting material. (x 0.4).

B. Embryogenic calli formed from a petiole explant cultured on B5 semi-solidified agar medium with 1.0 mg l^{-1} NAA and 4.0 mg l^{-1} BAP (BN16 medium) after 60 d in the dark. (x 1.2).

C. Staining of the medium by leaf-derived calli cultured on full-strength MS semi-solidified agar medium with 4.8 mg l^{-1} picloram (MSP2 medium, top two Petri dishes) and 7.2 mg l^{-1} picloram (MSP3 medium, botton two Petri dishes) in the dark. (x 0.93).

D. Pale yellow, watery, friable calli from leaf explants cultured on UM semi-solidified agar medium in the dark. (x 0.93).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D

3.3.3 Effects of CPPU and 2,4-D on embryogenesis induction

Leaf-derived callus was induced in 100% of explants on MS medium containing 0.24 mg l^{-1} CPPU in combination with 1.0 mg l^{-1} 2,4-D after a period of 30 d. Two types of calli were obtained: whitish-yellow, compact or yellow-green, nodular. Only the former type of callus was produced in the presence of 0.24 mg l^{-1} CPPU and 2.0 mg l^{-1} 2,4-D and for 70% of explants. On the medium with CPPU (as sole growth regulator), all explants became necrotic. Although calli appeared to be embryogenic, they failed to undergo embryogenesis. Petiole explants did not respond to these media.

3.3.4 Effects of TDZ on organogenesis induction

No organogenic response was observed for any of the treatments. Necrosis occurred in all leaf explants cultured on the highest concentrations of TDZ and in 50% of tissues with 0.01 and 0.001 mg l^{-1} TDZ. No response from petiole explants was observed.

3.3.5 Plant regeneration

3.3.5.1 Somatic embryogenesis on leaf and petiole-derived callus

After nine subcultures (every 4 weeks) of callus on UM medium containing kinetin and 2,4-D a population of compact cells exhibited the formation of embryogenic clusters. These embryogenic clusters developed somatic embryos, on leaf-derived calli, after 10 - 11 subcultures. This was observed when calli, attached to the original explant, became necrotic but still showed some fresh growth (Plate 3.2.A). The shape of embryos was mostly globular (> 80%) but misshapen structures were also obtained. After embryo formation, the callus was subcultured on fresh UM medium and kept for a further 40 d before transfer to regeneration medium (Section 3.3.5.2.1). Callus, with somatic embryos, cultured on MS medium with nitrate at half-strength did not differentiate or germinate.

Somatic embryos were also induced on callus from leaf explants on MSP media at the lowest concentrations of picloram tested (4.8 or 7.2 mg l^{-1}; MSP2 and MSP3 media). Differentiated embryos were observed at the surface of the callus after 11 - 12 passages on MSP2 medium (Plate 3.2.B,C). Most of somatic embryos developed from light brown callus (Plate 3.2.D). Of the embryos that appeared on the surface of callus cultured on MSP3 medium, about 90% originated from pale yellow callus (Plate 3.2.E) and about 10% from brownish-yellow and friable callus. Occasionally, secondary somatic embryos forming from primary embryos were observed (Plate 3.2.F). There were typically 15 embryos per callus when the original leaf explant was retained, and 7 embryos in the absence of the explant.

Petiole-derived compact calli differentiated to form embryogenic calli and somatic embryos on BN medium containing NAA and BAP. On BN16 and BN18 media, calli gave rise to somatic embryos that developed to cotyledonar stage on the same media (Plate 3.3.A, B).

3.3.5.2 Regeneration of somatic embryos from leaf-derived callus

3.3.5.2.1 Development and germination of somatic embryos

Calli derived from leaf explants cultured via repetitive passage on UM medium produced somatic embryos. When these calli were cultured on hormone-free MS medium the embryos started to become brown after 4 - 5 d in the dark. Greening and development of the somatic embryos were observed (7 d) after these calli were transferred to half-strength MS medium and maintained in the light. Embryos enlarged from 0.2 mm to 0.6 mm diam. after 15 d in culture but none developed further.

Somatic embryos on calli cultured on half-strength MS medium supplemented with BAP, BAP in combination with NAA or zeatin (see Section 3.2.4.1.2) enlarged in all treatments after 7 d. In treatments with 1.0 mg l^{-1} BAP in combination with 0.001 mg l^{1} NAA, there was an increase in the number of somatic embryos from 22 to 30 (scored from two calli). It became clear during these experiments that differentiation of the somatic embryos could not be achieved in these treatments tested. The first sign of differentiation was greening and enlargement of the somatic embryos after 15 d cultured on N6 medium with 0.5 mg l^{-1} GA_3. Phenolic compounds accumulated at the region of contact between callus and medium. For this reason, callus was subcultured on to fresh N6 medium lacking GA_3. The duration of culture on N6 medium was increased to 28 d in order to obtain maturity of the embryos. Germinated embryos characterized by the appearance of green cotyledons were observed within 24 d cultured on N6 medium (Plate 3.3.F). Occasionally, secondary somatic embryos forming from primary embryos were observed. Cultures were maintained on the same medium without subculture. The young plantlets (with a shoot system but without a well developed root system) obtained on half-strength MS medium and N6 medium were then transferred to media supplemented with IBA or IBA in combination with NAA to stimulate root development (Section 3.3.5.4).

3.3.5.2.2 Effects of ABA and partial desiccation on embryogenesis

In the presence of ABA (0.1 and 0.2 mg l^{-1}) the cream-coloured embryos became swollen and slightly brown. After transfer to MS6 medium, lacking ABA, an enlargement of the embryos was clearly visible. The beginning of germination was only observed in cultures pre-treated with 0.2 mg l^{-1} ABA; however, no further

Plate 3.2: Somatic embryogenesis of leaf-derived callus cultures of *Diphylleia cymosa*

A. Globular-stage somatic embryos from friable leaf calli after 11 months culture on UM semi-solidified agar medium in the dark. (x 3.0).

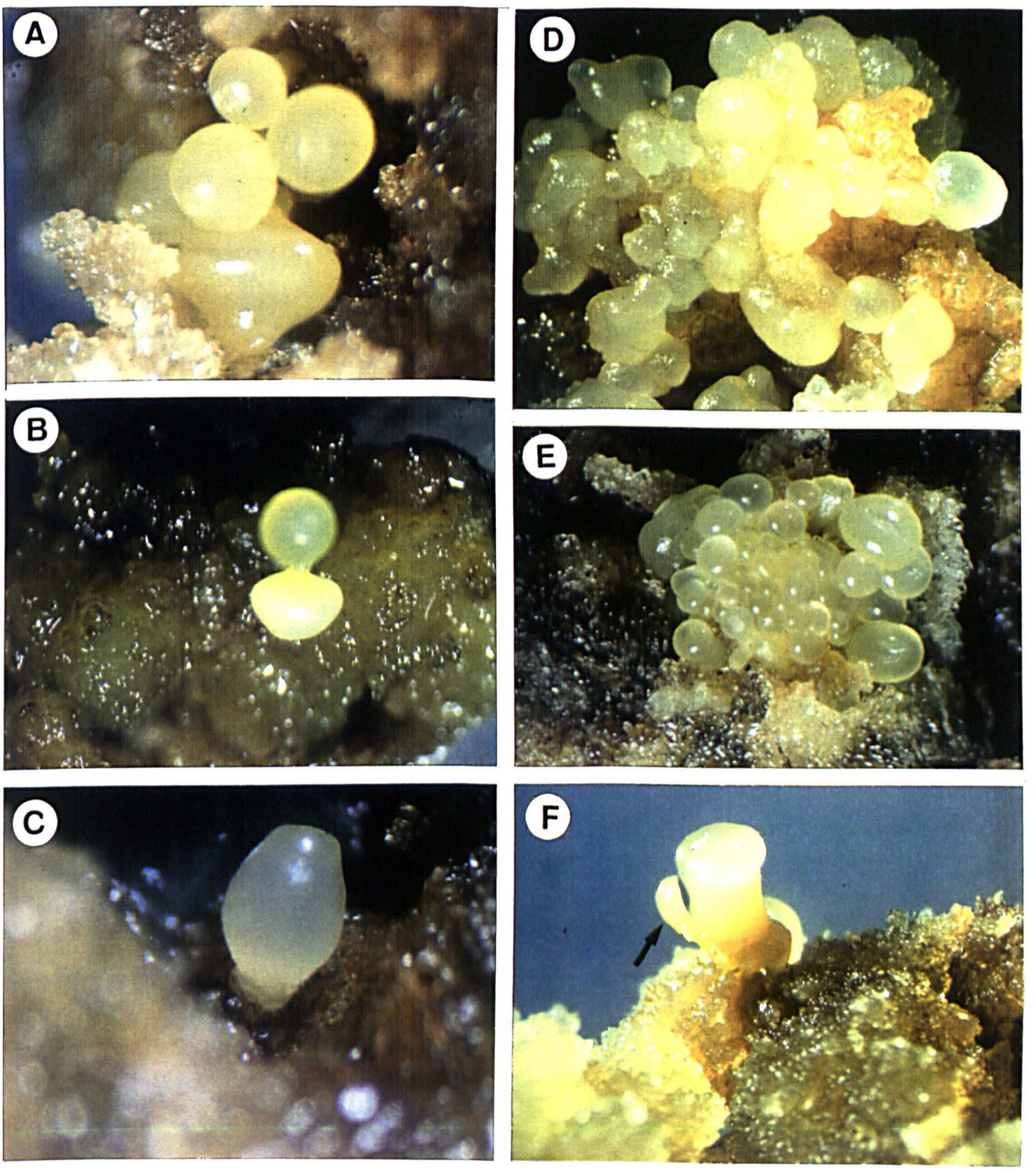
B. Production of differentiated somatic embryos formed after 11 - 12 passages on MSP2 semi-solidified agar medium in the dark. (x 2.0).

C. A single, late torpedo-stage somatic embryo formed from leaf-derived callus cultured on MSP2 in the dark. (x 3.5).

D. Different stages of somatic embryos (globular and heart) originated from light brown calli cultured on MSP2 medium for 11 - 12 months culture in the dark. (x 1.5).

E. Somatic embryos formed from pale yellow callus cultured on MSP3 semi-solidified agar medium in the dark. (x 1.5).

F. A torpedo-stage somatic embryo on callus cultured on MSP3 medium. Note secondary embryogenesis (arrow). (x 1.5).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development was obtained in the following 8 weeks. After pre-treatment with ABA, all calli cultured on MS6 medium were creamy-green whilst controls turned brown. Based on these results, desiccation (of embryos and calli) and the presence of ABA in the culture medium were not effective in the germination of somatic embryos on MS6 medium.

3.3.5.2.3 Effects of ABA and agarose on embryogenesis

When embryogenic calli subcultured on half-strength MS medium were pre-cultured on the same medium with 0.1 mg l^{-1} ABA and transferred to MS medium semi-solidified with 0.4% (w:v) agarose, green spots formed 15 d after transfer. Germinated embryos with developed shoot systems were transferred after 11 weeks to media with IBA, or IBA in combination with NAA in order to stimulate the development of roots (Section 3.3.5.4). Medium containing 0.2 mg l^{-1} ABA induced the formation of dark-green structures within 15 d. After 10 weeks, these structures were transferred to half-strength MS medium. Within 23 d, germinated somatic embryos with an abnormal appearance (two fused cotyledons) emerged and rhizogenesis was observed.

For embryogenic calli (100%) pre-cultured on half-strength MS medium supplemented with BAP and NAA, greening of the embryos was observed within 15 d on medium semi-solidified with 0.4% (w:v) agarose after treatment with 0.1 mg l^{-1} ABA. Differentiation of cotyledons occurred after 8 weeks even though cotyledons were fused. After 3 weeks with 0.2 mg l^{-1} ABA and transfer to half-strength MS medium lacking growth regulators, the formation of the same type of abnormal structures mentioned in this section was observed. No greening was observed for this treatment. Overall, the presence of ABA induced a few abnormalities even though germinated embryos with a normal appearance were obtained. After 11 weeks on medium lacking ABA and semi-solidified with agarose, all germinated embryos and embryo-like structures (with abnormal appearance) were transferred to half-strength MS medium with GA_3 for further development.

3.3.5.2.4 Effects of GA_3 on somatic embryos

The presence of GA_3 in the culture medium was important in order to promote the development of somatic embryos and embryo-like structures. Many germinated embryos (from control cultures on half-strength MS medium with agarose; Section 3.2.4.1.4) maintained their growth on half-strength MS medium solidified with 0.2% (w:v) Phytagel after 2 weeks. Root developing (0.5 - 1.0 cm in length) was observed in the third week. Germinated embryos with roots were subcultured every 4 weeks, but cotyledons remained fused. The embryo-like structures (formed on half-strength

MS medium after pre-treatment with 0.2 mg l^{-1} ABA) also developed on half-strength MS medium with Phytagel lacking GA_3. Root and shoot systems were formed within 2 weeks and 4 weeks respectively. These young developing plantlets grew normally and, after 8 weeks of culture, shoot elongation was between 1.0 to 2.0 cm.

3.3.5.3 Somatic embryogenesis on petiole-derived callus

3.3.5.3.1 Effects of zeatin

In the presence of zeatin, newly developing green portions of calli from BN15 medium was observed after 8 weeks but no embryo-like structures were produced. During embryo initiation, the surface of calli (approx.75%) became smooth, nodular and compact in appearance about 4 - 5 d after transfer to MSZ medium. Embryogenic calli, originally from BN16 medium, showed dedifferentiation and green globular projections with a reflective appearance on MSZ medium. These structures emerged from the callus and gradually became differentiated. Between 26 - 28 d in culture (in the light), embryogenic responses were observed (100% of calli gave rise to somatic embryos) (Plate 3.3.C). Callus was subcultured onto fresh aliquots of MSZ medium every 4 weeks. For a further 11 d, these structures continued to develop on top of the compact, yellowish-cream calli. Somatic embryos exhibited abnormalities which could be detected by visual observation (Plate 3.3.D). A few green embryo-like structures were also seen within 30 d in calli from BN17 and BN18 media when transferred to MSZ medium, however, they failed to undergo germination. There was no response of calli from BN20 medium after culture on MSZ medium.

Only somatic embryos derived from BN16 medium and cultured on MSZ medium in the light were capable of further development to cotyledonar stage even though no plants were obtained. These results suggested that induction and development of somatic embryos were callus-type dependent. Thus, the ratio of the auxin NAA to the cytokinin BAP on the previous medium where embryogenic callus was initially induced may later influence the embryogenic capacity. The abnormalities mentioned above are probably also responsible for the unsuccessful plant regeneration from petiole somatic embryos.

3.3.5.3.2 Effects of zeatin and BAP

Combined BAP (0 and 1.0 mg l^{-1}) and zeatin (0.5 and 1.0 mg l^{-1}) (BZ1, BZ2 and BZ3 media,) in the culture media provided a continuous source of newly developing whitish and compact calli. All calli turned brown and died at 1.0 mg l^{-1} of both BAP and zeatin (BZ4 medium; except those from BN18 medium). After 12 weeks, shiny green, abnormal embryo-like structures emerged on calli from BN18 medium transferred to BZ2 and BZ4 media. These structures formed on calli cultured

either in the dark or in the light but their growth was better in the light (Plate 3.3.E). None of the greenish embryo-like structures developed into plantlets.

The embryogenic response of calli from petioles occurred on medium containing the highest concentration of BAP and zeatin. The highest concentration was most effective in promoting development of the somatic embryos. Zeatin influenced somatic embryogenesis from petiole explants even though some abnormalities in embryos were observed.

3.3.5.4 Effects of IBA and NAA on stimulation of root development

Somatic embryos, pre-cultured on MS medium supplemented with 0.1 mg l^{-1} IBA with 0.1 mg l^{-1} NAA developed roots (100%) within 21 d when cultured either on half-strength MS medium lacking growth regulators or N3 medium. Mature somatic embryos cultured on half-strength MS medium containing 0.1 or 0.5 mg l^{-1} IBA or half-strength MS medium lacking auxins failed to develop roots. However, only those formed on half-strength MS medium with BAP and NAA and pre-cultured (8 d) on half-strength MS medium with 0.5 mg l^{-1} IBA gave roots (100%) after 21 d of culture on half-strength MS medium. In both treatments with 0.1 and 0.5 mg l^{-1} IBA, plantlets obtained from somatic embryos developed yellow-green leaves after 21 d culture. Rooting was also inhibited when germinated embryos were cultured on N3 medium with 0.1 and 0.5 mg l^{-1} IBA or without auxins even though they showed further growth and maintained a green colour. In *D. cymosa*, stimulation of rooting of somatic embryos was achieved by a combination of IBA and NAA in the pre-treatment of the well developed somatic embryos.

Plantlets, obtained from somatic embryos, cultured on half-strength MS medium supplemented with 0.2% (w:v) sucrose and solidified with 0.7% (w:v) agar or 0.2% (w:v) Phytagel and cultured on Nitsch medium (Appendix 2) with sucrose and Phytagel at the same concentrations remained green after 13 weeks in culture. Plantlets (100%) cultured on half-strength MS medium supplemented with agar tended to elongate from 0.9 cm (original size) to 2.0 cm (final size) after 14 weeks (Plate 3.3.G).

3.3.5.5 Histology

Light microscopy study confirmed the development of somatic embryos from the leaf-derived calli of *D. cymosa* cultured on UM medium. The somatic embryos appeared as isolated structures with no vascular connections to the input tissue. Somatic embryos showed a normal ontogenesis albeit a few abnormalities were detected. A normal somatic embryo at an advanced stage (cotyledonary stage) was observed showing distinct cotyledons and a central vascular bundle (Plate 3.4).

Plate 3.3: Somatic embryogenesis from petiole-derived callus and plant regeneration from leaf-derived callus of *Diphylleia cymosa*

A. Differentiated somatic embryo formed on petiole-derived callus cultured on B5 semi-solidified agar medium with 1.0 mg l^{-1} NAA and 4.0 mg l^{-1} BAP in the dark (BN16 medium). (x 2.5).

B. Embryo-like structure formed on yellowish-cream petiole-derived callus cultured on B5 semi-solidified agar medium with 2.0 mg l^{-1} NAA and 2.0 mg l^{-1} BAP (BN18 medium) in the dark. (x 0.66).

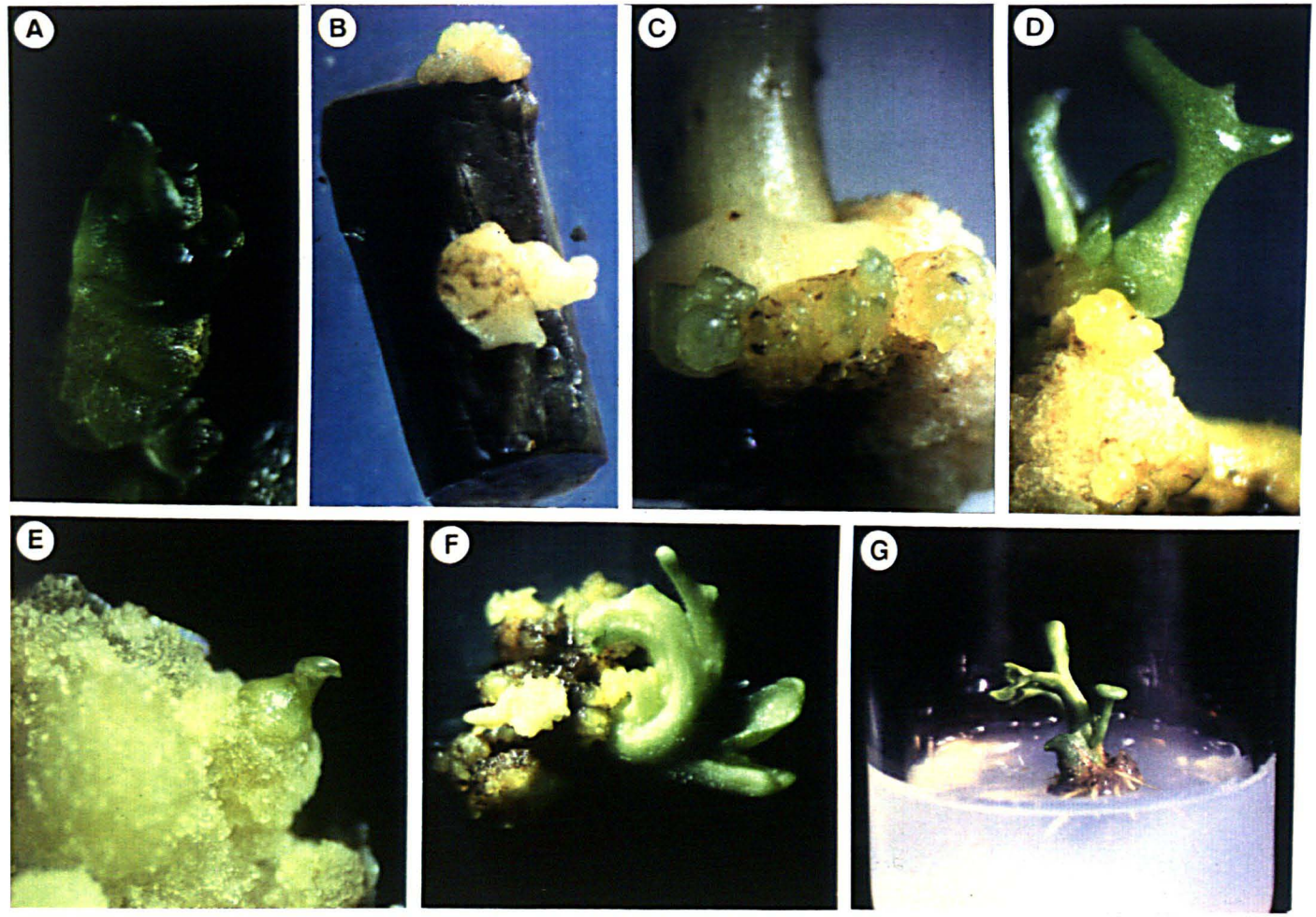
C. Differentiation of green globular embryo-like structures formed on pale yellow, embryogenic petiole-derived callus after 27 d in the light following transfer to MS semi-solidified agar medium with 1.0 mg l^{-1} zeatin (MSZ medium). (x 1.0).

D. Development and germination of green, abnormal embryo-like structures from embryogenic petiole-derived callus after 63 d culture in the light on MSZ medium. (x 0.66).

E. Development of a shiny green, abnormal embryo-like structure on pale green, frosted, embryogenic petiole-derived callus cultured on MS semi-solidified agar medium supplemented with 1.0 mg l^{-1} each of BAP and zeatin (BZ4 medium) for 12 weeks in the light. (x 3.0).

F. Germination and development of somatic embryos after treatment with 0.5 mg l^{-1} GA_3 and transfer for 24 d to N6 medium lacking growth regulators. Note the germinated somatic embryos attached to embryogenic leaf-derived callus. (x 0.66).

G. A plant cultured on half-strength MS medium lacking growth regulators, supplemented with 0.2% (w:v) sucrose and semi-solidified with 0.7% (w:v) agar. (x 1.6).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G

Plate 3.4: Longitudinal section of a somatic embryo at the cotyledonary stage from leaf-derived callus

Somatic embryo showing two cotyledons and the central vascular bundle. Cotyledons (C), epicotyl (E), vascular system (V), root initials (R), starch granules (ST). (x 152).


C
C
E
V
V
ST
V
V
R

A normal vascular system connected the cotyledon and the root poles. The central core of the somatic embryo was occupied by procambium which bifurcated into cotyledons at the shoot pole. The shoot apical meristem consisted of a small group of cytoplasmically-dense cells occupying the apical notch between the cotyledons. The root apical meristem was located at the region between the cap and the procambial pole. During somatic embryo maturation numerous small starch granules were formed in the subepidermal layers of the mature embryo. These granules were stained black following use of the Pizzolato method (Plate 3.5.A). The accumulation of starch in the cells at the cotyledonar region demonstrated that maturation of the somatic embryos was progressing. Embryos with well developed cotyledons and epidermal cell layers were also observed (Plate 3.5.B). The occurrence of somatic embryos with fused cotyledons (Section 3.3.5.2.3) was clearly detected by histological examination (Plate 3.5.C) even though such embryos had a normal hypocotyl and root system.

3.3.5.6 Cytology of cultivated and regenerant *D. cymosa* plants

Mitosis in root tip cells was observed by Feulgen and aceto-orcein squash techniques. The cytological examination of root tip squashes of cultivated plants at metaphase revealed *D. cymosa* to be diploid having $2n = 2x = 12$ chromosomes, confirming published data (Langlet, 1928). The complement comprised metacentric (m), sub-metacentric (sm) and acrocentric (a) chromosomes (Plate 3.6.A). The chromosomes were relatively large, ranging in length from 6 - 15 μm, with the metacentric chromosome being longer than the sub-metacentric ones. The NOR was located in the short arm of one of the sub-metacentric chromosomes and was observed as a large secondary constriction (Plate 3.6.A). Cytologically, the regenerant showed a low incidence of chromosomal abnormality compared to the cultivated (control) plants. The somatic chromosome number, analysed at pro-metaphase, was unaltered when compared with control plants although some structural variation was revealed. These alterations may have occurred during the process of plant regeneration and/or during development as the *Diphylleia* regenerant showed a number of somatic cells with gross aberrations. Such aberrations were mainly chromatid fractures and fragments (Plate 3.6.B). In both cultivated and the regenerant *D. cymosa*, chromosomes were easily stained as previously described for *P. hexandrum* (Chapter 2, Section 2.3.9).

Unfortunately, no more regenerants were available to replicate the cytological observations and even though other regenerants were produced mature root systems did not develop.

Plate 3.5: Development of somatic embryos from leaf-derived callus, matured on UM medium; histochemical staining for starch

A. Starch granules (arrows) in the subepidermal layers of a mature somatic embryo. Note the vascular cells (arrowheads). (x 760).

B. Median longitudinal section of a mature somatic embryo from leaf-derived callus showing cotyledons (C) and a vascular strand (VS). (x 152).

C. Longitudinal section of a somatic embryo showing a normal hypocotyl, the root pole (RP) and fused cotyledons (FC). (x 152).

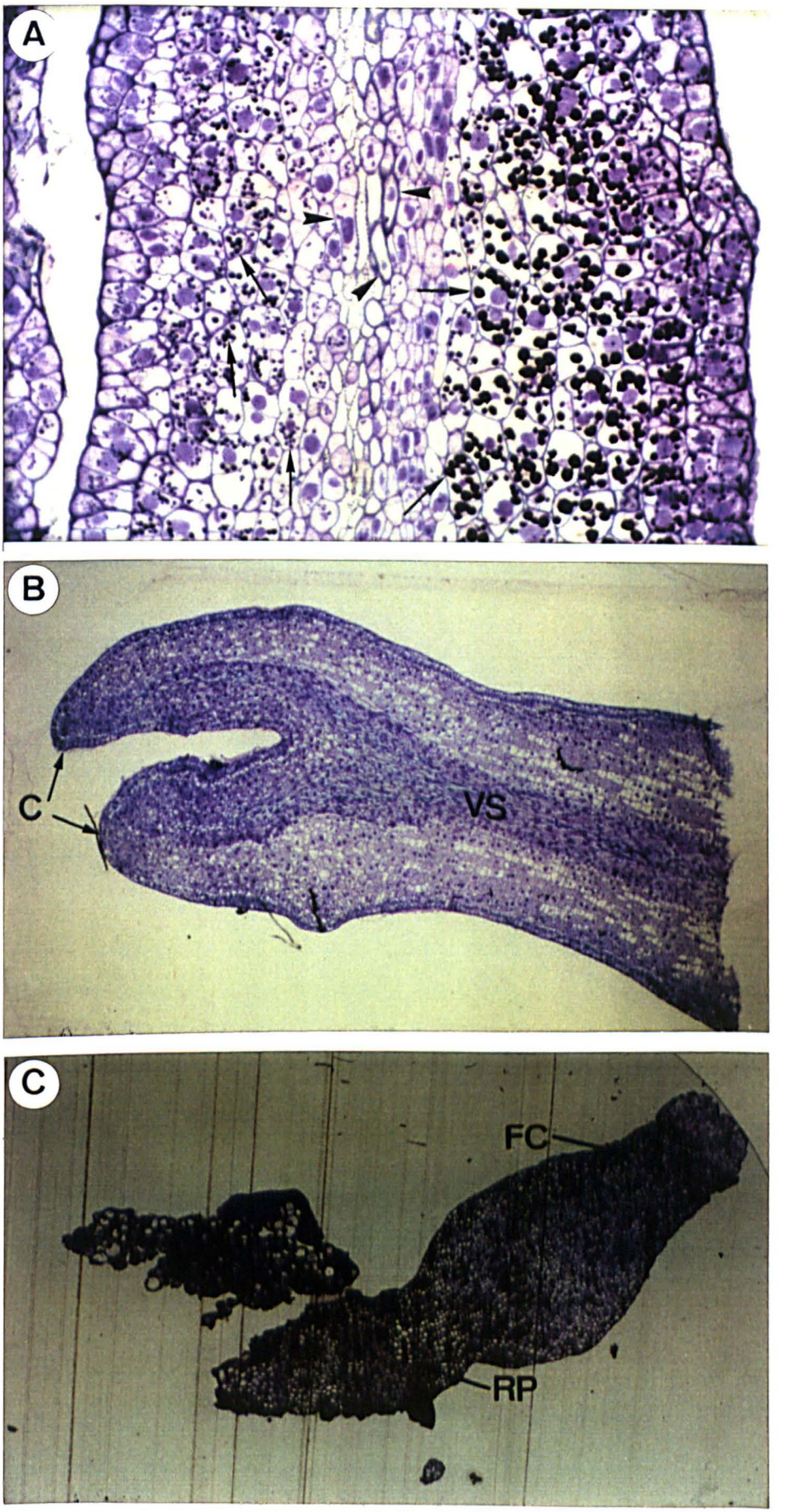
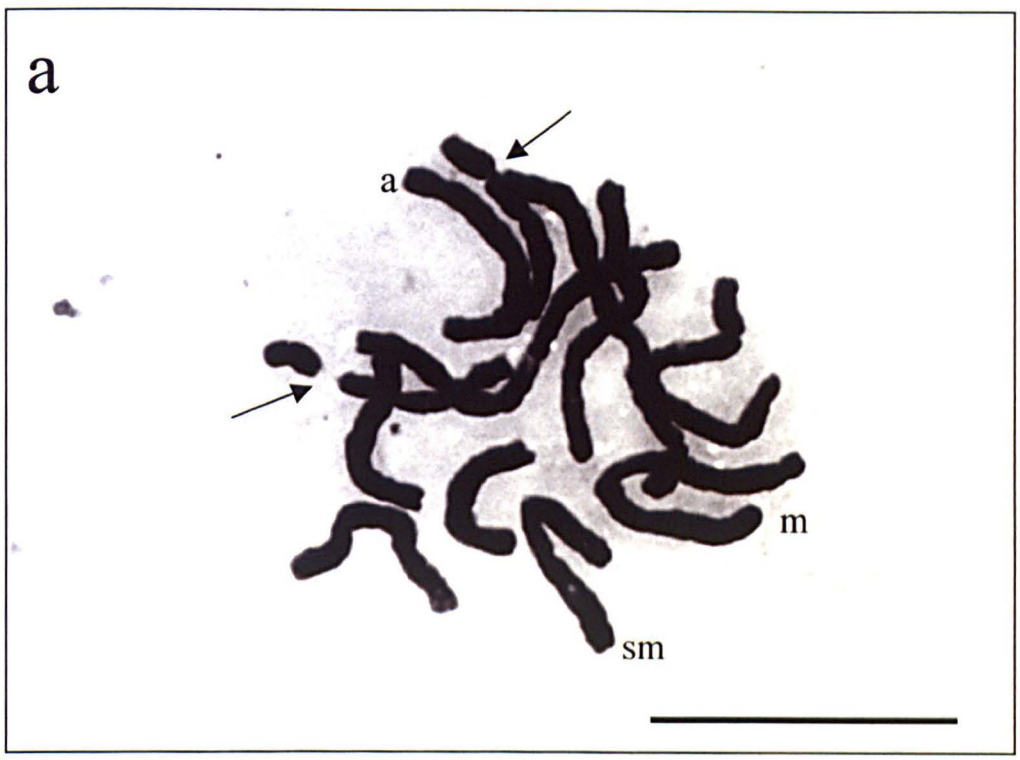
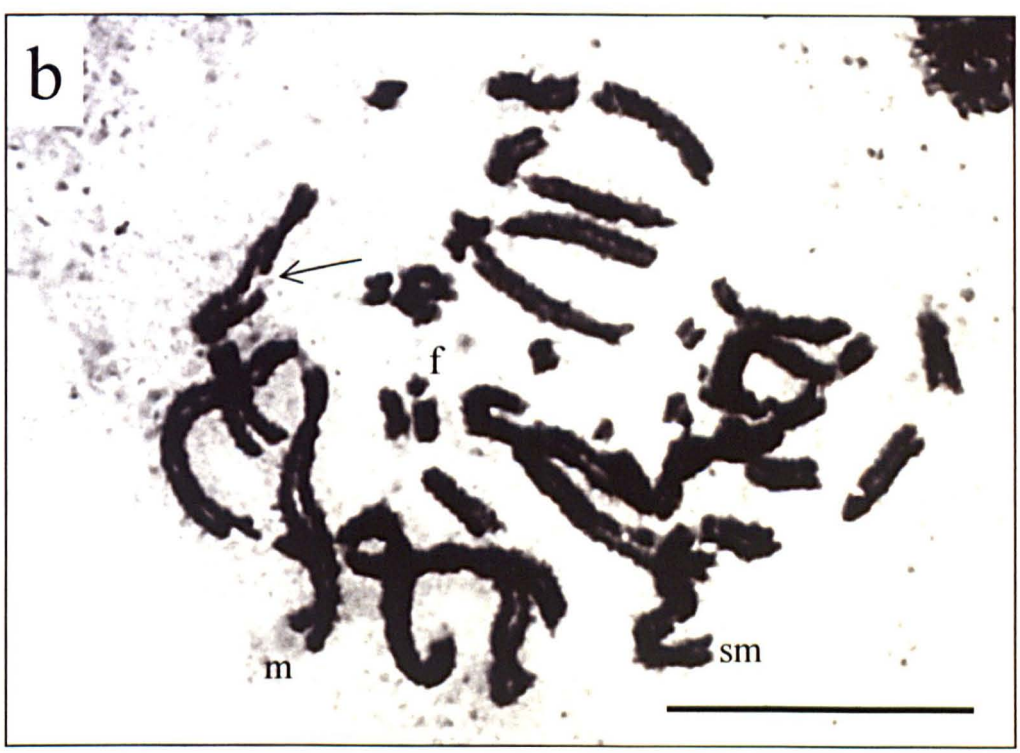

A
B
C
VS
C
FC
RP

Plate 3.6: Cytogenetic comparison of cultivated and regenerated *Diphylleia cymosa*

A. Metaphase in a control plant from a propagated clone showing a complement of $2n = 2x = 12$ somatic chromosomes, comprising metacentric (m), sub-metacentric (sm), acrocentric (a) chromosomes and the nucleolar organizer region (NOR) (arrows) stained with aceto-orcein reagent. (Bar = 10 μm).

B. Late pro-metaphase in the *Diphylleia* regenerant showing metacentric (m) and sub-metacentric (sm) chromosomes, a chromosome fragment (f) and the point of a chromatid fracture (arrowed), following staining with Feulgen and aceto-orcein reagents. (Bar = 10 μm).


a
a
m
sm


b
f
m
sm

3.3.5.7 Effects of *Pluronic*® F-68 and Tween 20 on callus biomass

After 30 d culture, calli cultured on UM medium with 0.01 and 0.1% (w:v) *Pluronic*® F-68 exhibited a significantly increased over control biomass production as for both fresh and dry weights ($P < 0.05$) (Table 3.3). In the first treatment, calli increased their size from 1.0 cm diam. (original callus size) to 1.5 cm, without any change in colour. The largest increase in biomass (f. wt. gain) was observed in calli, after 45 d culture, on medium containing 0.01% (w:v) *Pluronic*® F-68. These increased from 0.29 ± 0.07 g (initial f. wt.) to 0.48 ± 0.12 g compared to 0.15 ± 0.03 g (initial f. wt.) to 0.25 ± 0.04 g (45 d) for control cultures.

Inclusion of *Pluronic*® F-68 in UM medium also influenced phenolic oxidation of explants. The level and release of phenolic compounds was higher for cultures on UM medium with 0.1% (w:v) *Pluronic*® F-68 after 60 d. This result suggested that there was probably a correlation between browning of plant tissue *in vitro* and the concentration of *Pluronic*® F-68 used. In contrast, control cultures did not exhibit phenolic oxidation and all calli retained their initial colour (pale yellow).

The addition of Tween 20 to UM medium did not stimulate biomass production (f. wt. gain) as measured after 15 and 30 d culture. However, supplementation with 0.1% (v:v) Tween 20 did significantly ($P < 0.05$) increase cell growth compared to control [0% (v:v) surfactant] after 45 d culture, although this was not sustained over a further 15 d culture (Table 3.3).

3.3.5.8 Establishment and maintenance of cell suspensions

3.3.5.8.1 Effects of subculture régime and culture medium

Friable calli, derived from leaf and petiole explants, behaved comparably during the initiation of cell suspensions. Initially, the calli transferred to UM medium grew to form large clumps of cells (1.0 to 3.0 mm diam.). However, after several subcultures the inoculum separated into small cell aggregates and single cells. Cells of both cultures were pale-yellow in colour (Plate 3.7.A) and cytoplasmically dense, and spherical (Plate 3.7.B). Petiole-derived cell suspensions became necrotic in the absence of conditioned medium. Friable leaf callus was more abundant on MSP2 medium than other media evaluated to initiate cell suspensions. However, these calli failed to grow even though L-cysteine (85 µl of a stock solution, Sigma, UK) was added to the medium, in an attempt to control phenolic oxidation. Both the callus and culture medium turned brown and callus could not be rescued.

The percentage viability of cell suspensions measured 3 d after subculture in UM medium was 76% for leaf and 88 - 90% for petiole.

Table 3.3: Effects of *Pluronic*® F-68 and Tween 20 on biomass growth of callus from leaf explants of *D. cymosa* assessed after 15, 30, 45 and 60 days culture

Surfactant concentration (% w:v, v:v)[1]	Days of culture							
	15		30		45		60	
	f. wt	d. wt	f. wt	d. wt	f. wt	d. wt	f. wt	d. wt
Pluronic®								
0	0.15 ± 0.03	0.02 ± 0.01	0.13 ± 0.03	0.01 ± 0.01	0.25 ± 0.04	0.02 ± 0.01	0.30 ± 0.10	0.02 ± 0.01
0.001	0.11 ± 0.02	0.02 ± 0.01	0.09 ± 0.02	0.01 ± 0.01	0.15 ± 0.06	0.02 ± 0.02	0.28 ± 0.23	0.02 ± 0.02
0.01	0.29 ± 0.07*	0.02 ± 0.01	0.25 ± 0.07*	0.03 ± 0.01*	0.48 ± 0.12*	0.04 ± 0.02	0.42 ± 0.09	0.03 ± 0.01
0.1	0.23 ± 0.09*	0.02 ± 0.01	0.27 ± 0.13*	0.03 ± 0.01*	0.23 ± 0.13	0.02 ± 0.02	0.33 ± 0.19	0.03 ± 0.02
Tween 20								
0	0.13 ± 0.04	0.02 ± 0.01	0.14 ± 0.05	0.01 ± 0.01	0.10 ± 0.03	0.01 ± 0.01	0.09 ± 0.03	0.01 ± 0.01
0.001	0.12 ± 0.02	0.01 ± 0.01	0.16 ± 0.08	0.01 ± 0.01	0.13 ± 0.07	0.01 ± 0.01	0.11 ± 0.03	0.01 ± 0.01
0.01	0.12 ± 0.03	0.01 ± 0.01	0.12 ± 0.03	0.01 ± 0.01	0.11 ± 0.05	0.01 ± 0.01	0.08 ± 0.02	0.01 ± 0.01
0.1	0.12 ± 0.03	0.01 ± 0.01	0.13 ± 0.03	0.01 ± 0.01	0.16 ± 0.03*	0.01 ± 0.00	0.11 ± 0.03	0.01 ± 0.01

[1] Ratio (w:v) for *Pluronic*® F-68 and (v:v) for Tween 20; f. wt = fresh weight (g); d. wt = dry weight (g).
Values are mean ± s.d. of 5 replicates from experiments repeated 3 times; *$P < 0.05$ compared with corresponding mean control value (0% surfactant) using one-way ANOVAs incorporating a Tukey-HSD post-Hoc test; n = 10 calli were used for each treatment.

3.3.5.8.2 Growth curves of cell suspension cultures

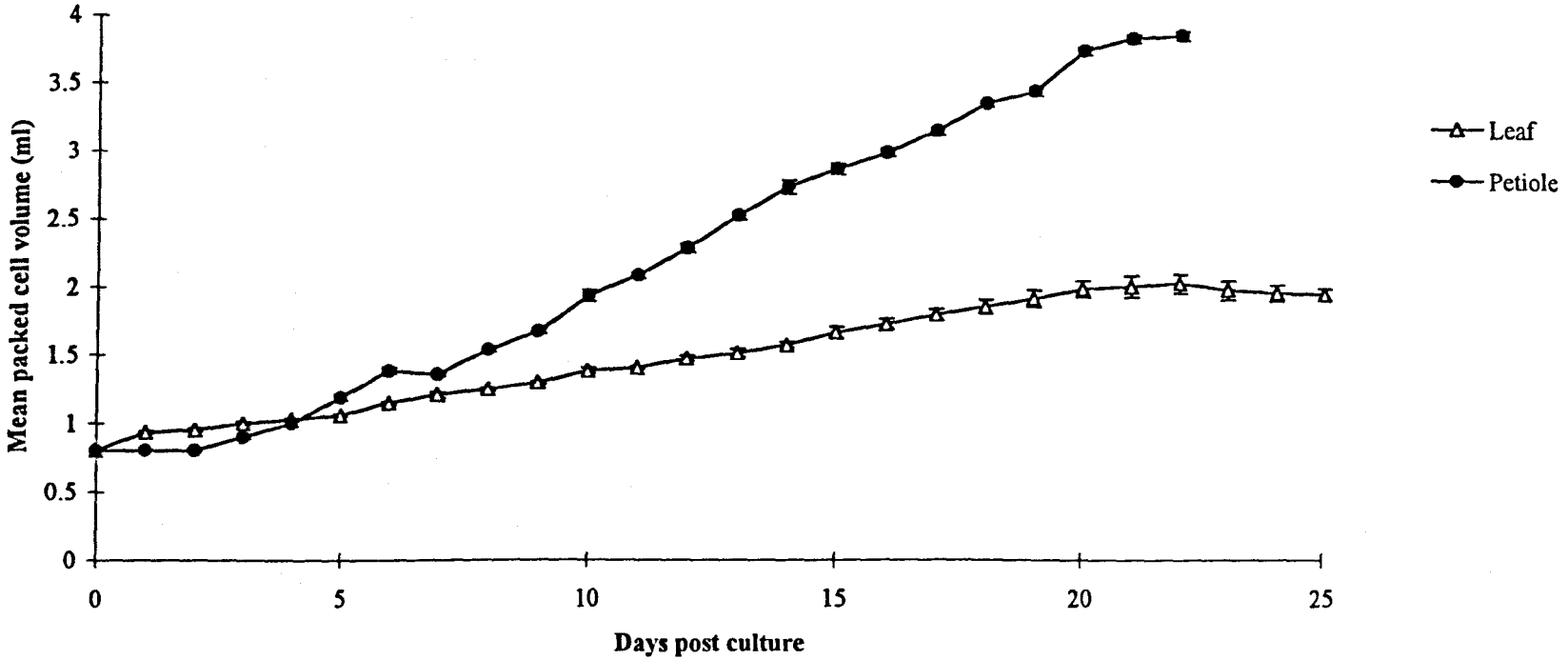
Cell suspensions of *D. cymosa* gave sigmoidal growth curves in UM medium. Petiole-derived cell suspensions in UM medium showed a faster growth compared to that of leaf cell suspensions. PCV doubled after 10 d for petiole cultures and after 15 d for leaf cell suspensions (Fig. 3.3). The PCV of the petiole cell suspensions increased more than 4.5 fold before the stationary phase was reached whereas the PCV of leaf cell suspensions increased up to 2.5 fold. As shown in Fig. 3.3 petiole cell suspensions showed a lag phase of 2 d and an exponential phase of 18 d, while leaf cell suspensions showed no lag phase and an exponential phase of 20 d.

3.3.5.9 Cryopreservation of petiole-derived cell cultures

The addition of 6% (w:v) mannitol during the pre-growth stage induced freeze tolerance in cells. In the initial experiment, the use of cryprotectant A did not significantly increase the mean post-thaw cell absorbance (TTC) assessed 4 d after thawing (0.34 ± 0.12; n = 20), compared with the mean absorbance of the cryopreserved cells lacking cryoprotectant (treatment 2; 0.49 ± 0.24). In the second experiment, the use of cryoprotectant B increased the mean viability of cryopreserved cells (0.62 ± 0.24; n = 20) compared with cryopreserved cells lacking cryoprotectant (treatment 2: 0.54 ± 0.22). However, only the viability of unfrozen cells (1.10 ± 0.36) was statistically significant ($P < 0.05$) compared to cells frozen in the presence of cryoprotectants A and B.

Cells cryopreserved in the presence of cryoprotectants A or B and subsequently cultured on semi-solid UM medium became dark-brown in appearance and did not increase in biomass (Plate 3.7.A,B). After 90 d, a smaller increase in biomass occurred for cryopreserved cells without cryoprotection (1.14 ± 0.78) (Plate 3.7.C). The greatest increase in biomass occurred with unfrozen cells (1.43 ± 1.17; n = 20) (Plate 3.7.D). Such callus was friable in appearance as described previously (see Results, Section 3.3.1.7) and retained the pale yellow colour typical of the cell suspensions. Although results of the two treatments were nor statistically significant, the results from these experiments indicated that callus growth was better for unfrozen cells. Suspension cultures were re-initiated from thawed cryopreserved cells (without cryoprotection, treatment 2) and unfrozen cells (treatment 3).

Fig. 3.3: ***Diphylleia cymosa*** **cell suspension growth curves in UM medium**



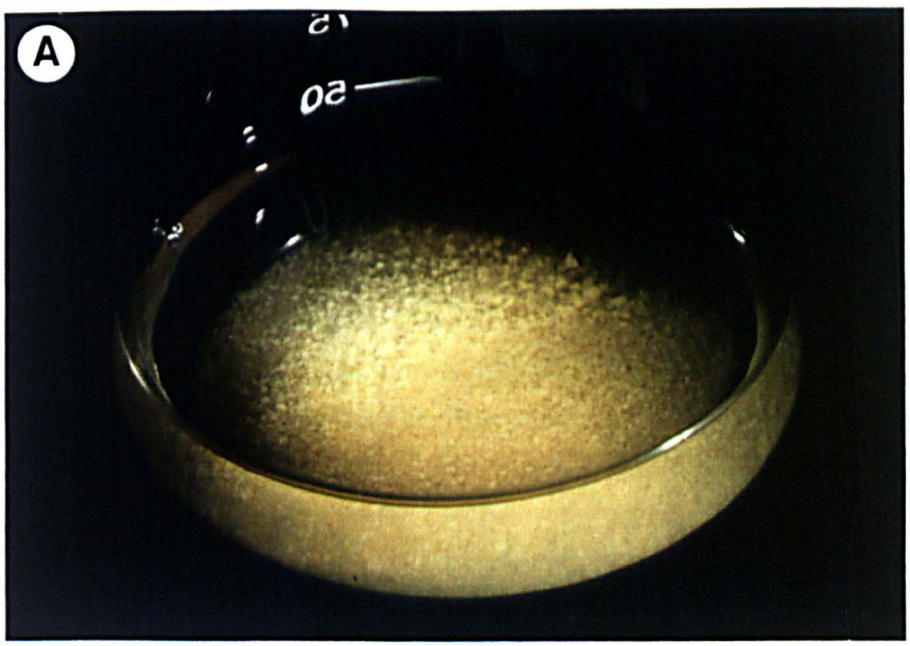
Values are mean ± s.e. of 5 readings for each flask (n = 4 throughout) and the experiment was repeated twice.
Leaf and petiole-derived cell suspension cultures were 6 and 13 months old respectively.

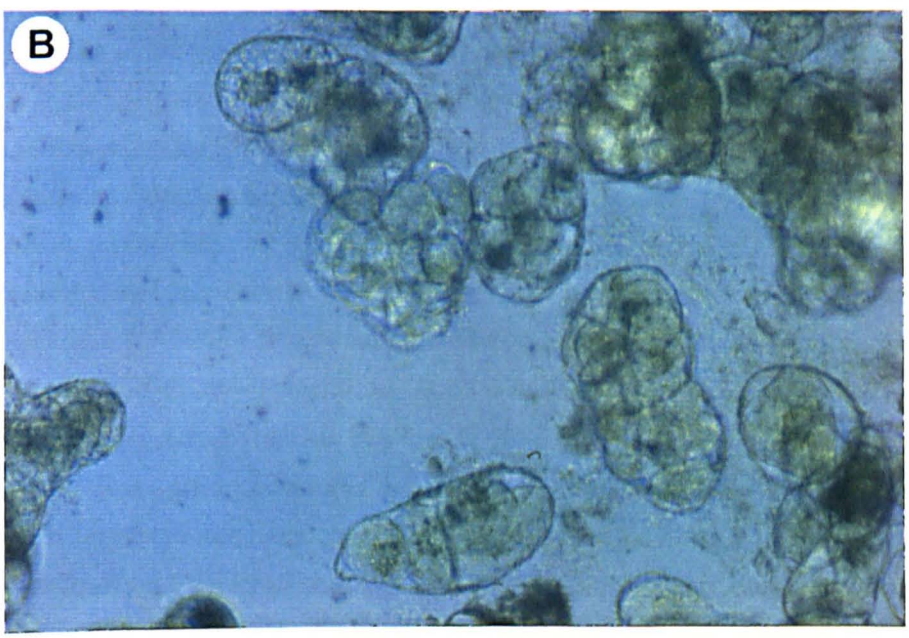
Plate 3.7: Leaf-derived callus cell suspension cultures of *Diphylleia cymosa*

A. Cell suspension cultures (6 months-old) in liquid UM medium. (x 1.12).

B. Leaf-derived callus cells in suspension culture in UM medium showing mitotic division. (x 385).

C. Growth of post-thaw cryopreserved cells with cryoprotectants A (top left) and B (top right), without cryoprotectants (bottom left) and unfrozen cells (bottom right). (x 0.44).


A


B


C

3.4 Discussion

These studies overall have demonstrated, for the first time, that *D. cymosa* can be introduced into tissue culture. However, during this study a number of difficulties were encountered including the limited amount of plant germplasm that was available. This was a major constraint in repeating the experiments. As discussed previously for *P. hexandrum* (Chapter 2), *D. cymosa* also displayed a short growing season. Due to problems of contamination, which were not overcome, it was not possible to establish axenic shoot cultures with the limited amount of material available (no seeds) to this study. The first leaves appeared in May and then started to die in September. The plant remained dormant in the ground during the winter. It was not possible to overcome dormancy by maintaining plants in the glasshouse during the winter period. Thus, germplasm collection for the tissue culture experiments could only be carried out during four months of the year. In these experiments, only a few types of explants can be used since there are no nodes, branches or multiple leaves on non-flowering shoots due to the aerial anatomy; seeds were difficult to obtain.

In *D. cymosa*, induction of embryogenic calli from leaf explants was observed at the lower concentrations of picloram evaluated (4.8 or 7.2 mg l^{-1}) even though cell suspensions could not be initiated. Picloram has been reported to promote embryogenic callus in young leaf explants of *Passiflora giberti* (Otoni, 1995), *Manihot esculenta* (cassava) (Taylor *et al.*, 1996) and *Saccharum* spp. (sugarcane) (Aftab *et al.*, 1996) from which embryogenic cell suspensions were successfully established. Petiole explants of *Diphylleia* responded to other growth regulator combinations tested such as NAA and BAP. In these experiments, the embryogenic response was more dependent on NAA concentration than that of BAP. The plant growth regulator combinations from which petiole embryogenic callus was originated also influenced induction and subsequent germination of somatic embryos.

Browning of the tissues and staining of the medium observed in the experiments for callus induction may be attributed to several factors as described previously (Chapter 2, Section 2.4). In higher plants, oxidation inhibits growth of excised tissues and their calli. Short interval subculture régimes have been suggested (Nomura *et al.*, 1998) in order to preserve cultures. In *D. cymosa*, regular subculture of tissues and calli to fresh medium at short intervals inhibited the staining of the culture medium and browning of the leaf and petiole explants and calli derived therefrom.

Somatic embryogenesis in *D. cymosa* was obtained indirectly through a callus phase. Somatic embryos were induced from leaf-derived callus on medium with 2,4-D in combination with 0.25 mg l^{-1} kinetin. In rice, lower concentrations of kinetin (0.1 mg l^{-1}) have been effective in promoting embryogenic calli (Yoshida *et al.*, 1994).

The cytokinin used can affect the induction of embryogenesis, although the exact effect of cytokinins during embryo induction is not clear (Merkle *et al.*, 1995). The auxins have an essential role in somatic embryogenesis; they act as a trigger for the dedifferentiation during which the tissues becomes competent to respond to the embryogenic stimulus and to form an embryo (DeKlerk *et al.*, 1997). The synthetic auxins 2,4-D and NAA and the natural auxin IAA are commonly employed as inducers for somatic embryogenesis. Somatic embryos of *D. cymosa* albeit at a low frequency were induced for leaf-derived callus in the presence of 2,4-D. Embryogenic cells though lose embryogenic competence becoming non-embryogenic after a long duration in culture medium (Kiyosue *et al.*, 1993) and the continued exposure to auxins such as 2,4-D leads to the induction of secondary embryogenesis (Emons, 1994). In *D. cymosa*, secondary embryogenesis was observed in somatic embryos at the cotyledonary stage when cultured with GA_3. Mature somatic embryos can be a source for secondary embryos (Raemakers *et al.*, 1995). The latter was also observed in embryogenic callus cultures derived from leaf explants after prolonged culture in picloram-containing medium. The same phenomenon was reported for embryogenic callus of *Hordeum vulgare* (barley) cultured with picloram (Kachhwaha *et al.*, 1997). Moreover, a significant interaction was observed between the callus and the attached progenitor leaf explant in terms of embryo production in *D. cymosa*. The number of embryos per callus was greater when the leaf tissue remained attached to the callus, indicating a role of the original explant in the triggering of somatic embryogenesis. In *Diphylleia*, embryogenesis only occurred in the dark as for *P. hexandrum* (Chapter 2, Section 2.4).

In *D. cymosa*, somatic embryos showed different degrees of morphological abnormalities, particularly fused cotyledons. Such abnormalities involving cotyledons has often been reported in somatic embryogenesis (Yeung, 1995) and occurs with many species such as *Theobroma cacao* (Alemanno *et al.*, 1996). The occurrence of abnormalities in the histodifferentiation of somatic embryos is affected by the choice of growth regulators used. Histodifferentiation of somatic embryos and their maturation requires the removal of growth regulators from the medium or their use at lower concentrations compared to the levels used for induction and proliferation (Merkle *et al.*, 1995). Of the growth regulators used, ABA and zeatin induced abnormalities in *Diphylleia* somatic embryo development.

In *D. cymosa* somatic embryos matured in response to an increase of sucrose concentration in the culture medium. Exogenously-supplied carbohydrates during maturation appear to be important in terms of embryo quality and extent of maturation (Merkle *et al.*, 1995). In *Diphylleia*, the process of embryo maturation was histochemically confirmed through the identification of starch granules in

cotyledons. The presence of starch has been shown to be linked to maturation of somatic embryos (Merkle *et al.*, 1995) and plantlet development (Camaño, 1999). Starch in the cotyledons of somatic embryos of *D. cymosa* was consistent with other reports whereby cotyledons and hypocotyls are sites of accumulation for starch, lipids and proteins (Gutmann *et al.*, 1996).

In this study, during post-maturation of somatic embryos, a residual ABA from maturation may have inhibited further development. Some auxins can inhibit shoot and root formation, thus their removal from the medium prior to germination of somatic embryos is important. In order to do this, GA_3 and water stress can be applied (Merkle *et al.*, 1995). In some species, a synchronous development of shoot and root axes in embryos has been attributed to desiccation (Szabados *et al.*, 1987). In this study with *D. cymosa*, the effect of water stress, induced by 1% (w:v) agarose, was assessed in order to perhaps promote plant regeneration. Even though only one plant was obtained, regeneration was observed after a two-step procedure with agarose. In rice, the same concentration of agarose and partial desiccation enhanced the frequency of somatic embryos and plant regeneration (Fernando, 1997). Plant regeneration from somatic embryos of cassava has also been improved by desiccation prior to germination (Mathews *et al.*, 1993). However, in the case of *D. cymosa*, desiccation followed by culture on medium with ABA prevented germination of the somatic embryos. Studies with other species, for example white clover (Parrot, 1991), have demonstrated that not all species require desiccation in order to obtain germination of somatic embryos (Merkle *et al.*, 1995). The presence of GA_3 had a positive effect on embryo differentiation and post-maturation although it may account for fused cotyledons. However, in cocoa the use of low concentrations of GA_3, NAA and zeatin have been shown to stimulate growth and differentiation of somatic embryos (Adu-Ampomah *et al.*, 1988).

These studies with *D. cymosa* indicated that none of the plant growth regulators tested were appropriate for obtaining an efficient and reproducible regeneration system from somatic embryos. In grape, the arrest of somatic embryo development has its origins at an early stage of development (Coutos-Thevenot *et al.*, 1992); traditional treatments have not been effective in the conversion of grape embryos to plants (Goebel-Tourand *et al.*, 1993). Studies with asparagus, soybean and sweet potato have shown that auxin levels affected the morphology of embryos and consequently their conversion to plantlets (Wetzstein and Baker, 1993). Genotype and choice may be the overriding factor here for the recalcitrance of *Diphylleia* (Chengalrayan *et al.*, 1998), whilst orientation of the embryo, during germination, and environmental manipulations (Merkle *et al.*, 1995) may also be relevant. More studies are certainly necessary in order to improve somatic embryogenesis in *D. cymosa*.

Another finding from this investigation was the non-responsiveness of leaf and petiole explants of *D. cymosa* to exogenously-applied phenylureas, in particular, CPPU and TDZ. Both are substituted phenylurea-based growth regulators that exhibit strong cytokinin-like activities in various physiological processes. CPPU has a role in the regulation of cell division and growth, and has been reported to stimulate direct somatic embryogenesis in peanut (Murthy and Saxena, 1994). In *D. cymosa*, callus formation was observed when leaf and petiole explants were cultured with CPPU (in combination with 2,4-D) and although calli had an embryogenic-like appearance, they failed to complete embryogenesis.

It was not possible to induce organogenesis as an alternative regeneration pathway. TDZ has been used in the tissue culture of *Mentha* x *piperita* and *M. spicata* (Faure *et al.*, 1998) in order to induce organogenesis and can also promote somatic embryogenesis in peanut (Murthy *et al.*, 1995). In leaf and petiole explants of *Diphylleia*, failure of an organogenic response may not only be due to the low concentrations of TDZ used. It appears that species and tissue type are determining factors in the ability to respond to phenylurea. A recent report has demonstrated that nodal explants of *Bambusa edulis* cultured with 0.1 mg l^{-1} TDZ produced multiple shoots (Lin and Chang, 1998), whilst at higher concentrations it has stimulated somatic embryogenesis in callus from seeds of *Azadirachta indica* (Murthy and Saxena, 1998).

Based on cytological studies, the somatic chromosome number of $2n = 2x = 12$ was constant for all control plants analysed. This was consistent with an earlier study by Langlet (1928) which has, to date, been the only published chromosome data for *D. cymosa*. Other authors (Ying *et al.*, 1984) have raised doubts as to the identity of the material examined by Langlet as no herbarium voucher specimens can now be traced. In this study, the cultivated plants appeared comparable to specimens studied by Langlet; cytological studies revealed a presence of relatively large chromosomes (6 - 15 μm).

Loss or gain of somatic chromosomes (and structure) in plant tissue cultures (cell and regenerants) have been suggested to be one of the main causes of a lack or loss of regeneration potential (Ronchi, 1995). This was observed in the current studies; however, since only one plant was obtained thus, the possible degree of somaclonal variation could not be adequately assessed for *Diphylleia*. The occurrence of a few abnormalities such as fragments in some cells suggests that somaclonal variation may be significant. However, these results must be regarded as insufficient to draw any firm conclusions in relation to chromosome stability in the regenerant of *D. cymosa*.

The results of the assessments of *Pluronic*® F-68 and Tween 20 supported the general observation that the concentration of surfactants can influence biomass, with lower concentrations of *Pluronic*® F-68 being ellective for *Diphylleia*. Such variation in responsiveness of cells, tissues and organs to *Pluronic*® F-68 is already known (Lowe *et al.*, 1993) and is almost certainly genotypically-dependent (Brutovská *et al.*, 1994).

Browning of calli was minimal in the presence of the lowest concentrations of *Pluronic*® F-68; higher levels led to cell death. *Pluronic*® F-68 has been reported to inhibit the onset of necrosis in transformed roots of jute (Khatun *et al.*, 1993b) and chrysanthemum explants (Khehra *et al.*, 1995) which may be due, at least in part, to enzymatic changes. Previous studies on *S. dulcamara* suggested an activation of key metabolic enzymes in root and callus cultured in the presence of this surfactant (Kumar *et al.*, 1992).

The experiments involving cryopreservation demonstrated that *Diphylleia* cell suspensions showed freeze tolerance with mannitol treatment during the pre-growth phase. Cell size, vacuolar volume, water content and other factors may affect survival of cells in liquid nitrogen (Friesen *et al.*, 1991). Studies using mannitol as a pre-growth additive have given different responses, for example, *Catharanthus roseus* cells exhibited intolerance to mannitol treatment (Chen *et al.*, 1984). However, use of mannitol increased the amount of viable cells recovered following *D. lanata* cryopreservation of suspension cultures (Diettrich *et al.*, 1982).

Although cryopreserved cells lacking cryoprotectants and unfrozen cells were partially dehydrated, they still underwent mitotic division which was reflected by an increase in biomass even though this was not statistically significant.

Chapter Four

Isolation and Identifcation of Lignans from Rhizomes and Roots of *Podophyllum* Species

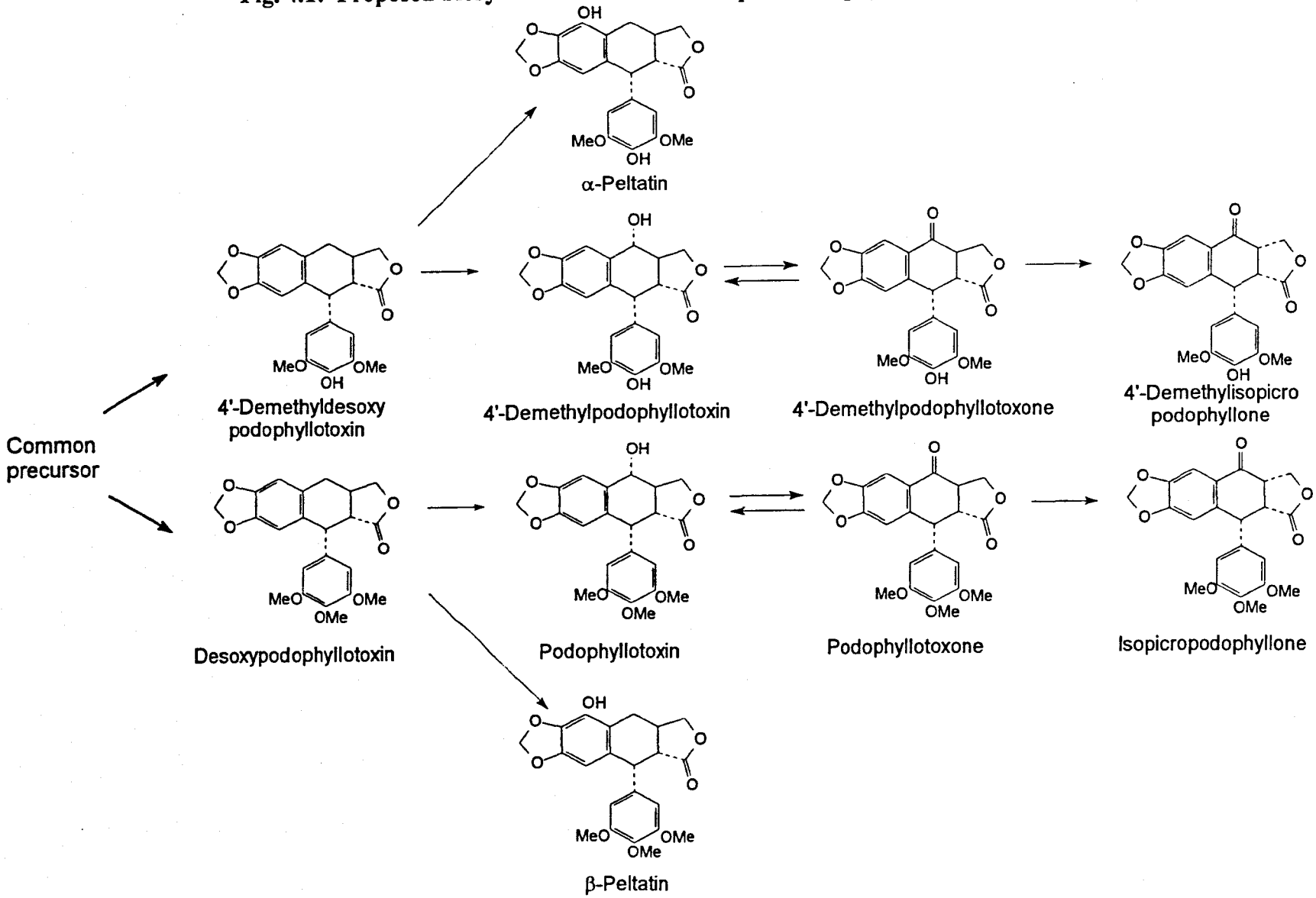
4.1 Introduction

The successful development of clinical anticancer drugs derived from the natural aryltetralin lactone lignan podophyllotoxin has drawn more attention to *Podophyllum*-based lignans. Lignans and their glucosides have been isolated and identified from the roots of *P. hexandrum* and *P. peltatum*; both species contain the same ten tumour-inhibitory lignans (Fig. 4.1) based on an aryltetralin lactone skeleton. In *P. hexandrum*, the podophyllotoxin content (4.3% d. wt.) is higher than that of *P. peltatum* (0.25% d. wt.) (Jackson and Dewick, 1984c). Seasonal variations and the age of the plants have been reported to influence the lignan contents of both species (Ayres and Loike, 1990). In *P. peltatum* roots, the contents of peltatins are low during the growing season probably due to metabolic turnover and reach maximum during dormancy (Kamil and Dewick, 1986a).

4.1.1 Biosynthesis of *Podophyllum* lignans

Studies on the biosynthetic pathways of *Podophyllum* lignans started with phytochemical investigations of the lignans present in *P. hexandrum* and *P. peltatum* (Jackson and Dewick, 1984c). They reported the presence of two groups of lignans: the first formed by lignans with 3',4',5'-trimethoxy substitution in the pendent aromatic ring (*e.g.* podophyllotoxin) whilst the second has a 4'-hydroxy-3',5'-dimethoxy substituted pendent ring as in 4'-demethylpodophyllotoxin. Supply of *P. hexandrum* with labelled aryltetralin lignans demonstrated that the two lignan groups were biogenetically distinct (Jackson and Dewick, 1984a, 1984b). Hydroxylation of desoxypodophyllotoxin resulted in podophyllotoxin, which oxidizes to the ketone podophyllotoxone. A similar sequence occurs with the 4'-demethyl series: the lignans 4'-demethylpodophyllotoxin and 4'-demethylpodophyllotoxone derived via 4'-demethyldesoxypodophyllotoxin. The hydroxylation of desoxypodophyllotoxin or 4'-demethyldesoxypodophyllotoxin at C-5 position leads to the formation of the peltatins β-peltatin and α-peltatin, respectively (Kamil and Dewick, 1986a). The two groups of lignans are biogenetically distinct, however, they probably arise from a common precursor. A scheme has been proposed (Dewick, 1989) showing the biosynthetic interrelationship for all the *Podophyllum* aryltetralin lignan aglycones (Fig. 4.1).

Fig. 4.1: Proposed biosynthetic interrelationship for *Podophyllum*-based lignans



Modified from Dewick (1989).

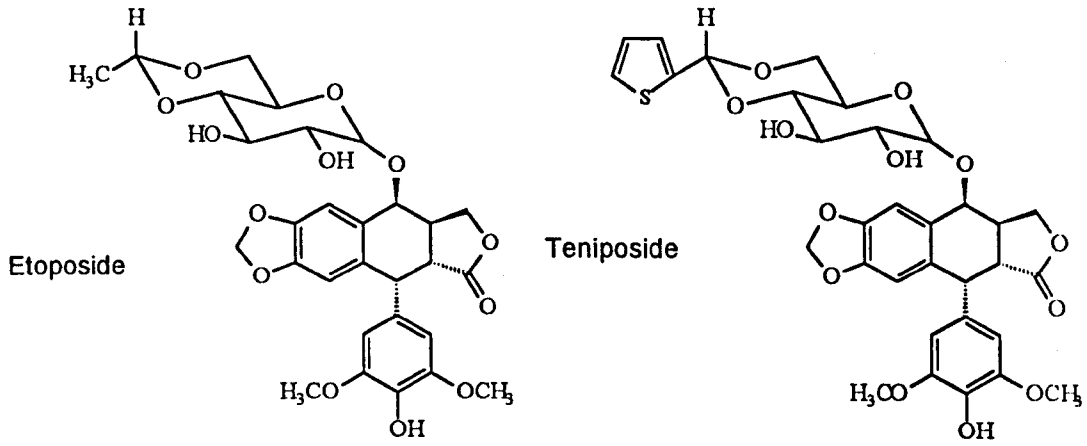
The ketones 4'-demethylpodophyllotoxone, 4'-demethylisopicropodophyllone and isopicropodophyllone (Fig. 4.1) have been reported as extraction artefacts (Jackson and Dewick, 1985). The results of feeding experiments with *P. hexandrum*, revealed that the dibenzylbutyrolactone lignan, yatein, was a satisfactory precursor of podophyllotoxin (Kamil and Dewick, 1986b). Moreover, matairesinol has been reported probably as being the branch-point compound to the two groups of *Podophyllum* lignans (Broomhead *et al.*, 1991). The latter study demonstrated that matairesinol was a common precursor of the 3', 4', 5'-trimethoxy and 4'-hydroxy-3', 5'-dimethoxy groups in *P. hexandrum*, *P. peltatum* and also in *Diphylleia cymosa*.

4.1.2 Biological and clinical properties

Due to the important nature of the *Podophyllum* lignans, they have been exhaustively investigated and show a wide range of biological activities. Although toxic, podophyllin resin is topically applied to treat external genital or perianal lesions as well as vaginal and anal warts (Speight and Holford, 1997). Podophyllotoxin and podophyllin are both used for self-treatment of penile warts. The use of podophyllin has reduced the treatment costs and thus it is recommended in countries with limited resources for healthcare (White *et al.*, 1997). Podophyllotoxin has shown antiviral activity (Bedows and Hatfield, 1982) and it may also be useful for treating psoriasis (Ayres and Loike, 1990). 4'-*O*-Demethyldehydropodophyllotoxin and picropodophyllone exhibit strong antifungal activity (Rahman *et al.*, 1995) whilst novel derivatives, related to podophyllotoxin, have shown inhibition of growth of *Saccharomyces cerevisiae* and *Aspergillus niger* (Salako, 1996).

Podophyllotoxin is an anticancer agent, however, due to toxic side effects its use to treat human neoplasia is limited (Speicher and Tew, 1993). Several studies have been undertaken on chemical modification of the podophyllotoxin structure in order to obtain derivatives that could be more clinically satisfactory. The drugs etoposide (or VP-16-213), 4'-demethyl-7-[4,6-*O*-ethylidene-β-D-glucopyranosyl epipodophyllotoxin] and teniposide (or VM-26), 4'-demethyl-7-[4,6-*O*-thenilidene-β-D-glucopyranosyl epipodophyllotoxin] are semi-synthetic derivatives of *Podophyllum* lignans (Fig. 4.2) used in cancer chemoterapy (Lewis *et al.*, 1995). Both compounds arrest cell growth by inhibiting DNA topoisomerase II which causes double-strand breaks in DNA. They are active against Hodgkin's disease and non-Hodgkin's lymphomas (Jardine, 1980). Etoposide is an effective agent for the treatment of a variety of tumours (Pratt *et al.*, 1994). It is used in combination with cisplatin in the treatment of germinal testicular cancer (Ayres and Loike, 1990), small cell lung cancers and brain neoplasmas (Muggia and von Hoff, 1997).

Fig. 4.2: Structures of etoposide and teniposide



Teniposide showed activity in neuroblastoma and acute lymphocytic leukaemias (Pratt *et al.*, 1994), predominantly child leukaemias. It is also an alternative drug, combined with carmustine, to treat adult brain neoplasmas (Muggia and von Hoff, 1997). The development of podophyllotoxin derivatives, which can act as topoisomerase inhibitors, has been suggested as a promising area of research (Dewick, 1997). Recent studies of structure-activity of podophyllotoxin have revealed new chemically modified congeners with potential antitumour activity such as NK 611, which is now in clinical trial (Damayanthi and Low, 1998).

4.1.3 Analytical techniques and approaches for *Podophyllum* lignans

Chromatographic methods such as PLC, TLC, column chromatography and HPLC have been used to separate and identify aryltetralin lignans from the roots of *Podophyllum* species (Jackson and Dewick, 1984c; Konuklugil, 1993). TLC has some advantages over other methods including simplicity, low cost and versatility in the choice of stationary and mobile phases, allowing simultaneous development of a number of samples and standards on the same plate (Sherma and Fried, 1996). However, TLC is more efficient as an analytical method for *Podophyllum* lignans when coupled with HPLC and spectroscopic methods. Studies on the quantification of podophyllotoxin and related compounds in rhizomes/roots of *Podophyllum* species by reverse-phase HPLC have been reported (Broomhead, 1989). This latter method provided an efficient separation of podophyllotoxin and related compounds in *Podophyllum* with a Taxsil® column and gradient elution (Bastos *et al.*, 1995). The same system was also used to evaluate the aryltetralin lignan contents of different tissues of *P. peltatum*, utilising added acetylpodophyllotoxin as internal standard (Bastos *et al.*, 1996). NMR and mass spectral data have provided complementary information for the identification and structural elucidation of *Podophyllum* lignans.

This chapter reports on the isolation and purification of aryltetralin lignans from the rhizomes and roots of *P. hexandrum* and *P. peltatum*. These studies were necessary in order to obtain authentic standards which were further used for the qualitative analysis of *D. cymosa* plants and tissue culture material (Chapter 5). In addition, these studies were also found important in order to gain experience in the isolation and identification of *Podophyllum* lignans. The isolated compounds were identified by comparison with standards on TLC plates and HPLC, ^{1}H NMR and by mass spectroscopy.

4.2 Materials and methods

4.2.1 Plant materials

A mixture of dried rhizomes and roots of *P. hexandrum* were purchased from United Chemical and Allied Products, Calcutta, India whilst those for *P. peltatum* were obtained from Joseph Flach, London, UK.

Rhizomes/roots of both *Podophyllum* species were ground in an electric mill and powdered plant material stored in light-proof jars until required.

4.2.2 Extraction and isolation of *Podophyllum* lignans

4.2.2.1 Extraction procedure

Powdered material of *P. hexandrum* (10 g d. wt.) was extracted with 30 ml 95% (v:v) hot Analar ethanol and the mixture was stirred (10 min) on a hot plate. The resulting mixture was cooled to room temperature, filtered (Whatman No. 1 filter paper) and the residue washed with a further aliquot of 95% (v:v) ethanol (3 x 30 ml). The organic extracts were combined and concentrated to dryness under reduced pressure in a rotatory evaporator at 35°C (Jackson and Dewick, 1984c). The resulting residue was redissolved in 3.5 ml acetone and subjected to PLC to separate lignans. The same procedure was followed for *P. peltatum*.

4.2.2.2 Preparative layer chromatography

The ethanolic extracts containing the lignan aglycones were applied as bands onto preparative glass plates (20 x 20 cm) precoated with a 1.0 mm layer of silica gel (Merck Kieselgel GF_{254}, Merck Ltd, Lutterworth, UK) and transferred to a glass tank containing saturated choroform:methanol (25:1, v:v). The development of the plates was carried out in the ascending direction. The chromatographic zones (bands) were located by visualisation under UV light at wavelengths of 254 and 365 nm or by a TLC spray reagent (Section 4.2.3.3). The relevant bands were individually scraped from the plates for purification of the lignans.

4.2.2.3 TLC spray reagent for detection of *Podophyllum* lignans

An alternative method of detection used a solution of concentrated nitric acid in acetic acid (3:10, v:v) (Jackson and Dewick, 1985) which was sprayed onto the preparative glass plates in order to identify the *Podophyllum* lignans. The plates were left (1.0 min; room temperature) followed by heating (1.0 min) with a hot air dryer to obtain a colour reaction.

4.2.2.4 Purification of lignans

The silica aliquots containing each band separated by PLC were individually transferred to a small sintered-glass columns and eluted with 30 ml acetone (Analar). All collected fractions were evaporated, purified (TLC) and recrystallized.

4.2.2.5 Thin layer chromatography

The fractions eluted (Section 4.2.2.4) were resuspended in acetone and applied to TLC aluminium sheets (20 x 20 cm) precoated with a layer (0.20 mm) of silica gel (Merck Kieselgel GF_{254}). The analytes and standards were spotted, using a glass capillary, and the plates were developed in a saturated chamber with suitable solvent (Results, Section 4.3.1) and air-dried.

4.2.3 Crystallization of compounds

Whenever possible isolated compounds were crystallized (once or twice) from a small volume (0.5 ml) of ethanol or methanol (Sigma), and kept at 0°C for several days prior to characterization by spectroscopic methods.

4.2.4 Identification and characterization of lignans

The isolated compounds were characterized by different methods and their properties compared against authentic standards.

4.2.4.1 Determination of melting point

The melting points were assessed with a Gallenkamp melting point apparatus and are uncorrected.

4.2.4.2 High performance liquid chromatography

4.2.4.2.1 Source of the standard compounds

Standards for desoxypodophyllotoxin and podophyllotoxone were kindly provided by Dr A B de Oliveira (Faculdade de Farmácia, UFMG, Belo Horizonte, Brazil), and for podophyllotoxin, 4'-demethypodophyllotoxin, picropodophyllin and

α-peltatin by Dr A A Salako (Department of Pharmaceutical Sciences, University of Nottingham, Nottingham, UK).

4.2.4.2.2 Standard sample preparation

The lignan standard solutions were prepared at 1.0 mg ml^{-1} (w:v) in 1.0 ml methanol (HPLC grade; Sigma).

4.2.4.2.3 HPLC protocol

Analytical HPLC was carried out using an instrument from Merck-Hitachi (Model D-2500). Samples were filtered by passage through a 0.45 μm single-use filter (Millex). Aliquots (5.0, 15 or 25 μl) were applied with an auto-sampler injection unit (AS - 2000 A) with a 100 μl loop onto a Lichrospher 100 RP-18 column (250 mm x 4.0 mm i.d.; 5 μm) with a pre-column of the same packing (Lichrospher 100 RP-18 column; 5 μm). The solvent was freshly prepared, filtered using a membrane filter (SUPOR) and a GN-6 White/Plain aqueous filter and degassed under vacuum prior to use. The solvent [acetonitrile:water:methanol (37:58:5, v:v:v)] was driven by an L-6200A HPLC pump and was passed through the column (1 h) prior to sample injection. The solvent flow rate was 1.2 ml min^{-1} and peaks were analysed (230 nm) by a UV-VIS spectrophotometer detector (Model L-4250) connected to a D-2500 Chromato-integrator. A minimum of three aliquots were prepared per compound or lignan standard; each aliquot was analysed in triplicate. The retention times were recorded for all samples. The HPLC chromatograms were scanned onto computer and the images enhanced using a combination of the softwares Photoshop® and/or Power Point (Appendix 4).

4.2.4.3 Proton nuclear magnetic resonance spectrometry

Complete assignments of the proton chemical shifts for the individual compounds were accomplished via high resolution ^1H NMR spectra recorded at 400 MHz using a Bruker AM 400 spectrometer at room temperature. Most of the solutions were generally prepared in deuteriochloroform ($CDCl_3$) but for samples of 4'-demethylpodophyllotoxin, in hexadeuterioacetone [$(CD_3)_2CO$]. Tetramethylsilane (TMS) was used as an internal reference.

4.2.4.4 Mass spectrometry

Electron impact (EI) mass spectra were recorded by direct (probe) insertion into an AEI MS 902 mass spectrometer.

4.3 Results

4.3.1 Extraction and isolation of lignans

The ethanolic extracts (495 mg) from rhizomes and roots of *P. hexandrum* and *P. peltatum*, fractionated by PLC, showed under UV light the presence of eight fluorescent chromatographic zones (bands) for both species. Not all bands though were isolated. Bands with their respective R_f (Appendix 5) and the lignans isolated from each species were:

Band 1 (R_f = 0.90)

This, isolated from *P. hexandrum*, was found to be a mixture of desoxypodophyllotoxin and podophyllotoxone, which was successfully separated by further PLC, developed with ether (Broomhead, 1989). Based on comparisons with authenticated materials (on TLC plates), the upper band was identified as podophyllotoxone whilst the lower band was desoxypodophyllotoxin. Both compounds were crystallized from ethanol but some impurities were observed when compared to known, pure standards. Thus they were recrystallized from ethanol yielding 2.2 mg podophyllotoxone and 1.8 mg desoxypodophyllotoxin respectively and identified by HPLC and spectroscopic methods.

Band 2 (R_f = 0.80)

This band isolated from *P. peltatum* was further purified (TLC) using chloroform:isopropanol (10:1, v:v) and resulted in a light brown gum residue. Even though crystallization (from ethanol) was attempted it was not possible to obtain crystals. However, isopicropodophyllone was obtained (yield 0.9 mg) and although it was not pure was identified as such by ^{1}H NMR and HPLC.

Band 3 (R_f = 0.75)

Band 3 (*P. hexandrum*) was initially isolated as a mixture of two bands. In order to separate these bands the fractions were further purified (PLC) with ether (Broomhead, 1989). The upper band (R_f = 0.74) was distinct to the lower band (R_f = 0.63). These bands appeared to be 4′-demethyldesoxypodophyllotoxin (lower R_f) and 4′-demethylpodophyllotoxone (higher R_f). These two compounds were not isolated separately and their subsequent characterization by spectroscopic techniques was not possible.

Band 4 (R_f = 0.60)

This band appeared as a minor band compared with the other bands in both *Podophyllum* species and thus was not isolated.

Band 5 (R_f = 0.50)

This was a major band for *P. peltatum* but minor for *P. hexandrum* and thus was separated for the former species. Further purification by TLC using chloroform:isopropanol (10:1, v:v) and crystallization from ethanol resulted in the compound β-peltatin (yield 11 mg) which was confirmed by spectral data.

Bands 6 (R_f = 0.30) and 7 (R_f = 0.25)

These bands were isolated as a mixture from extracts of *P. hexandrum*. Band 6 was the strongest chromatographic zone observed in this species. It was thus, necessary to further purify (by TLC) this with acetone:petrol [60 - 80°, (1:1, v:v)] in order to separate bands 6 and 7. Podophyllotoxin was isolated and crystallized twice from ethanol (yield 253 mg). This compound was later confirmed as such by spectral data and HPLC. Band 6 was the second major chromatographic zone in *P. peltatum* and was also isolated together with band 7. Band 6 was separated from band 7 in the same way (see earlier this Section) and podophyllotoxin was obtained. From band 7 α-peltatin was isolated which, after crystallization, from ethanol (yield 6.3 mg) was identified by HPLC and spectrocospic techniques.

Band 8 (R_f = 0.20)

This was the second major chromatographic band found for *P. hexandrum*. It was purified (TLC) with acetone:petrol [60 - 80°, (1:1, v:v)]. The aryltetralin lignan 4′-demethylpodophyllotoxin was isolated and crystallized from ethanol (yield 19.5 mg). This compound was identified by HPLC and spectroscopic methods. In extracts of *P. peltatum*, this was found as a minor band.

Lignans were also identified by the TLC spray reagent. The lignans reacted with the spray reagent and the colours red or brown (for the peltatins) were observed on the TLC plates. 4′-Demethylpodophylltoxin, 4′-demethyldesoxypodophyllotoxin, α-peltatin and 4′-demethylpodophyllotoxone reacted immediately with the chromogenic spray reagent. However, podophyllotoxin, desoxypodophyllotoxin, podophyllotoxone, β-peltatin and isopicropodophyllone only reacted after heating the plates. A picro compound (or a picro derivative of podophyllotoxin) in the form of white crystals was obtained (yield 1.5 mg) during the extraction and isolation of the lignans from *P. hexandrum*. This compound was later identified (TLC, HPLC and spectroscopic methods) as picropodophyllin. All compounds isolated (crystallized or not) were maintained at 4°C until required for spectroscopic studies.

4.3.2 Identification and characterization of the isolated lignans

4.3.2.1 Melting points

Melting points for the aryltetralin lignans from *P. hexandrum* and *P. peltatum* are given in Table 4.1. Since isopicropodophyllone was obtained as a gum and therefore was not pure, it was impossible to determine its melting point. A sample of picropodophyllin melted at 218 - 220°C which was consistent with other reports (Salako, 1996).

Table 4.1: Melting points of lignans isolated from *P. hexandrum* and *P. peltatum*

Lignan	mp of isolated lignan (°C)	mp as cited (°C)
Desoxypodophyllotoxin	140 - 142	146 - 152[1]
Podophyllotoxin	183 - 184	182 - 184[2]
Podophyllotoxone	185 - 187	189 - 192[3]
α-Peltatin	208 - 210	221 - 223[1]
β-Peltatin	233 - 235	232 - 235[2]
4′-Demethypodophyllotoxin	239 - 241	250 - 251[2]

mp values indicate the melting points; [1] Salako (1996); [2] Jackson and Dewick (1984c); [3] Dewick and Jackson (1981).

4.3.2.2 HPLC

The retention times of some isolated lignans as well as those of the standards used are shown in Table 4.2.

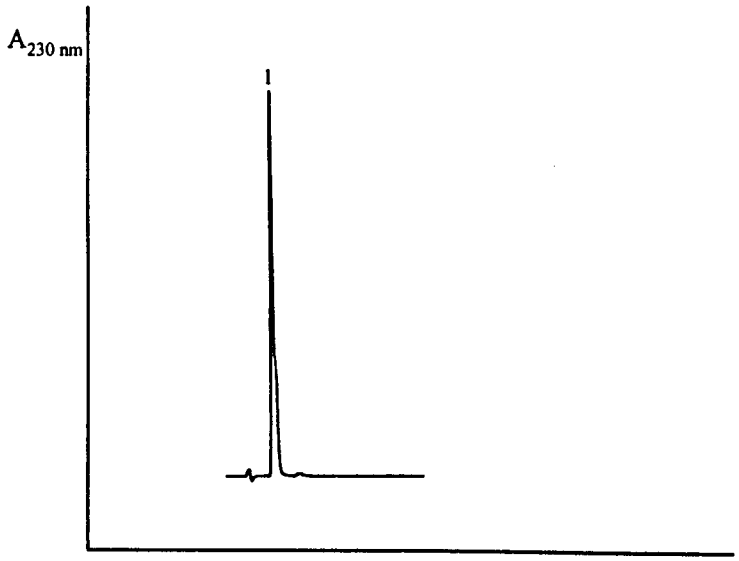
Table 4.2: Retention times of isolated lignans[1] and standards

Lignan	Rt of the isolated lignan (min)	Rt of lignan standard (min)
4′-Demethylpodophyllotoxin	3.50	3.52
α-Peltatin	3.62	3.64
Podophyllotoxin	5.60	5.66
Isopicropodophyllone	7.48	7.49
Podophyllotoxone	10.40	10.44
Desoxypodophyllotoxin	15.94	16.07

[1] System solvent: Acetonitrile:water:methanol (37:58:5, v:v:v).

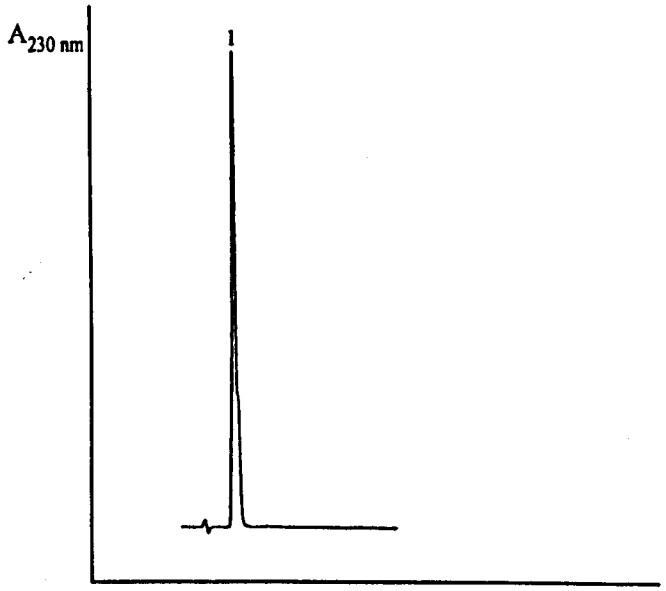
Only one prominent peak was observed in the chromatograms of some of the lignans. 4′-Demethylpodopophyllotoxin (Fig. 4.3), α-peltatin (Fig. 4.4), podophyllotoxin (Fig. 4.5) and desoxypodophyllotoxin (Fig. 4.8) corresponded to the respective lignans.

Fig. 4.3: HPLC chromatogram of 4′-demethylpodophyllotoxin



Peak (1) 4′-demethylpodophyllotoxin.

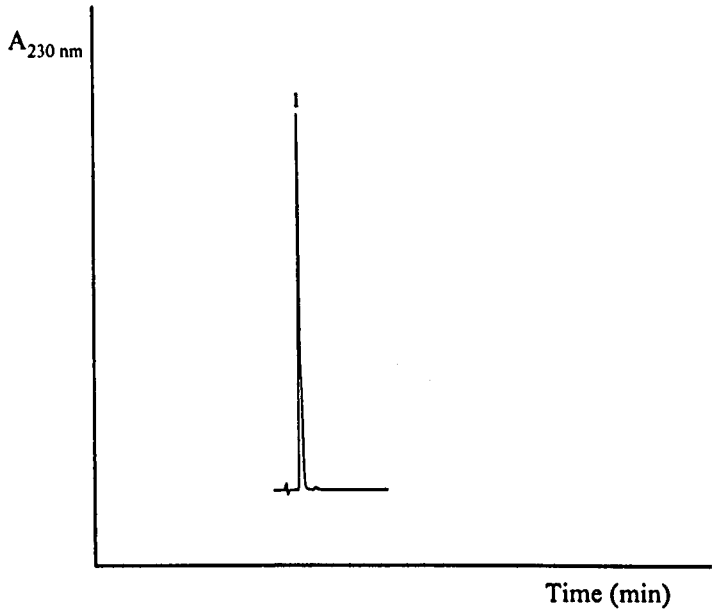
Fig. 4.4: HPLC chromatogram of α -peltatin



Peak (1) α-peltatin.

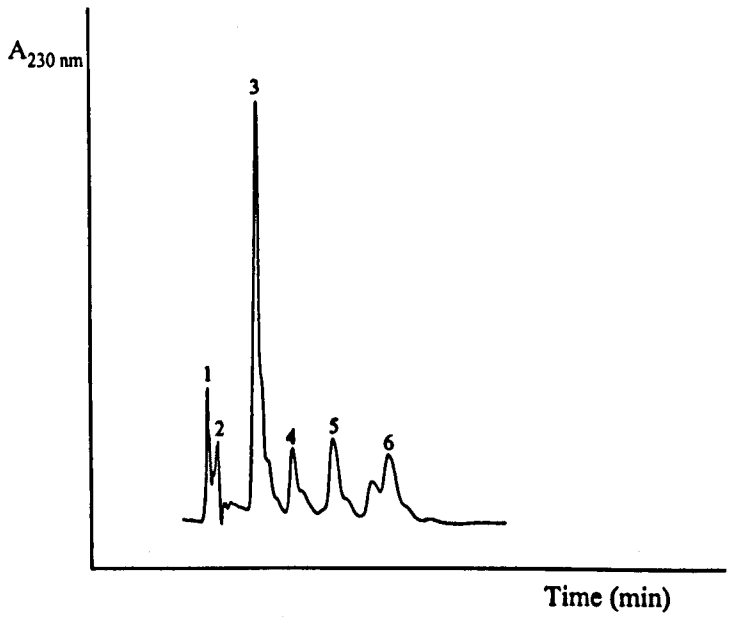
The chromatogram of the compound identified as isopicropodophyllone (Fig. 4.6) revealed some impurities peaks (1), (2), (3), (5) and (6) with Rt values of 1.74, 3.60, 5.46, 9.41 and 10.24 respectively. Peak (4) probably indicated the presence of isopicropodophyllone since its standard eluted at Rt = 7.79 (Table 4.2).

Fig. 4.5: HPLC chromatogram of podophyllotoxin



Peak (1) podophyllotoxin.

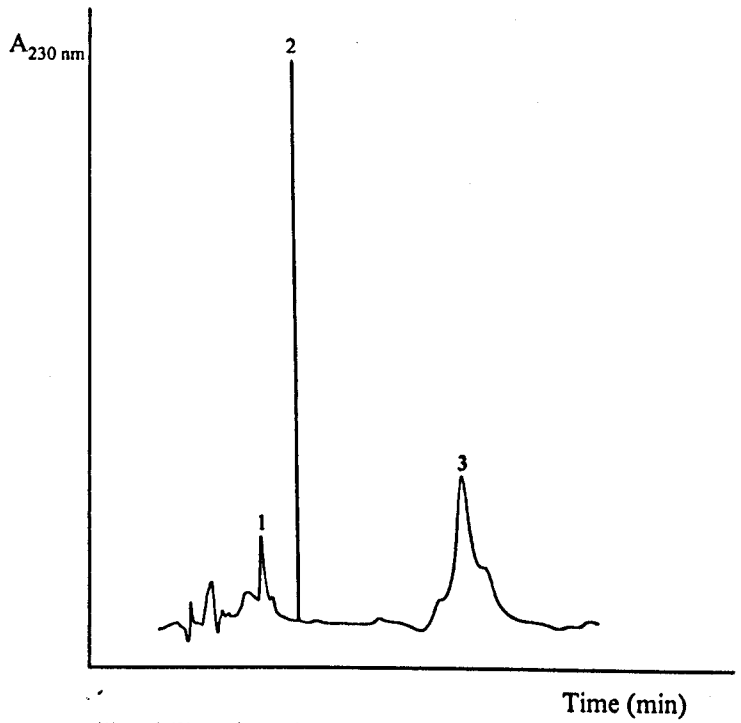
Fig. 4.6: HPLC chromatogram of isopicropodophyllone



(1), (2), (3), (5) and (6) are impurity peaks; (4) isopicropodophyllone.

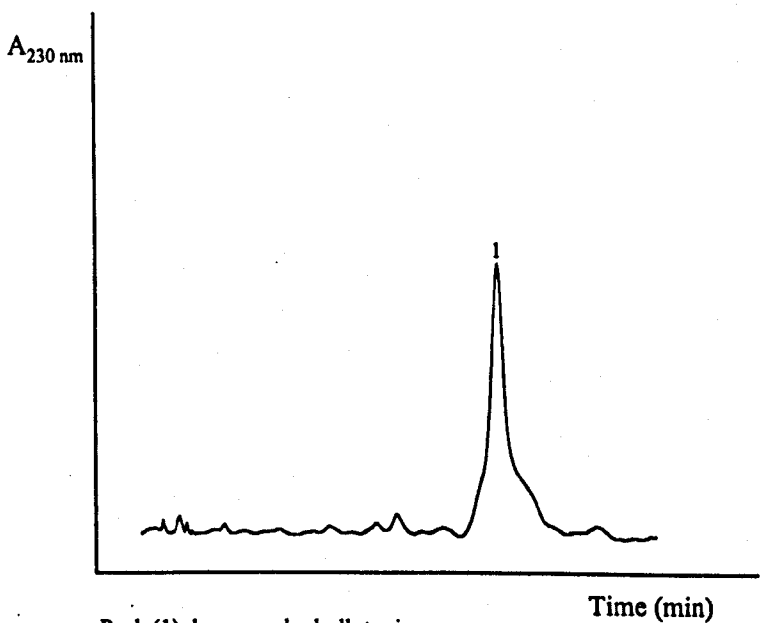
In the chromatogram of lignan podophyllotoxone (Fig. 4.7), two impurity peaks (1 and 2) with Rt = 3.55 and 4.0 respectively were noted. Peak (3) had an Rt = 10.40 and was possibly podophyllotoxone when this Rt value was compared with that of the authenticated standard (Table 4.2).

Fig. 4.7: HPLC chromatogram of podophyllotoxone



(1) and (2) are impurity peaks; (3) podophyllotoxone.

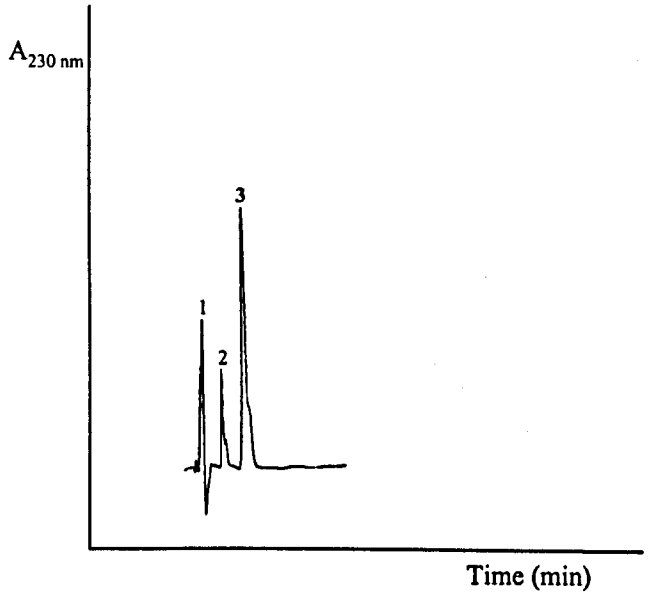
Fig. 4.8: HPLC chromatogram of desoxypodophyllotoxin



Peak (1) desoxypodophyllotoxin.

Peak (3) was possibly picropodophyllin (Fig. 4.9) and eluted at Rt = 5.50 even though two peaks (1) and (2) appeared with an Rt = 1.73 and 2.87 respectively. The retention time for the isolated picropodophyllin was similar to that of the reference compound used (Rt = 5.54).

Fig. 4.9: HPLC chromatogram of picropodophyllin



Peaks (1) and (2) are unknown; (3) picropodophyllin.

4.3.2.3 Proton nuclear magnetic resonance spectrometry

The structural assignments of compounds isolated from *P. hexandrum* and *P. peltatum*, based on their ^{1}H NMR spectral data, are summarized in Tables 4.3 and 4.4. ^{1}H NMR spectra are for podophyllotoxone (Appendix 6.1.1), desoxypodophyllotoxin (Appendix 6.1.2), β-peltatin (Appendix 6.1.3), α-peltatin (Appendix 6.1.4), podophyllotoxin (Appendix 6.1.5), 4′-demethylpodophyllotoxin (Appendix 6.1.6), picropodophyllin (Appendix 6.1.7) and isopicropodophyllone (Appendix 6.1.8).

Table 4.3: ^{1}H NMR data for podophyllotoxone, desoxypodophyllotoxin, β-peltatin and α-peltatin

Proton(s)	Podophyllotoxone[1]	Desoxypodophyllotoxin[2]	β-Peltatin[3]	α-Peltatin[4]
H-5	7.56 (s)	6.67 (s)	-	-
H-8	6.70 (s)	6.52 (s)	6.24 (s)	6.23 (s)
H-2′; H-6′	6.39 (s)	6.35 (s)	6.36 (s)	6.36 (s)
OCH_2O	6.10 (d), J = 1.1	5.95 (d), J = 1.4	5.95 (d), J = 1.3	5.96 (d), *J
	6.08 (d), J = 1.1	5.93 (d), J = 1.3	5.94 (d), J = 1.3	
OH	-	-	-	-
H-1	4.85 (d), J = 4.2	4.61 (brs)	4.61 (d), *J	4.74 (brs)
H-2	3.30 (dd), J = 15.5, 4.3	2.73 (m)	2.70 (m)	2.68 (m)
H-3	3.54 (ddd), J = 15.5, 10.4, 9.3	2.73 (m)	2.70 (m)	2.68 (m)
H-3aα	4.56 (dd), J = 9.3, 9.2	**	4.48 (m)	**
H-3aβ	4.36 (dd), J = 10.4, 9.3	3.92 (dd), *J	3.98 (m)	3.95 (dd), *J
H-4α	-	3.06 (m)	3.24 (m)	**
H-4β	-	2.75 (m)	2.50 (m)	**
4′-OMe	3.81 (s)	3.82 (s)	3.81 (s)	-
3′-OMe; 5′-OMe	3.74 (s)	3.75 (s)	3.76 (s)	3.79 (s)

[1] Podophyllotoxone (Appendix 6.1.1), [2] desoxypodophyllotoxin (Appendix 6.1.2), [3] β-peltatin (Appendix 6.1.3) and [4] α-peltatin (Appendix 6.1.4).
The values shown indicated the chemical shift positions of protons expressed as δ (in ppm) relative to the internal standard TMS (tetramethylsilane) in $CDCl_3$ solution. The signals are represented by (s) singlet, (brs) broad singlet, (d) doublet, (dd) double doublet, (ddd) doublet of double doublets, (m) multiplet and (t) triplet. J represents the coupling constants (in Hz). * J values not available; ** δ values not available. Spectral data of compounds were recorded at 400 MHz.

Table 4.4: ^{1}H NMR data for podophyllotoxin, 4′-demethylpodophyllotoxin, picropodophyllin and isopicropodophyllone

Proton(s)	Podophyllotoxin[1]	4′-Demethylpodophyllotoxin[2]	Picropodophyllin[3]	Isopicropodophyllone[4]
H-5	7.11 (s)	7.17 (s)	7.04 (s)	7.36 (s)
H-8	6.51 (s)	6.49 (s)	6.38 (s)	6.62 (s)
H-2′; H-6′	6.37 (s)	6.43 (s)	6.45 (s)	6.21 (s)
OCH_2O	5.99 (d), J = 1.2	5.97 (d), J = 1.0	5.94 (m)	6.01 (d), J = 1.0
	5.97 (d), J = 1.3	5.96 (d), J = 1.0		5.99 (d), J = 1.0
OH	-	-	-	-
H-1	4.59 (m)	**	4.12 (d), J = 5.3	4.50 (d), J = 5.8
H-2	2.87 - 2.75 (m)	**	3.24 (dd), J = 5.3, 9.3	3.57 - 3.50 (m)
H-3	2.87 - 2.75 (m)	2.78 (m)	2.75 (m)	3.57 - 3.50 (m)
H-3aα	4.59 (m)	**	4.52 - 4.41 (m)	4.45 (m)
H-3aβ	4.06 (m)	**	4.52 - 4.41 (m)	3.85 (m)
H-4β	4.77 (d), J = 9.1	**	4.52 - 4.41 (m)	-
4′-OMe	3.81 (s)	-	3.85 (s)	3.80 (s)
3′-OMe; 5′-OMe	3.74 (s)	3.69 (s)	3.82 (s)	3.73 (s)

[1] Podophyllotoxin (Appendix 6.1.5), [2] 4′-demethylpodophyllotoxin (Appendix 6.1.6), [3] picropodophyllin (Appendix 6.1.7) and [4] isopicropodophyllone (Appendix 6.1.8). The values shown indicated the chemical shift positions of protons expressed as δ (in ppm) relative to the internal standard TMS (tetramethylsilane) in $CDCl_3$ solution and in $(CD_3)_2CO$ for 4′-demethylpodophyllotoxin. J is the coupling constant (in Hz). The signals are represented by (s) singlet, (d) doublet, (dd) double doublet and (m) multiplet. * J values not available; ** δ values not available. Spectral data of compounds were recorded at 400 MHz.

4.3.2.4 Mass spectrometry

The identities of the aryltetralin lignans isolated from *P. hexandrum* and *P. peltatum* were also confirmed by electron impact mass spectrometry (Table 4.5). It was not possible to obtain the mass spectrum of isopicropodophyllone although it was previously identified (Table 4.4) by ^{1}H NMR.

Table 4.5: Mass spectral data for *Podophyllum* lignans

Compound	m/z[1](%)[2]	Spectrum
Podophyllotoxone	412 (M^+, 62 %), 367 (16), 353 (5), 337 (7), 336 (5), 313 (6), 200 (5), 168 (24), 153 (11), 149 (100) 115 (6)	Appendix 7.1.1
Desoxypodophyllotoxin	398 (M^+, 100 %), 230 (11), 185 (20), 181 (35), 173 (27), 168 (12)	Appendix 7.1.2
β-Peltatin	414 (M^+, 100 %), 246 (10), 201 (12), 189 (22), 181 (22), 168 (7)	Appendix 7.1.3
Podophyllotoxin	414 (M^+, 100 %), 399 (7), 189 (10), 181 (9), 168 (25), 153 (13)	Appendix 7.1.4
α-Peltatin	400 (M^+, 100 %), 246 (24), 201 (34), 189 (23), 167 (20), 154 (12)	Appendix 7.1.5
4'-Demethylpodophyllotoxin	400 (M^+, 100 %), 201 (12), 200 (10), 189 (8), 167 (13), 155 (9), 154 (36)	Appendix 7.1.6

[1] m/z values are the expression of the mass of ion divided by the charge; [2] Percentage values given in parentheses indicate the intensities of peaks relative to the base peak.

In the mass spectral analysis of podophyllotoxone (Appendix 7.1.1), the molecular ion peak (m/z 412) was usually the highest intensity peak, but appeared at a relatively low intensity (62 %) whilst the fragment m/z 149 came in as an intense peak (100 %); this was probably due to an impurity in the sample. Mass spectrometric fragmentation of picropodophyllin (Appendix 7.1.7) showed a molecular ion peak at m/z 414 (M^+, 69%) and a base peak at m/z 312 (100 %). Other fragments appeared at m/z 396 (54), 299 (5), 297 (39), 201 (11), 185 (4), 181 (7), 169 (29), 168 (48), 153 (9).

4.4 Discussion

Results of this study showed that, the pattern of lignan aglycones detected in rhizomes and roots of *P. hexandrum* and *P. peltatum* were similar to each other but the intensities of the chromatographic zones, under UV light, were found to be different. These results are in general agreement with earlier studies (Jackson and Dewick, 1984c) which showed that lignans present in both species appeared in different relative proportions. In the *Podophyllum* extracts, band 2 (see Results, Section 4.3.1) was observed as a faint zone and isopicropodophyllone was isolated even though it could not be purified. Picropodophyllin was obtained and characterized in the present study. It is a picro isomer of podophyllotoxin formed through epimerisation at C-2 in the presence of a mild base(s). Although it was obtained during the extraction of lignans this picro derivative is also considered an artefact resulting from traces of basic reagents.

Alternatively, the lignans were identified via TLC plates using a chromogenic spray reagent which gives red or brown colour to *o*-quinone derivatives (Jackson and Dewick, 1985). This reagent proved useful in identifing 4′-demethyl compounds such as 4′-demethylpodophylltoxin from those of the trimethoxy group (podophylltoxin). The lignans from the latter group required additional heating of the plates which were previously sprayed with the chromogenic reagent and corresponded to the demethylation step prior to *o*-quinone formation.

During the identification of the organic compounds in the target *Podophyllum* species it was important to know whether such compounds were of high purity. One possibility was therefore to determine melting point since crucial information can be gained from such values when the samples are crystalline. The melting points of the samples of desoxypodophyllotoxin, 4′-demethylpodophyllotoxin, podophyllotoxone and α-peltatin however differed from the values cited in the literature (Dewick and Jackson, 1981; Jackson and Dewick, 1984c; Salako, 1996). This problem of variation in the melting points of lignans has been previously recognized and attributed to the formation of polymorphic forms (Salako, 1996).

The compounds from each purified zone were however characterized by spectroscopic methods and their identities confirmed by comparing with authentic standards. Proton nuclear magnetic resonance spectroscopy was particularly useful here for structural elucidation of the *Podophyllum*-specific lignans (Jackson and Dewick, 1984c). The mass spectrometric data were in agreement with previous studies (Jackson and Dewick, 1984c; Salako, 1996) even though impurity peaks were shown to be present in the spectra for podophyllotoxone and picropodophyllin. The presence of such impurities which gave rise to the prominent peaks may be due to an extensive handling of samples on the preparative and thin layer plates or columns and

may also be attributed to traces of solvents being present prior to mass spectrometry (Williams and Fleming, 1995), which may give false signals in the molecular ion region which can in turn interfere with a correct determination of molecular mass.

In the present study, the lignans 4'-demethylpodophyllotoxin and podophyllotoxin obtained from rhizomes and roots extracts of *P. hexandrum* were used in the phytochemical analysis of whole plants and tissue culture materials of *D. cymosa* (Chapter 5, Section 5.2.5.1). These two compounds were relevant as authentic aryltetralin lactone lignans for the investigation of the presence of lignans in the calli and cell suspensions of *D. cymosa* as well as in the starting tissue explants (leaves and petioles).

Chapter Five

Phytochemical Analysis of Lignans in *Diphylleia cymosa*

5.1 Introduction

To date, only a few phytochemical studies concerning the production of secondary metabolites in the genus *Diphylleia* have been published, but have not included studies of tissue-cultured materials. According to these studies flavonoids (quercetin and kaempferol), wall polysaccharides and lignans (podophylltoxin, dehydropodophyllotoxin and diphyllin) have been identified in *D. grayi* and *D. sinensis* (Xingrue *et al.*, 1980; Ying *et al.*, 1984; Iriki *et al.*, 1984; Ma *et al.*, 1993). *Podophyllum* lignans are the most important group of natural products, in terms of pharmacological interest, reported in the genus *Diphylleia*.

A number of published studies concerning the lignan content of *D. cymosa* have been undertaken (Broomhead, 1989; Broomhead and Dewick, 1990, Konuklugil, 1993). Lignans and their corresponding glucosides were found in its roots and leaves (Broomhead and Dewick, 1990; Dewick 1997). These phytochemical studies have also shown that lignan contents varied with season and also depended on which tissue was used. The podophyllotoxin level was lower in roots harvested in the winter compared to leaves harvested before senescence (Table 5.1). The highest level of podophyllotoxin (*ca.* 5.4 mg g^{-1} d. wt.) was found in the first leaves (from young plants) harvested during the active growing season. The leaves of *D. cymosa* have proved to be a significant source of aryltetralin lactone lignans, and, although a quantitative seasonal lignan variation was observed; this species is though a promising source of medicinally useful lignans. Diphyllin, an arylnaphthalene lignan (Fig. 5.1), and its glucosides have been identified and quantified in its leaves and roots (Table 5.1) and also in *D. grayi* (Broomhead and Dewick, 1990).

No phytochemical studies dealing with the analysis of lignan content in tissue cultures of *D. cymosa* have been performed to date.

Fig. 5.1: Chemical structure of the lignan diphyllin

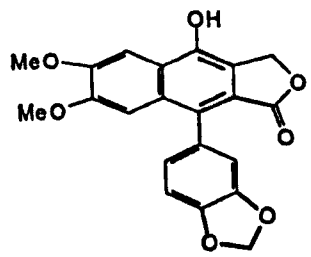


Table 5.1: Lignan content in leaf and root tissues of whole plants of *D. cymosa*[1]

Natural lignans	Explant	
	Leaf[2] (mg g^{-1} d. wt.)	Root[3] (mg g^{-1} d. wt.)
Podophyllotoxin	5.4 - 1.15	0.07
4'-Demethylpodophyllotoxin	0.40 - 0.97	0.04
4'-Demethyldesoxypodophyllotoxin	0.58 - 1.15	-
α-Peltatin	-	0.03
β-Peltatin	-	0.89
Diphyllin	0.31 - 1.3	0.07
4'-Demethyldesoxypodophyllotoxin-4'-*O*-glucoside	+	
α-Peltatin 5-*O*-glucoside		+
β-Peltatin 5-*O*-glucoside		+
Diphyllin glucoside	+	+
Diphyllin diglucoside	+	+

[1] Modified from Broomhead and Dewick (1990); [2] Leaf tissues were harvested during the growing season in May (higher values) and in September before senescence (lower values); [3] Root tissues were harvested in the winter at December.
(-) Indicates that the lignan was not detected; (+) Indicates that the lignan was detected, but not quantified.

5.1.1 Production of antitumour compounds in plant tissue cultures

A wide variety of antitumour compounds are produced by higher plants, but are generally found at very low concentrations. Therefore, the tissue culture approach has been explored in order to produce higher levels of these compounds. In this context, different classes of secondary metabolites and some of their derivatives have been synthesized in plant tissue culture (Table 5.2).

The production of cytotoxic lignans has received considerable attention. The lignan podophyllotoxin and its derivatives have been identified and quantified in *Podophyllum* tissue cultures and *Diphylleia* species (Chapters 1, 2). In *D. cymosa*, the lignans synthesized belong mainly to the aryltetralin and arylnaphthalene groups both derived from the phenylpropanoid pathway. In *P. hexandrum* callus cultures, the levels of demethylpodophyllotxin, podophyllotoxin 4-*O*-glucoside and podophyllotoxin assessed by HPLC were found to be comparable to those of intact mature root materials (Heyenga *et al.*, 1990). Podophyllotoxin has been also found in tissue cultures of *Callitris drummondii* (Van Uden *et al.*, 1990a). Root cultures of *Linum flavum* have been shown to accumulate podophyllotoxin (0.7 - 1.3% d. wt.) (Berlin et al., 1988) whilst cell suspension cultures were found to produce the lignan

Table 5.2: Production of antitumour compounds in plant tissue cultures[1]

Plant source	Compounds	Class
Baccharis megapotamica	Baccharin	Sesquiterpene
Camptotheca acuminata	Camptothecin	Alkaloid
Ochrosia elliptica	Ellipticine	Alkaloid
Cephalotaxus harringtonia	Homoharringtonine	Alkaloid
Heliotropium indicum	Indicine-*N*-oxide	Alkaloid
Putterlickia verrucosa	Maytansine	Alkaloid
Podophyllum hexandrum and *P. peltatum*	Podophyllotoxin	Lignan
Taxus brevifolia	Taxol	Diterpene
Thalictrum dasycarpum	Thalicarpine	Alkaloid
Trypterygium wilfordii	Tripdiolide	Diterpene
Catharanthus roseus	Vinblastine, vincristine	Alkaloid

[1] Modified from Misawa and Nakanishi (1988) and Dewick (1996).

5-methoxypodophyllotoxin (5-MPT) (up to 0.004% d. wt.) (Van Uden *et al.*, 1990a). The latter was noted to increase (0.16 - 0.20% d. wt.) when cell suspensions were transferred to culture medium lacking growth regulators. Regenerated *L. flavum* plantlets from callus or cell supensions have been shown to accumulate higher 5-MPT concentrations (0.7% d. wt.) in the roots than in the stems and leaves (0.4% d. wt.) (Van Uden *et al.*, 1994). The cytotoxic activity of 5-MPT against human tumour cells has been compared with podophyllotoxin and the antitumour drugs etoposide and teniposide (Van Uden *et al.*, 1992). Studies have demonstrated that some lines of leaf- and stem-derived cell suspension cultures of *L. album* have accumulated high concentrations of podophyllotoxin (0.3% d. wt.) and traces of 5-MPT after three weeks culture. In contrast, other lines from the same explants produced higher amounts of 5-MPT compared with podophyllotoxin. In these studies, a few lignans (5'-demethoxy-5-methoxypodophyllotoxin, lariciresinol and pinoresinol) were identified by mass spectroscopy whilst others (matairesinol, α- and β-peltatins and coniferin) were only determined by HPLC-cochromatography (Smollny *et al.*, 1992). In the cell suspension cultures of *L. nodiflorum*, podophyllotoxin, 5-MPT and deoxypodophyllotoxin were identified by TLC, HPLC and ^{1}H NMR (Konuklugil *et al.*, 1999). These cultures accumulated 5-MPT as the main lignan. Moreover, podophyllotoxin has been reported in leaf-derived calli of *Juniperus chinensis* (Muranaka *et al.*, 1998). The amount of lignan was twice that produced from the intact leaves.

Furthermore, callus and cell suspensions of *Forsythia intermedia* accumulate dibenzylbutyrolactone and furofuran lignans (Rahman *et al.*, 1990). According to these studies, the lignan profile was dependent on the choice of culture medium instead of explant source although lignan patterns in the explants were different. Matairesinol was the predominant lignan in leaf- and stem-derived callus cultures. In cell suspension cultures, matairesinol 4′-*O*-glucoside or epipinoresinol 4′-*O*-glucoside were the major lignans.

5.1.2 Analytical procedures for the analysis of lignans in *D. cymosa*

5.1.2.1 TLC

Roots and leaves of *D. cymosa* have been shown to exhibit different patterns of lignan accumulation (Broomhead and Dewick, 1990). Dried, powdered root and leaf materials were extracted with hot ethanol (Broomhead and Dewick, 1990). The ethanolic extracts were separated by PLC in chloroform:methanol (25:1, v:v) following the protocol previously described for *P. hexandrum* (see also Chapter 4, Section 4.2.3.2). β-Peltatin was the main lignan found in roots of *D. cymosa;* however it was not observed in leaves. The major lignans detected in leaves were 4′-demethylpodophyllotoxin, podophyllotoxin and 4′-demethyldesoxypodophyllotoxin. The latter compound has not been found in roots. Identification of the lignans was confirmed by spectral data and also by comparison with authentic standards. The results from these studies have also shown that the lignans diphyllin, 4′-demethyldesoxypodophyllotoxin 4′-*O*-D-glucoside, diphyllin glucoside and diphyllin diglucoside present in *D. cymosa* have been not detected in *Podophyllum* species.

5.1.2.2 HPLC

Several HPLC protocols are available to assess lignans present in the genera *Podophyllum* (Chapter 4, Section 4.1.3) and *Linum* (Konuklugil, 1993). However, in the genus *Diphylleia*, particularly *D. cymosa*, there is only one study (Broomhead, 1989) focussed on the assessement of lignans by HPLC. In this study, root and leaf extracts of *D. cymosa* were evaluated separately by reverse-phase HPLC coupled with a UV detector and the solvent system used was acetonitrile:water (45:55, v:v). Both root and leaf extracts were compared with individual standards in order to identify the lignans. The results showed some variation in the HPLC profiles between roots and leaves of *D. cymosa*. Such variation, as for example, the presence of α- and β-peltatin in the roots but not in the leaves, has also confirmed the results of earlier studies, obtained by TLC, as previously described (Section 5.1.2.1).

5.2 Materials and methods

5.2.1 Plant and tissue culture materials

For the present study, leaves and petioles of plants cultivated at the University of Nottingham (Chapter 3, Section 3.2.2.1) were utilised. Leaf laminae (10 d old after emergence above the ground), was excised and stored as previously described (Section 3.2.2.1) in order to assess the contents of lignans as control for the tissue cultures. Petiole-derived friable calli (6 months old) cultured on UM medium in the dark (Section 3.3.2) were harvested and analysed. Cell suspensions (derived from leaf and petiole explants) cultured in liquid UM medium (Section 3.2.8.3) were harvested at 5 d intervals during their growth cycle (day 0 - 25) and also analysed for lignan content.

5.2.2 Sample preparation

Ten petiole-derived friable calli (approx. 1.5 cm diam.) cultured on UM medium were weighed and stored overnight (-70°C) before lyophilization. Cultures were then transferred to a freeze-drier (Model SB4, ChemLab, Hornchurch, UK) fitted with a double-stage high vacuum pump (Model JD 60, Javac, Farnham, UK) and maintained overnight. Dried samples were powdered using a pestle and mortar. The same protocol was followed for the preparation of leaf explants. Samples of both leaf and petiole-derived cell suspensions cultured in UM liquid medium were filtered (Whatman No. 1 filter paper) under reduced pressure. The filtrate was treated in a similar manner to calli as described earlier in this Section. All samples were stored (-20°C) in Eppendorf tubes (Sarstedt, Numbrecht, Germany) for up to several months before use.

5.2.3 Extraction of lignans

An aliquot (200 mg d. wt.) of each freeze-dried sample was extracted by ultrasonification (ultrasonic bath, Model Bandelin Sonorex Super RK103H, Bandelin Company, Berlin, Germany) with 2 ml analytical grade ethanol (Sigma) for 30 s (2 x 30 s) before the crude ethanolic extracts were hydrolysed.

5.2.4 Hydrolysis of lignan glycosides

The aglycones were liberated by enzymic hydrolysis by adding 6 ml distilled water and 1.0 mg β-D-glucosidase (from almonds, 7.1 U/mg, Serva, Heidelberg, Germany) in solution. The pH was adjusted to 5.0 with orthophosphoric acid. After 1 h incubation at 35°C (in water bath), the reaction mixture was extracted with 12 ml analytical grade ethanol and then sonicated for 10 min at 70°C. This mixture was centrifuged (Hettich centrifuge, Model Hettich Universal, rotor no. 1323, Hettich

Company, Tuttlingen, Germany) at 2000 rpm (20 min). The supernatant (1.0 ml) was pipetted off and then stored (-20°C) until required.

5.2.5 Standard compounds

5.2.5.1 Source of standard compounds

The isolation and structural identification of the standard lignan aglycones podophyllotxin (Ptox) and 4′-demethylpodophyllotoxin obtained from *P. hexandrum* was as described in Chapter 4 (Section 4.3.1). The standard for diphyllin was kindly provided by Dr R S Ward (Chemistry Department, University of Wales, Swansea, UK), 5-MPT by Dr W Van Uden (Laboratory for Pharmacognosy, University of Groningen, Groningen, The Netherlands) and 4′-demethyldesoxypodophyllotoxin by Prof A San Feliciano (Department of Organic Chemistry, University of Salamanca, Salamanca, Spain).

5.2.5.2 Standard sample preparation

Lignan standard solutions were prepared to a concentration of 0.05 mg ml^{-1} (w:v) in 1 ml of methanol (HPLC grade; Sigma).

5.2.6 HPLC protocol

Qualitative HPLC analyses were performed using an instrument from LDC Analytical (Gelnhausen, Germany) equipped with an injection valve with a 20 µm loop. Samples were filtered (FP 030/AS, Schleicher and Schuell) and 10 µl aliquots were injected by syringe onto a reverse-phase C-18 column (250 mm x 4.6 mm i.d.) with pre-column (50 mm) packed with Nucleosil 100 (5 µm; Macherey and Nagel, Dürer, Germany). The mobile phase consisted of two solvent systems: solvent A [water (100%)] and solvent B [acetonitrile:orthophosphoric acid (15:85, v:v)]. Both solvents were freshly prepared, filtered through a 0.22 µm Millipore filter and degassed under vacuum prior to use. The solvents were driven by two chromatography pumps (ConstaMetric III and ConstaMetric I, pumps A and B respectively). The mobile phase was passed through the column for 1 h prior to sample injection. The flow-rate of the solvents was 0.8 ml min^{-1}; the gradient conditions are described in Table 5.3. The peaks were detected by a UV spectrophotometric detector (Model SM 4000) at a wavelength of 290 nm. The chromatograms were recorded on a computer integrator (MPD Model 3000-E). A minimum of three aliquots were prepared from each sample (leaf tissues, calli, cell suspensions and culture medium) and standard; each aliquot was analysed three times under the same conditions. The retention times were recorded for all samples and were compared with the standards. Calibration curves were generated every 25 min of HPLC run time. The

chromatograms were scanned as previously mentioned (Chapter 4, Section 4.2.5.4.3; Appendix 4).

Table 5.3: Gradient conditions for HPLC of lignans

Time (min)	Flow-rate (ml min^{-1})	Pump A water (%)	Pump B acetonitrile:ortho-phosphoric acid (%)
0	0.8	50	50
17	0.8	33	67
18	0.8	50	50
25	0.8	50	50

5.3 Results

5.3.1 HPLC profile of the lignan standards

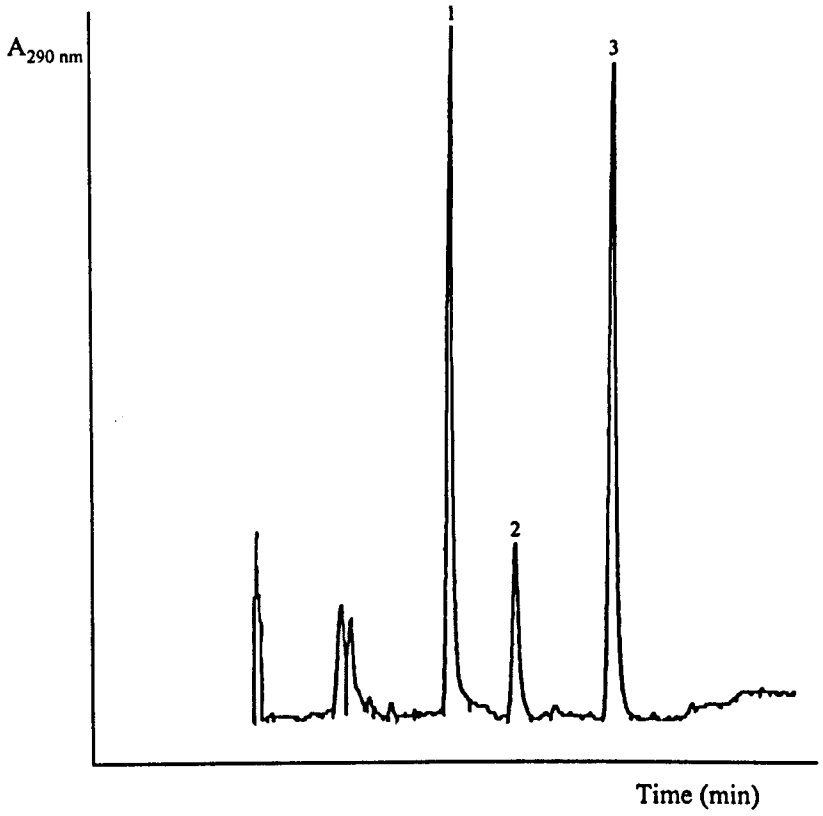
The chromatogram of the standard lignan mixture [Ptox compound (1), 5-MPT compound (2) and 4′-demethyldesoxypodophyllotoxin compound (3)] is shown in Fig. 5.2. The optimum separation was obtained by using a gradient of acetonitrile containing ortho-phosphoric acid (15:85, v:v) (solvent B) and water (solvent A) starting from 50 to 67% of B during 17 min. The elution gradient was decreased to 50% of B within 1 min and was maintained as constant for 25 min. The three lignans appeared well separated from all other peaks in the chromatogram and were easily identified by their respective peaks and retention times. The retention time values for the compounds (1) to (3) under the stated conditions are given in Table 5.4.

Table 5.4: Retention times of a standard lignan mixture on the HPLC[1]

Peak number	Substance	Retention time (min)
1	Podophyllotoxin	6.61
2	5-Methoxypodophyllotoxin	8.89
3	4′-Demethyldesoxypodophyllotoxin	12.24

[1] (see also Fig. 5.2).

Fig. 5.2: HPLC chromatogram of the lignans Ptox , 5-MPT and 4′-demethyldesoxypodophyllotoxin in a standard mixture



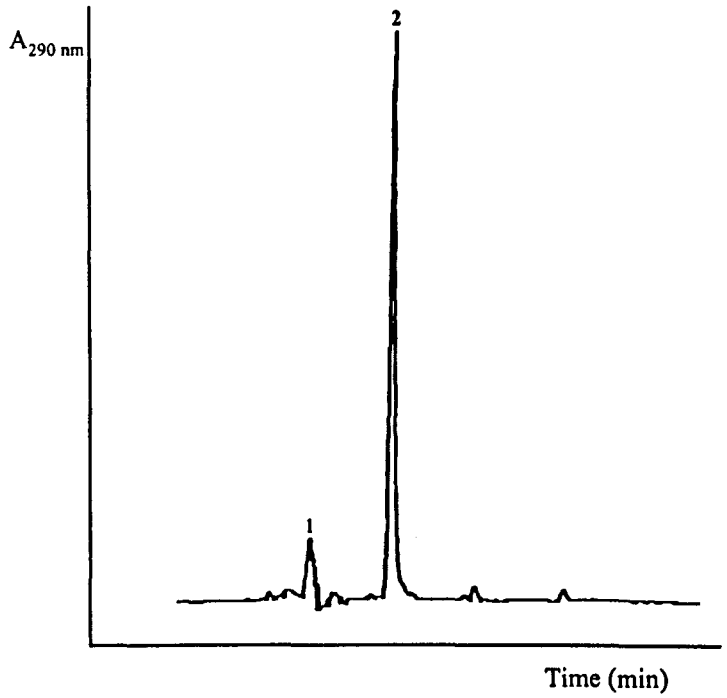
Peak (1) Ptox; (2) 5-MPT; (3) 4′-demethylpodohyllotoxin.

The other two standard solutions (4′-demethylpodophyllotoxin and diphyllin) were separately eluted with the same elution gradient. An impurity peak [(1) in Fig. 5.3] was observed at Rt = 4.08 but the 4′-demethylpodophyllotoxin peak (2) was seen at Rt = 6.40. In Fig. 5.4, diphyllin (2) showed satisfactory resolution (Rt = 12.48) with the same elution gradient even though an impurity peak (1) appeared (Rt = 4.05) in the chromatogram.

5.3.2 HPLC profile of leaf explants

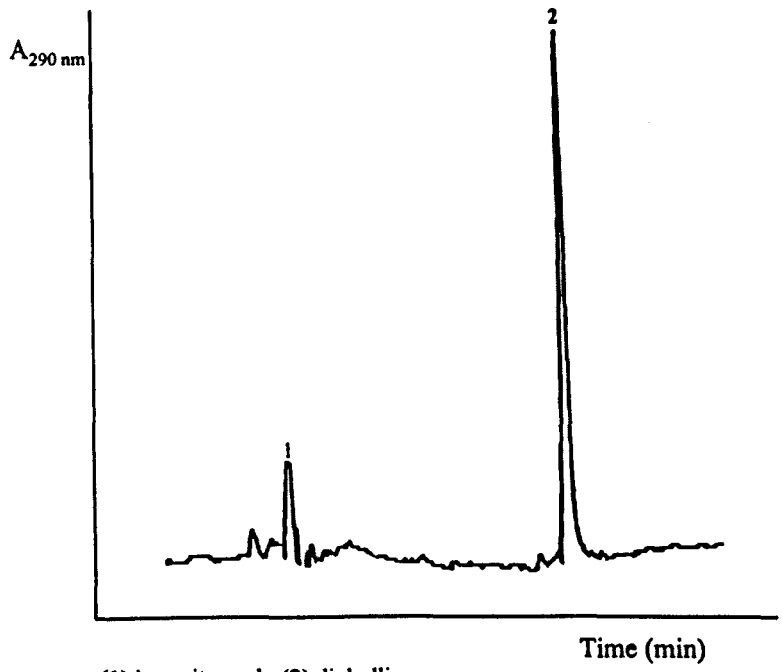
In the leaf extract from cultivated plants, an unidentified peak (1), eluting (Rt = 6.29) just before podophyllotoxin (2), was observed (Fig. 5.5). Separation of the podophyllotoxin peak (2) from the peak (1) was incomplete. Podophyllotoxin was identified by comparing its retention time value (Rt = 6.60) with that of the authentic compound used in the standard mixture as previously shown (Table 5.4). Two unknown peaks (3) and (4) eluted at retention time values 8.40 and 10.00 respectively. Peak (5) which had a Rt = of 12.09 was possibly diphyllin; however the retention time was less than that of the diphyllin standard (Rt = 12.48) (Fig. 5.4) .

Fig. 5.3: HPLC chromatogram of the 4′-demethylpodophyllotoxin standard



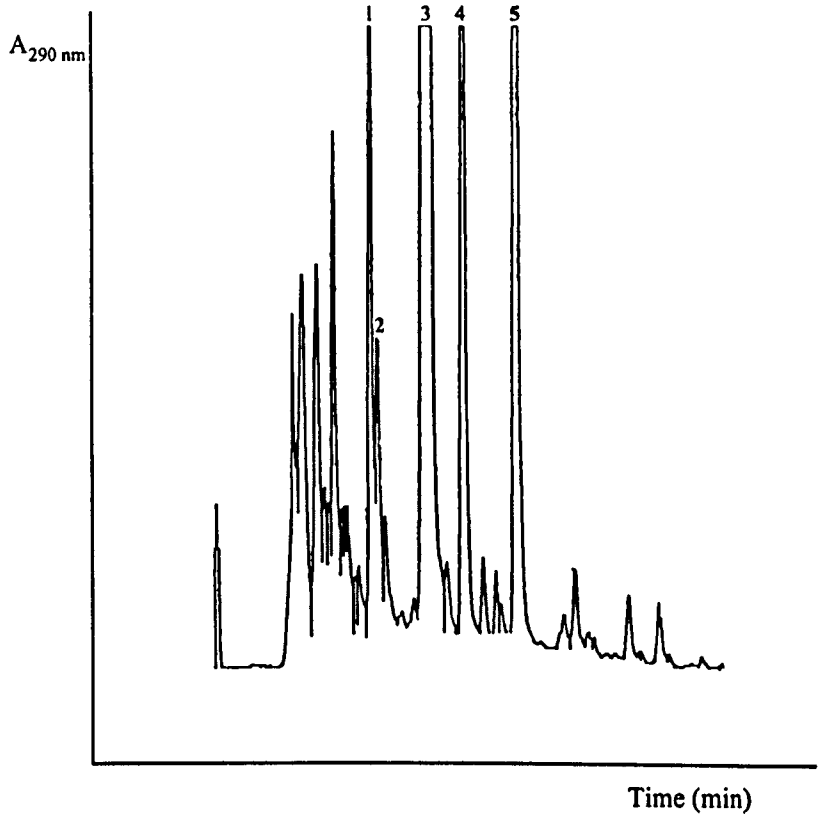
(1) impurity peak; (2) 4′-demethylpodophyllotoxin.

Fig. 5.4: HPLC chromatogram of the diphyllin standard



(1) impurity peak; (2) diphyllin.

Fig. 5.5: HPLC profile of the leaf extracts of *Diphylleia cymosa*

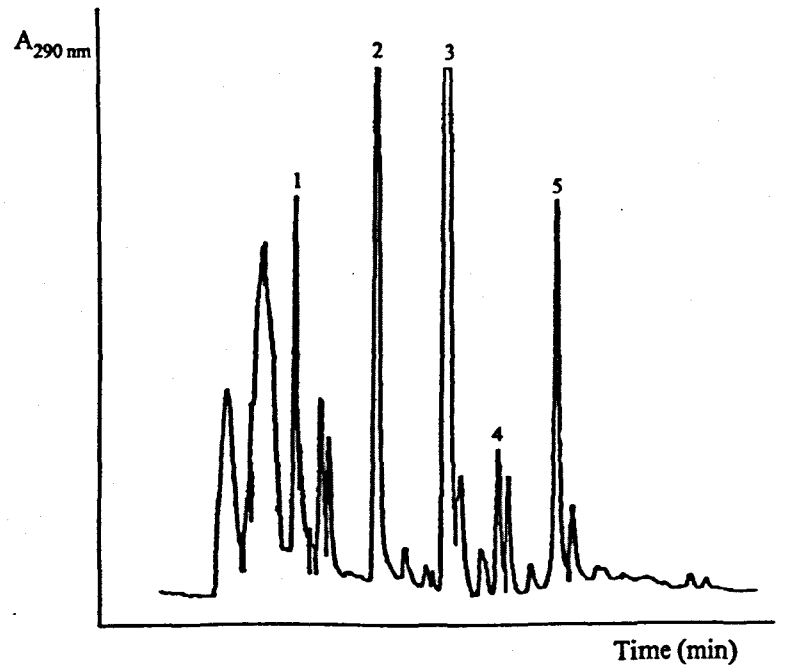


Peaks (1), (3) and (4) are unknown; (2) podophyllotoxin; (5) diphyllin.

5.3.3 HPLC profile of leaf- and petiole-derived cell suspensions and calli

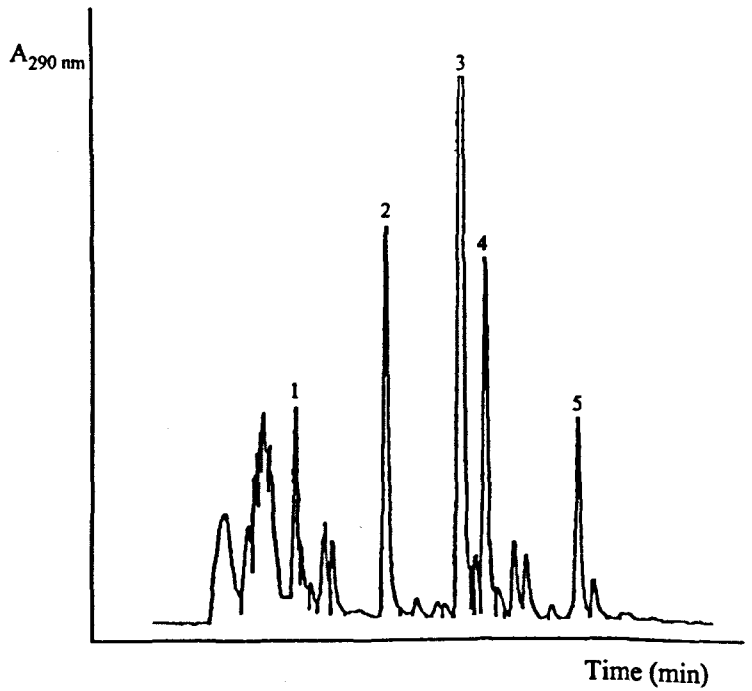
Samples of leaf-derived cell suspensions, after 15 d post subculture, showed five major peaks (Fig. 5.6.A). Peaks (1), (2), (3) (4) and (5) had Rt = 5.79, 8.89, 11.55, 13.52 and 15.77 respectively. The same sample source after being spiked with the diphyllin standard revealed five major peaks (Fig. 5.6.B) which are: peaks (1), (2) and (3) with Rt = 5.75, 8.88 and 11.53 respectively; however they could not be identified. Diphyllin (peak 4) was clearly seen only in this spiked sample (Rt = 12.42) followed by an unknown peak (5) with a Rt = 15.71.

Fig. 5.6: HPLC profile of leaf-derived cell suspensions harvested at 15 d post culture (A) and spiked with diphyllin standard (B)



Peaks (1), (2), (3), (4) and (5) are unknown.

A

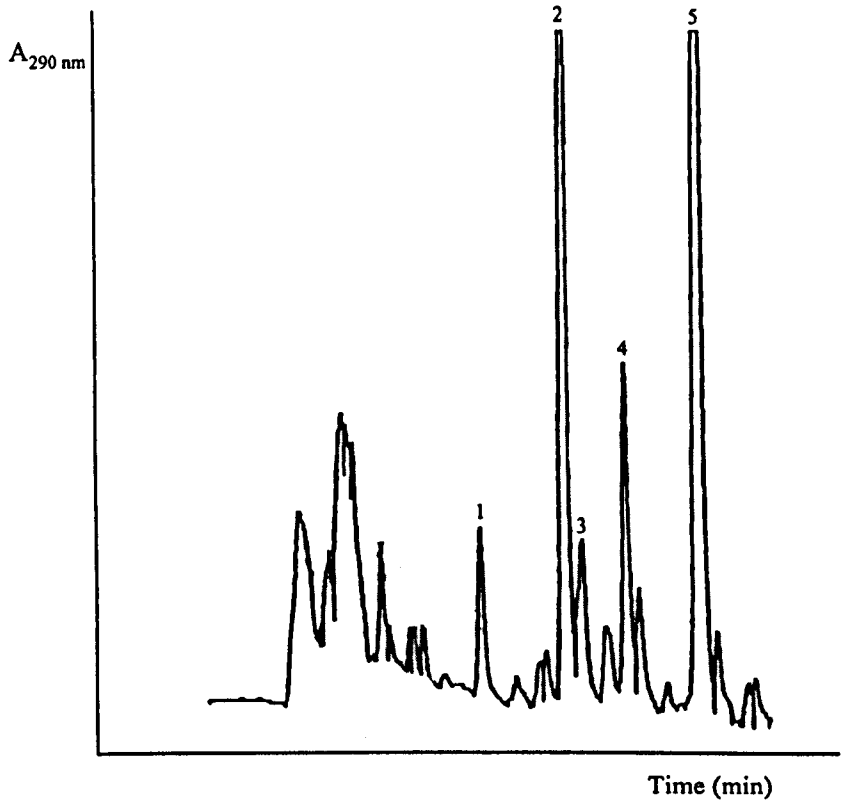


Peaks (1), (2), (3) and (5) are unknown; (4) diphyllin.

B

In leaf-derived cell suspension samples harvested at 25 d post subculture (Fig. 5.7), although it was not spiked, most of the peaks eluted at similar Rt values to those from leaf cell suspension extracts harvested at 15 d post culture (Fig. 5.6.A). HPLC analysis revealed peaks (1), (2), (3), (4) and (5) with Rt = 5.75, 8.89, 11.53, 13.49 and 15.74 respectively. Peak 5 (Fig. 5.7) exhibited a five-fold increase in percentage area (data not given) at 25 d post culture compared to peak 5 (Fig. 5.6.A) from cell suspension extracts harvested at 15 d.

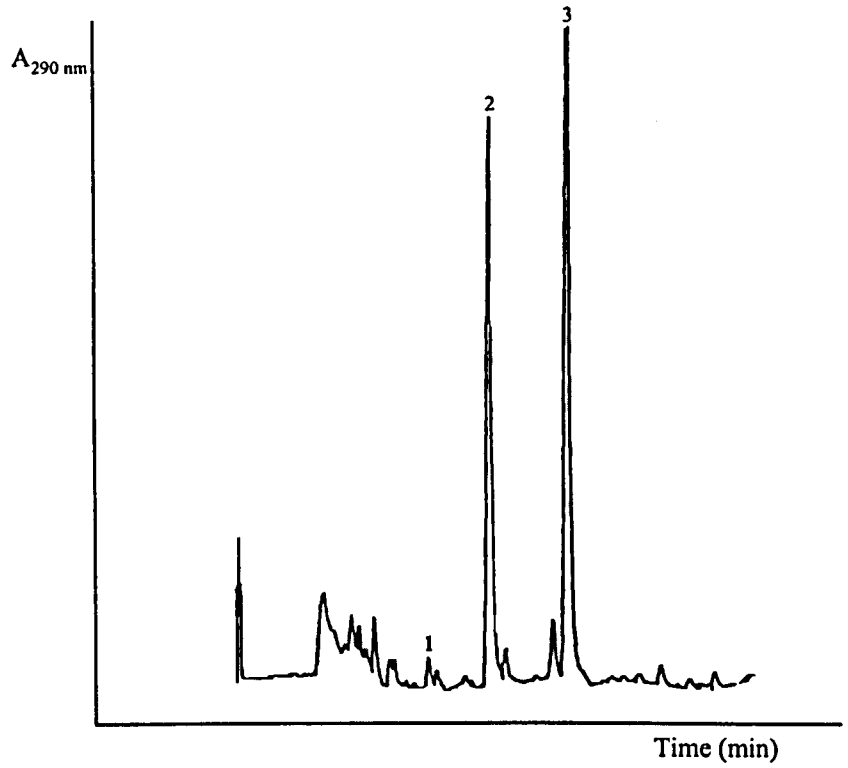
Fig. 5.7: HPLC profile of leaf-derived cell suspensions harvested after 25 d post subculture



Peaks (1), (2), (3), (4) and (5) are unknown.

The HPLC pattern of petiole-derived calli (Fig. 5.8) showed a minor peak (1) [Rt = 6.60] which corresponded to the same retention time shown by the podophyllotoxin standard (Table 5.4). The two major peaks, (2) and (3), had a retention time of 8.60 and 11.24 respectively but did not match with any of the standards used. The Rt values of peaks (2) and (3) were similar to those from cell cultures harvested at 15 d post culture (Fig. 5.6.A).

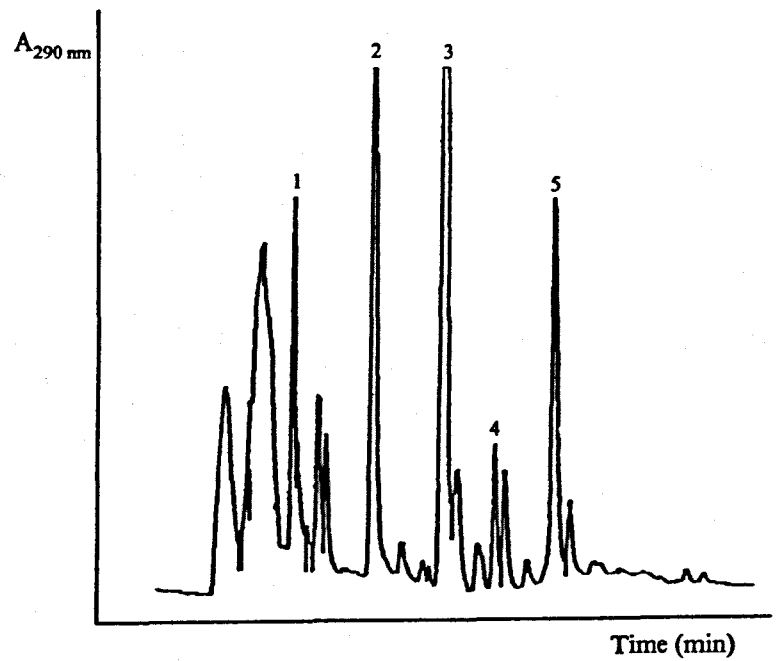
Fig. 5.8: HPLC profile of petiole-derived calli cultured on UM medium



Peak (1) podophyllotoxin; (2) and (3) are unknown.

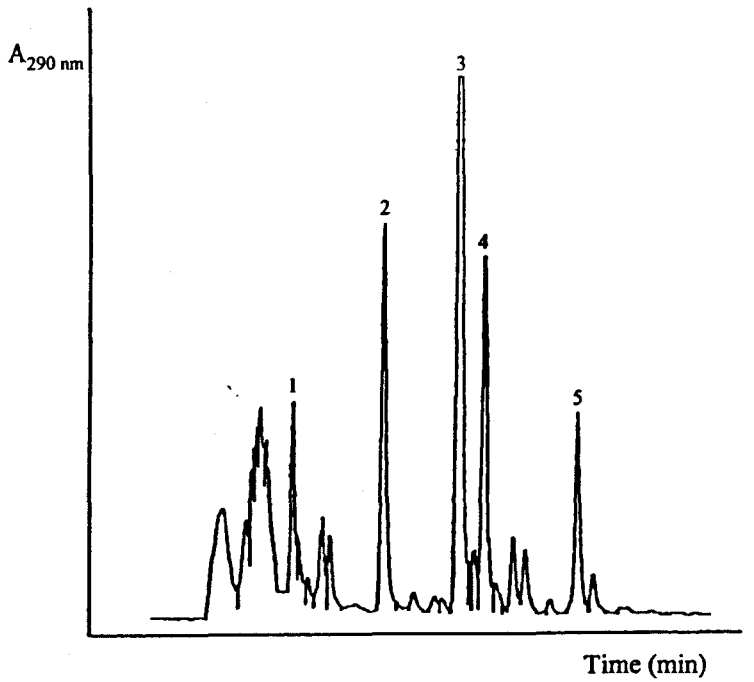
The five peaks observed in the chromatogram of petiole-derived cell suspensions harvested at 10 d post culture were: peaks (1), (2), (3) (4) and (5) with Rt values of 4.92, 8.86, 11.59, 13.59 and 15.84 respectively (Fig. 5.9.A). In petiole-derived cell suspensions harvested at 25 d post culture, peaks (1), (2), (3), (4) and (5) had the following Rt values 4.90, 8.59, 11.26, 13.25 and 15.56 (Fig. 5.9.B). HPLC analysis revealed that an unknown peak 1 (Fig. 5.9.A) showed approximately a two-fold percentage increase of area (data are not given) than peak 1 from petiole-derived cell suspension extracts harvested at 25 d post culture (Fig. 5.9.B) even though both peaks had similar Rt values.

Fig. 5.9: HPLC profile of petiole-derived cell suspensions harvested at 10 d (A) and 25 d (B) post culture



Peaks (1), (2), (3), (4) and (5) are unknown.

A



Peaks (1), (2), (3) and (5) are unknown; (4) diphyllin.

B

5.4 Discussion

In this study the gradient used was effective in separating the mixture of the lignan aglycones podophyllotoxin, 5-MPT and 4'-demethyldesoxypodophyllotoxin in a single HPLC run. The elution order was podophyllotoxin, 5-MPT and 4'-demethyldesoxypodophyllotoxin according to their polarity. Although the lignan 5-MPT was not found in *D. cymosa*, it was used as a marker in the lignan mixture. The presence of additional peaks in the chromatograms of the 4'-demethylpodophyllotoxin and diphyllin standards may be attributed to impurities in the samples but these did not interfere with the analyses of both lignans.

Extracts of whole plant tissues (leaves), leaf and petiole-derived cell supensions and calli were only injected on the reverse-phase HPLC column after enzymic hydrolysis with β-D-glucosidase. This method was found to be suitable for lignan screening since it increased the aglycone content of the extracts, after hydrolysis by liberating them of the corresponding glycosides. The use of enzymes to bring about hydrolysis is of interest because of its potential specificity for removing particular sugars from particular sites on the lignans. Moreover, the combination of gradient conditions and mobile phases resulted in an efficient and rapid separation of peaks.

In *D. cymosa*, the aryltetralin lactone lignan, podophyllotoxin, was identified in young leaf tissues from cultivated plants used as starting material in the tissue culture experiments (Chapter 3). This result was in agreement with earlier studies which demonstrated the presence of podophyllotoxin in *D. cymosa* (Broomhead and Dewick, 1990). According to the aforementioned study, the highest amounts of this lignan were detected in young leaves which appeared in May. Therefore, these tissues appeared to provide a high-yielding culture.

HPLC elution of extracts from petiole-derived friable calli cultured on solid UM medium in the dark conditions revealed the presence of podophyllotoxin with the same retention time value (6.60 min) as that of the authentic standard. In the present study, the peak corresponding to podophyllotoxin indicated that the lignan was accumulated at low concentrations in petiole-derived calli, which lacked the original petiole explant. These cultures differentiated into the embryogenic state (Chapter 3). In *D. cymosa*, the extent of tissue differentiation did not appear to influence lignan production because undifferentiated cultures (calli) synthesized lignan. However, it would be interesting to assess the lignan content in the embryogenic callus cultures. This was not possible during the present study since the HPLC facilities were not often available for the analysis. Since podophyllotoxin levels were not quantified in the present study, its occurrence in leaf tissues and petiole-derived calli were analysed purely in terms of aglycones liberated after enzymic hydrolysis of crude extracts.

HPLC analysis of harvested cell suspension cultures, derived from calli of both leaf and petiole explants, showed that they were not capable of lignan biosynthesis. Since both cell cultures were grown in liquid UM medium containing 2,4-D and kinetin, it may be possible that this factor affected lignan accumulation by increasing catabolic enzymes. The presence of synthetic auxins such as 2,4-D in the culture medium has been shown to support growth but inhibits secondary metabolism in different cultures (Arias-Castro *et al.*, 1993). Choice of genotype has also been reported to play an important role in production levels of secondary metabolites in tissue cultures (Zenk *et al.*, 1977), for example lignans in cell cultures of *Linum album* (Smollny *et al.*, 1998). Podophyllotoxin was the main product together with other lignans such as 5-MPT. The latter was found at low levels and was dependent on the genotype used to initiate the cell cultures.

The efficient production of secondary metabolites via calli or cell suspension cultures requires two growth stages which consist initially of a period for biomass production followed by a period of (limited) growth for secondary metabolite production and accumulation (Collin and Edwards, 1998). Therefore, secondary metabolite production becomes important after a growth stage when conditions are growth limiting. Several growth regulators such as auxins have been reported to strongly influence culture growth and secondary metabolism in tissue culture (Whitmer *et al.*, 1998). In *C. roseus* cell suspension cultures, 2,4-D negatively influenced the production of terpenoid indole alkaloids by reducing the source of supply of the biosynthetic precursors such as tryptamine which ultimately resulted in low yields of the medicinally important alkaloids (Whitmer *et al.*, 1998). The auxin 2,4-D is also known to inhibit cell differentiation. In the present work, the peak corresponding to podophyllotoxin in petiole-derived calli of *D. cymosa* suggested that the lignan may be present at low levels in these cultures even when they grown in the presence of 2,4-D and kinetin. However, when these calli were used as a source of cell suspension cultures maintained in liquid UM medium the presence of 2,4-D and kinetin in the culture medium ultimately inhibited lignan synthesis. This may be due to an increased cellular uptake of the auxin when cells are grown in liquid medium.

The capacity of plant cell cultures to produce secondary metabolites has been reported to be dependent on the nature of these metabolites (Marty *et al.*, 1997). Production could also be attributed to the key enzymes between primary and secondary metabolism being absent or inhibited in plant cell suspension cultures. Thus, it is important to isolate, purify and characterize the enzymes involved in the regulation of specific pathways (Alfermann and Petersen, 1995). For the isolation of enzymes, plant cell cultures are better sources than whole plants. This knowledge

could be useful for identifying the corresponding plant genes and for biotechnological use.

In the present study, HPLC analysis of *D. cymosa* leaf extracts revealed a peak which perhaps indicated the presence of diphyllin even though its retention time was less than that shown by the known diphyllin standard. Diphyllin was clearly seen in the spiked sample of leaf-derived cell suspensions harvested at 15 d post culture but was not detected in the sample without added diphyllin. Therefore, diphyllin was not found in leaf-derived cell cultures.

It was noteworthy that unknown peaks were detected in the chromatograms of calli and cell culture extracts of *D. cymosa*. Since the retention values did not correspond to any of the standards used, it would be crucial, in future studies, to isolate and purify these compounds in order to identify them based on spectral data. They could be secondary metabolites as for example new lignans from tissue cultures of *D. cymosa*.

These are the first phytochemical studies undertaken with tissue cultures of *D. cymosa*. Overall these studies have demonstrated that the HPLC system used was highly suitable for determining the presence of lignans in leaf tissues, as well as in petiole-derived calli of *D. cymosa*. The method used proved to be simple, very rapid and sensitive for screening lignans. In addition, the present study demonstrated that friable calli, derived from petiole explants, even when cultured on UM medium supplemented with 2,4-D were able to synthesizing and to accumulating podophyllotoxin; a valuable precursor for the chemical synthesis of the anticancer drugs etoposide and teniposide.

Chapter Six

Fingerprint Analyses of *C*-Glycosylflavonoids in *P. edulis*, *P. incarnata* and their Somatic Hybrids

6.1 Introduction

The extracts of *P. edulis* and *P. incarnata* are used in sedative preparations for oral administration as previously described (Chapter 1, Sections 1.2.2.7 and 1.2.2.8). Despite numerous studies, the active compounds responsible for their pharmacological activities remain unknown to date (Speroni *et al.*, 1996). Flavonoids and harman alkaloids have been proposed to be the active compounds. In *P. incarnata*, the harman alkaloids were not detected in sixteen out of seventeen different samples evaluated by reverse-phase HPLC (Rehwald *et al.*, 1995). Within the flavonoids, *C*-glycosylflavonoids are the main components in the aerial parts of *P. incarnata*.

In the early 1980s, an effort was made in Brazil to evaluate traditional herbal medicines. A programme supported by the Brazilian Federal Government in collaboration with some universities investigated Brazilian plants used by the local population for their primary health care (CEME, 1988). Relatively few plants had their medicinal applications validated by this programme. However, extracts from the leaves of *P. edulis* fv. *flavicarpa* displayed psychopharmacological activity in laboratory (Valle and Leite, 1978; Valle and Leite, 1983) and human clinical trials (CEME, 1988). The results obtained led to the recommendation of the tea, made from the leaves of this species, as a mild tranquilizer (Matos, 1991).

Only a few reports are known of adverse reactions to herbal preparations containing *Passiflora* extracts. For example, a patient taking tablets derived from *P. incarnata* for insomnia showed symptoms of urticaria and cutaneous vasculitis (Smith *et al.*, 1993). Recently, a case has been reported of a patient with occupational allergic respiratory diseases (asthma and rhinitis) caused by *Passiflora* (Bianchi *et al.*, 1997).

Of the *C*-glycosylflavonoids that have been reported in *Passiflora* extracts, the group of *C*-glycosylflavones is of therapeutic importance (Meier, 1995a). Therefore, in this chapter, attention was focussed on the analyses of *C*-glycosylflavones of the species *P. edulis* fv. *flavicarpa* and *P. incarnata* and their somatic hybrids. Future studies may allow exploitation of such novel germplasm.

6.1.1 Naturally occurring *C*-glycosylflavonoids

More than 300 natural *C*-glycosylflavonoids have been reported (Bruneton, 1995). Different structural types can be distinguished; the mono-*C*-glycosyl-, the di-*C*-glycosyl-, the *O*-glycosyl-*C*-glycosyl- and the *O*-acyl-*C*-glycosylflavonoids (Jay, 1994). They have sugar substituents bonded to a carbon in general at 6-*C* and/or 8-*C* positions of the aglycone. Phytochemical studies of leaves of *P. edulis* and *P. incarnata* have revealed complex patterns of *C*-glycosylflavonoids (Table 6.1) based on the aglycones apigenin and luteolin as shown in Fig. 6.1.

6.1.2 Biological and medical properties of *C*-glycosylflavonoids: an overview

Some *C*-glycosylflavonoids are of interest because they have biological and pharmacological properties. Some colourless *C*-glycosylflavones, such as swertisin and isoorientin can act as co-pigments to anthocyanins contributing to flower colour due to bathochromic shifts and increasing intensities of absorption (Asen *et al.*, 1986). *C*-glycosylflavones may also have a role as ultraviolet light screens (Les and Sheridan, 1990). They have been found in floating leaves of *Potamogeton* species but not in submerged leaves even though both were growing under the same environmental conditions. This has been attributed to the ability of floating leaves to filter UV radiation for their protection against its damaging effects.

With regard to the medicinal uses of *C*-glycosylflavonoids, isoorientin has been reported to have an antihepatotoxic activity (Hattori *et al.*, 1988). It can be metabolized by human intestinal bacteria according to studies on the metabolism of flavonoids by intestinal flora (Hattori *et al.*, 1988). Vitexin-2″-*O*-rhamnoside can increase the coronary blood flow (Rehwald *et al.*, 1994b). 2″-*O*-xylosylvitexin and vicenin-2 have been reported to exhibit a strong hypotensive activity (Jay, 1994). Glucosylorientin and vitexin are inhibitors of thyroid peroxidase and are responsible for the goitrogenic and antithyroid activities of *Pennisetum americanum* (Harborne and Baxter, 1993). Luteolin 4′-*O*-glucopyranoside, apigenin and luteolin have demonstrated an inhibitory effect on interleukin-5 bioactivity which is a therapeutic target for development of bronchial anti-inflammatory drugs (Park *et al.*, 1999).

6.1.3 Isolation and identification of *C*-glycosylflavonoids

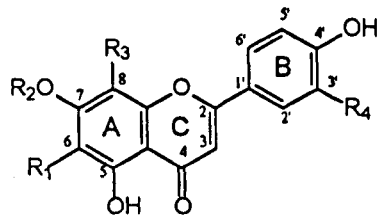
6.1.3.1 Extraction

C-glycosylflavonoids can be extracted at high temperature using acetone, ethanol or methanol mixed with water (20 to 50%) (Bruneton, 1995). Supercritical fluid extraction, which is an alternative to conventional liquid extraction, has been suggested as an option for flavonoid extraction from leaves of *P. alata*, *P. edulis* and *P. incarnata* (Moraes *et al.*, 1997). According to this study, carbon dioxide modified

Table 6.1: *C*-glycosylflavonoids from leaf extracts of *P. edulis* and *P. incarnata*

C-Glycosylflavonoid type	Compound	Reference
Mono *C*-glycosylflavones	Isoorientin, isovitexin, orientin, vitexin, luteolin-6-*C*-chinovoside, luteolin-6-*C*-fucoside, swertisin	Mareck *et al.*, 1991; Jay, 1994; Rehwald *et al.*, 1994a
Di-*C*-glycosylflavones	Lucenin-2, isoschaftoside, schaftoside, vicenin-2	Proliac and Raynaud, 1986; Li *et al.*, 1991
O-glycosyl-*C*-glycosylflavones	Isovitexin 2″-*O*-β-gluco-pyranoside, isoorientin 2″-*O*-β-gluco-pyranoside, isoscoparin 2″-*O*-glucoside	Li *et al.*, 1991; Rahman *et al.*, 1997

Fig. 6.1: Structures of *C*-glycosylflavonoids from *Passiflora*



Compound	No.	Substituents[a]			
		R_1	R_2	R_3	R_4
Vitexin	I	H	H	glc	H
Isovitexin (saponaretin, homovitexin)	II	glc	H	H	H
Orientin	III	H	H	glc	OH
Isoorientin (homoorientin)	IV	glc	H	H	OH
Saponarin	V	glc	glc	H	H
Schaftoside	VI	glc	H	ara	H
Isoschaftoside	VII	ara	H	glc	H
Vicenin-2	VIII	glc	H	glc	H
Isovitexin-2″-*O*-β-glucopyranoside	IX	soph	H	H	H
Isoorientin-2″-*O*-β-glucopyranoside	X	soph	H	H	OH
Isoscoparin-2″-*O*-glucoside	XI	glc	H	H	OCH_3
Swertisin (7-*O*-methyl isovitexin)	XII	glc	CH_3	H	H
Lucenin-2	XIII	glc	H	glc	OH

[a] glc = β-D-glucopyranosyl; ara = α-L-arabinopyranosyl; soph = sophorose.
Modified from Li *et al.* (1991).

with methanol was the extraction fluid and rutin, orientin and vitexin were identified in the leaves extracts of *Passiflora* by HPLC-DAD. Acid hydrolysis, coupled with an appropriate chromatographic system, has been suggested as a method for separating mono-*C*-glycosyl-, di-*C*-glycosyl- and *O*-glycosyl-*C*-glycosylflavones (Jay, 1994).

6.1.3.2 Chromatographic techniques

TLC is often used for characterization of the aglycones because there are no general reactions of flavonoids. Some of its applications in flavonoid studies include rapid microanalyses with small amounts of material (useful in the selection of solvents prior to column elution), analytical monitoring of fractions from column chromatography and identification of flavonoids by cochromatography (Markham, 1982). Table 6.2 summarizes some TLC systems used in the separation of flavonoid aglycones and their glycosides. For flavonoid aglycones, addition of an acid to methanol-water mixtures is required to avoid tailing (Hostettmann and Hostettmann, 1982). The use of conventional TLC with silica gel and very polar eluents (water, acetic acid, ethyl acetate and acetone) has been proven suitable for the analysis of *C*-glycosylflavonoids (Pothier, 1996). The replacement of silica gel with polyamide requires the use of less polar solvents (MEK and methanol) and acids are not used. The application of newer TLC techniques such as HPTLC for the qualitative and quantitative analysis of flavonoids which may be responsible for the anxiolytic properties of *P. coerulea* has been reported (Pastene *et al.*, 1997). In the latter, isoorientin and other minor flavonoids were detected from the methanolic extract by an optimized HPTLC method.

For detection of flavonoids on TLC plates there are several spray reagents, including a methanolic solution of the aminoethyl ester of diphenylboric acid (NP) followed by ethanolic polyethylene glycol (PEG). NP enables the different substitution patterns to be distinguished: under UV light at 365 nm the flavones and flavonols with 4′-hydroxylation or 3′, 4′- or 3′, 4′, 5′-hydroxylation reveal green, yellow and orange spots respectively (Andersen and Francis, 1996). PEG lowers the detection sensitivity of flavonoids from 10 ng to 0.5 ng (Wagner *et al.*, 1984). In addition, other chromogenic spray reagents such as, for example aluminium chloride, antimony chloride in 5% (v:v) alcohol (Pothier, 1996) and diphenyltin dichloride (Andersen and Francis, 1996), have also been used for detection of flavonoids.

HPLC has been proved to be a powerful technique for the qualitative and quantitative determination of *C*-glycosylflavones (aglycones and glycosides). The development of HPLC procedures for their analysis is particularly significant for *Passiflora* and has opened up new possibilities for the separation of other *C*-glycosylflavones. In this context, swertisin has been found for the first time in the

methanolic extract of *P. incarnata* (Rehwald *et al.*, 1994a). Several HPLC studies of *C*-glycosylflavonoids have been reported, and some of them are shown in Table 6.3. Furthermore, HPLC studies coupled with ionspray ionization tandem mass spectrometry and nuclear magnetic resonance have been undertaken for the identification and characterization of *C*-glycosylflavonoids from extracts of *P. incarnata* as well as for the structural elucidation of isolated unknown compounds (Raffaelli *et al.*, 1997; Chimichi *et al.*, 1998). In these studies, ten known flavonoids and a new di-*C*-glycosylflavonoid were identified and characterized.

Table 6.2: TLC systems for the separation of polar flavonoid aglycones and glycosides

Flavonoid type	Stationary phase	Mobile phase	Detection[1]	Reference
Polar Aglycone	Silica gel[2]	$C_6H_5CH_3$:HCO_2Et:HCO_2H (5:4:1)	UV	Andersen and Francis, 1996
	Cellulose	HOAc (50%)	UV	Markham and Bloor, 1998
	Polyamide[3]	$C_6H_5CH_3$:MEK:MeOH (12:5:3)	UV	Fried and Sherma, 1996
	RP-18[4]	MeOH:HCO_2H:H_2O (58:10:16)	NP/PEG and UV	Andersen and Francis, 1996
Flavonoid Glycoside	Silica gel[2]	$CH_3CO_2C_2H_5$:HCO_2H: HOAc:H_2O (100:11:11:27)	NP/PEG and UV	Andersen and Francis, 1996
	Cellulose	n-BuOH:HOAc:H_2O (4:1:5)	UV	Markham, 1982

[1] The use of a spray reagent (NP/PEG 4000) may improve detection; [2] Silica gel (60 F_{254}) plates; [3] Polyamide (DC-Alufolien F_{254}) plates; [4] RP-18 (octadecyl-bonded silica) plates.

6.1.4 Aims of these studies

The aim of the present study was to provide chromatographic evidence to supplement the identification of the *P. edulis* and *P. incarnata* genome in novel somatic hybrids. For that purpose, studies were undertaken to determine the TLC and HPLC flavonoid profiles of four putative somatic hybrids [*P. incarnata* (+) *P. edulis*]. In addition, studies mainly on *C*-glycosylflavones, aimed to assess whether they were synthesized and accumulated by both *Passiflora* parental species as well as their somatic hybrids. This would provide evidence that such novel hybrids have potential medicinal value.

Table 6.3: HPLC studies for the analysis of *C*-glycosylflavonoids in *Passiflora*

Analytical technique	Summary of protocol	Reference
HPLC	On Bondapack C_{18} in solvent A (5% HOAc in H_2O) and solvent B (1% HOAc in MeCN), g/e [1]	Forni, 1980
HPLC	On Bondapack C_{18} in iPrOH:THF:H_2O (5:15:85), i/e[2]	Pietta *et al.*, 1986
HPLC (reverse-phase), MS, ^{13}C NMR	On Lichrosorb RP-18 in 12% MeOH (i/e[2] for 2 min); 5% HCO_2H in H_2O, g/e[1]), 25 and 40% MeOH (after 8 and 25 min respectively)	Li *et al.*, 1991
HPLC (reverse-phase, DAD detector)	On Bondapack C_{18} in solvent A [THF:iPrOH:MeCN (10:2:3)] and solvent B (0.5% H_3PO_4), i/e[2] (0 to 15 min 12% A in B)	Rehwald *et al.*, 1994a
Rapid-HPLC (reverse-phase, DAD detector)	Kaempferol-7neohesperidoside in MeOH added before HPLC on Nucleosil RP-18 in solvent A (MeCN) and solvent B (0.15% THF in H_2O), linear g/e[1]	Krenn *et al.*, 1995 [cited by Meier, 1995a]
HPLC (reverse-phase)	On Apex ODS in iPrOH:THF:H_2O (5:15:85) or HOAc:MeCN:H_2O (10:180:820), i/e[2]	Silva *et al.*, 1995

[1] g/e = gradient elution; [2] i/e = isocratic elution.

6.2 Materials and methods

6.2.1 Plant materials

This study utilised plant germplasm maintained at the University of Nottingham. *P. edulis* fv. *flavicarpa* and *P. incarnata* plants were propagated by seed-grown in a mixture of Levinton M3 soil-less and John Innes No. 3 composts (1:1, v:v) (Otoni *et al.*, 1995). Seedlings were transferred to the glasshouse and grown under natural daylight at 28°C. Somatic hybrid plants (designated SH1, SH2, SH3 and SH4) of independent origin were obtained by electrofusion of leaf mesophyll protoplasts of both *Passiflora* species mentioned above (Otoni *et al.*, 1995) and were clonally propagated by vegetative cuttings.

6.2.2 Sample preparation

Fully expanded leaves from *P. edulis* fv. *flavicarpa*, *P. incarnata* and their somatic hybrids [SH1, SH2, SH3 and SH4] (Plate 6.1.A) were harvested and pooled from the entire length of the vines (3 years old) grown in the glasshouse. Leaves were transferred to steel baskets and dried for 24 h in an oven at 40 - 45°C. Dried leaves

were ground in an electric mill and stored in brown jars, protected from light and humidity, until required.

6.2.3 Source of standards

The pure standard of chlorogenic acid was kindly supplied by Dr MA Paulo (Faculdade de Farmácia, Universidade de Lisboa, Lisboa, Portugal), and a reference mixture of isovitexin with vitexin by Dr S Bladt (Institut für Pharmazeutische Biologie, Universität München, München, Germany). Standards for isoorientin (homoorientin), isovitexin, orientin and vitexin (HPLC grade) were purchased from Extrasynthèse (Genay, France). Since these *C*-glycosylflavonoids are the main flavonoids which have been reported in *P. edulis* and *P. incarnata*, they were used as standards in this study.

6.2.4 Analysis of flavonoid glycosides

6.2.4.1 Extraction from plant tissue

In the present studies, *C*-glycosylflavonoids were investigated according to published methods (Silva *et al.*, 1995). Powdered leaves (1.0 g d.wt) of each sample (from six lines) were extracted with 10 ml pre-warmed analytical grade methanol (Sigma) for 5 min on a water bath at 60°C. After cooling, the methanolic extract was filtered (Whatman No. 1 filter paper) and the residue washed with a further portion of 2 ml methanol. The clear filtrates were combined and concentrated to dryness under reduced pressure using a rotary evaporator at 30°C. The green residues were used for TLC analysis and flavonoid isolation.

6.2.4.2 Separation of flavonoids by TLC

For analytical TLC, the green residue above was resuspended in 1 ml methanol and applied on TLC aluminium sheets (10.0 x 10.0 cm) precoated with a layer (0.20 mm) of silica gel (Merck Kieselgel GF_{254}). The analytes and standards were applied (10 µl and 20 µl) as bands (approx. 1.0 cm in length), using a glass capillary, and the plates were developed in a saturated tank containing 150 ml of ethyl acetate (BDH):formic acid (Sigma):glacial acetic acid (BDH):water [100:11:11:27, (v:v:v:v); Solvent A] or ethyl acetate:formic acid:water [90:11:11, (v:v:v); Solvent B] as the mobile phase then and air-dried.

6.2.4.3 Detection of flavonoids

Flavonoids separated by TLC (Section 6.2.3.2) were detected either without chemical treatment or by the use of spray reagents.

6.2.4.3.1 Without chemical treatment (fluorescence)

The natural UV-absorbance of flavonoids was observed under UV light at 254 and 365 nm.

6.2.4.3.2 TLC spray reagents

After development, TLC plates were dried in a fume cupboard to completely remove the mobile phase. Flavonoid bands were examined under UV light at 365 nm after spraying the dried plates with 1% (v:v) methanolic solution of the aminoethyl ester of diphenylboric acid (NP) (Sigma) followed by 5% (w:v) polyethylene glycol MW 4000 (PEG) in absolute ethanol. The bands were characterized by their colours and R_f values in relation to reference standards. Photographs of plates were taken with a Leica camera using Kodak Gold 100 film and a UV filter system using Y1(yellow) for UV light at 365 nm.

6.2.5 Isolation and characterization of *C*-glycosylflavonoids

6.2.5.1 Isolation from leaf tissue

Attempts to isolate and identify some of the *C*-glycosylflavonoids from *P. incarnata* and SH1 were carried out. SH1 was randomly selected from within the somatic hybrids. Leaf extracts from these plants were analysed by PLC, TLC and mass spectrometry as part of this study. The green residues of both samples (Section 6.2.4.1) were treated with 1 ml methanol (Analar) and applied to the PLC plates (20 x 20 cm) precoated with a layer (1.0 mm) of silica gel (Merck Kieselgel GF_{254}). Development of plates was performed in a saturated glass tank containing 150 ml of solvent A as previously described (Section 6.2.4.2). Bands were visualised under UV light at 254 and 365 nm, and relevant bands were individually scraped from the plates for purification of the flavonoids.

6.2.5.2 Purification of flavonoids

Aliquots of silica containing the bands that were removed from the plates were stirred overnight with 30 ml methanol (Analar). This process was used to maximize the extraction of the compounds from the silica. The resulting extracts were filtered under vacuum and concentrated to dryness under reduced pressure (30°C). To ensure the purity of the isolated compounds additonal TLC was carried out against flavonoid standards. The purified compounds were then characterized by mass spectrometry.

6.2.5.3 Mass spectral characterization of flavonoids

For structural characterization and identification of the isolated compounds from *P. incarnata* and SH1, electron impact and fast atom bombardment (FAB) mass

spectrometry were applied. The FAB mass spectra were recorded on a KRATOS (Model MS890MS) spectrometer. Samples were dissolved in methanol and 1 µl of the solution was added to thioglycerol that was the liquid matrix used .

6.2.6 HPLC of leaf extracts of all lines of *Passiflora*

Initially, the HPLC analysis of flavonoids in the leaf extracts of *P. edulis*, *P. incarnata* and their four somatic hybrids were based on published methods (Rehwald *et al.*, 1994a) using an isocratic separation. However, the chromatograms showed broadened peaks and good separations were not achieved (chromatograms are not shown). Therefore, some modifications were made to this separation protocol as described in Section 6.2.6.3.

6.2.6.1 Standards and solvents

Authentic standards (Section 6.2.3) were used for HPLC analyis of *Passiflora* leaf extracts. The solvents acetonitrile, isopropanol, methanol and tetrahydrofuran were of HPLC grade whilst orthophosphoric acid was of analytical grade.

6.2.6.2 Preparation of standard solutions

The standard solutions were prepared at different concentrations in 2 or 5 ml methanol as described in Table 6.4.

Table 6.4: Standard solutions for HPLC analysis

Substance	Weight (mg)	Solvent (methanol) (ml)
chlorogenic acid	2.983	2
isoorientin	1.045	5
isovitexin	1.164	5
orientin	1.790	5
isovitexin/vitexin mixture	0.085	2
vitexin	1.320	5

6.2.6.3 HPLC conditions

Qualitative analyses were performed using a Hewlett Packard 1100 instrument fitted with a reverse-phase C-18 column (150 mm x 3.9 mm i.d.; 5 µm) (Nova-Pak; Waters, Bedford, USA). Samples (5 µl) were injected by the Hewlett Packard autosampler (Model G1313A) into the HPLC system. Eluent flow-rate was 1 ml min^{-1} and the delivery was provided by an HP 1100 binary pump (Model G1312A). The

column temperature was maintained at 23°C by the HP 1100 thermostat (Model G1316A). For chromatographic separation, a gradient elution was used and the conditions are described in Table 6.5. The UV spectra at 336 nm were recorded by a Hewlett Packard diode array detector (Model G1315A). Samples of each *Passiflora* extract (all six lines) were prepared in triplicate and each aliquot was analysed up to three times under the same conditions. The Rt values were recorded for all samples and standards. The chromatograms were scanned as previously described (Chapter 4, Section 4.2.5.4.3; see also Appendix 4).

Table 6.5: Gradient conditions for HPLC

Time (min)	Flow-rate (ml min^{-1})	Solvent A H_3PO_4[1] (%)	Solvent B THF:iPrOH:MeCN[2] (%)
0	1.0	92	8
25	1.0	80	20
30	1.0	92	8
35	1.0	92	8

[1] H_3PO_4 is orthophosphoric acid; [2] THF:iPrOH:MeCN corresponds to tetrahydrofuran:isopropanol:acetonitrile.

6.3 Results

6.3.1 TLC assessments of *C*-glycosylflavonoids

6.3.1.1 Before and after chemical treatment

The fluorescence of flavonoids in UV light at 254 nm was seen as dark blue bands on a yellow background on the TLC plates without chemical treatment. Using both solvent systems A or B, typical fluorescent zones were observed after spraying. Flavonoids bands were differentiated on the basis of their colour and R_f (Appendix 5) and compared to bands of the standards used. Comparisons in UV light at 365 nm of plates from the six lines of *Passiflora* showed the following chromatographic bands:

P. edulis

The chromatogram (Plate 6.1.B) was characterized by an orange fluorescent zone in UV light at 365 nm showing the same R_f as the isoorientin standard (R_f = 0.80). There was a yellowish fluorescent zone in the same R_f region as vitexin (R_f = 0.89) that may have masked this flavone zone. A yellowish fluorescent zone was visualised in the region of isovitexin (R_f = 0.85). The same chromatogram also showed five orange fluorescent zones below isoorientin which could not be identified with any of the standards used.

P. incarnata

The chromatogram (Plate 6.1.B) showed a yellowish-green fluorescent zone in the same R_f region as the vitexin standard (R_f = 0.89). Isovitexin was only detectable as a narrow yellowish-green fluorescent zone (R_f = 0.85). A pale blue fluorescent zone directly below isovitexin was possibly due to phenol carboxylic acids (*e.g.* chlorogenic acid). There were also two further yellowish-green fluorescent zones (R_f = 0.66 and 0.70 respectively) below isovitexin which did not match with the standards used. In the two parental species, the zone corresponding to chlorophyll fluoresced red in UV light at 365 nm (Plate 6.1.B).

Somatic hybrids

The extracts of the four somatic hybrids showed similar patterns of flavonoids (Plate 6.1.C) characterized by a main orange fluorescent zone of isoorientin (R_f = 0.72). This flavone appeared as a strong zone which could be an indication of its high concentration. The size of the isoorientin spot was similar between the somatic hybrids except in SH1. In the latter, the spot size was somewhat higher than of that in the other three. Five more orange fluorescent zones were present in the low and intermediate R_f range (0.49 - 0.69). Among them, an intense orange zone (R_f = 0.56), possibly corresponding to flavonoid aglycones was seen at the solvent front. These zones were also visualised in *P. edulis* (Plate 6.1.B). It is possible that the two middle zones were masking those yellowish-green fluorescent zones (R_f = 0.66 and 0.70 respectively) observed in *P. incarnata* (Plate 6.1.B). In SH1, a yellowish-green fluorescent zone of vitexin (R_f = 0.86) was detected. This zone was weak in SH3 and SH4 and seemed to be only present in traces in SH2. In SH1, a pale yellow-green zone (R_f = 0.81) migrated in the same region as the orientin standard, whilst only traces were observed in this zone in SH3 and SH4. The red zone of the chlorophyll fraction was observed in each of the the somatic hybrids at the upper R_f region.

6.3.2 Identification and characterization of *C*-glycosylflavonoids

6.3.2.1 Isolation and purification of flavonoids

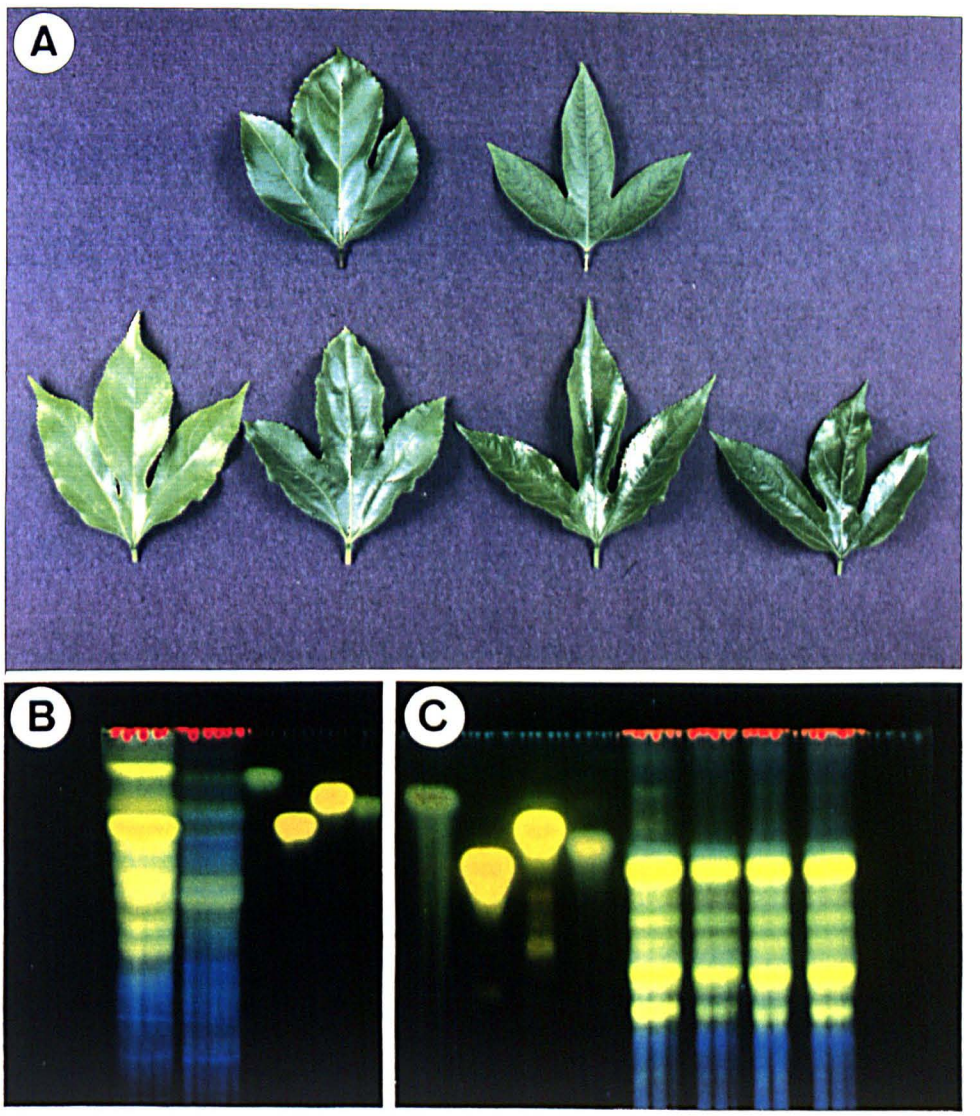
Three bands (1, 2 and 3) separated from the methanolic leaf extracts of *P. incarnata* by PLC were located under UV light. Band 1 appeared as a mixture of two zones showing yellow-green and blue fluorescence in the long wavelength (365 nm). Band 2 was intense in the short wavelength (254 nm) irradiation and showed a strong yellow-green fluorescence at 365 nm. Band 3 was characterized by two intense orange and yellow-green fluorescent zones and a narrow blue fluorescent zone at 365 nm. Only the zones showing the stronger fluorescence were separated from each band for further purification. When the zones showing the blue fluorescence were

Plate 6.1: Flavonoid banding patterns of leaf extracts of *Passiflora* species and their somatic hybrids

A. Mature leaves were harvested from *P. edulis* fv. *flavicarpa*, *P. incarnata* and their novel somatic hybrids (designated SH1, SH2, SH3 and SH4) which were maintained in the glasshouse with natural daylight supplemented by a 16 h photoperiod (180 μmol m^{-2} s^{-1}) provided by "Daylight" fluorescent tubes. Top row, left to right: *P. edulis* and *P. incarnata*, botton row, left to right: SH1, SH2, SH3 and SH4.

B. TLC separation of *C*-glycosylflavonoids from leaf extracts of *P. edulis* fv. *flavicarpa* and *P. incarnata*, together with reference flavonoids vitexin, isoorientin, orientin and isovitexin. Mobile phase: ethyl acetate:formic acid:glacial acetic acid:water (100:11:11:27, by volume). Stationary phase: sylica gel 60 F_{254} (0.20 mm). Spotting volume: 10 μl. Developing distance: 7.2 cm. Detection: spraying with 1% (v:v) methanolic solution of the aminoethyl ester of diphyenylboric acid, followed by 5% (w:v) polyethylene glycol MW 4000 and visualisation under UV at 365 nm. Left to right, *P. edulis* and *P. incarnata*, vitexin, isoorientin, orientin and isovitexin.

C. TLC separation of *C*-glycosylflavonoids from leaf extracts of the somatic hybrids SH1, SH2, SH3 and SH4. The chromatographic conditions were the same as described in Plate 6.1.B. Left to right, reference flavonoids vitexin, isoorientin, orientin, isovitexin, and extracts from somatic hybrids SH1, SH2, SH3 and SH4.


A
B
C

cochromatographed on the same chromatogram against flavonoids standards these zones appeared in the region of the chlorogenic acid standard. Three compounds (PI/1, PI/2 and PI/3) were isolated and purified from the *P. incarnata* extracts. After further TLC, which improved purification of the isolated compounds, PI/1 and P1/2 revealed a yellow-green fluorescence on the same chromatogram. When isolated, compound PI/2 was light yellow whilst the other two were off-white.

From the extracts of SH1, three bands (2B, 3A and 3B) showing only a intense yellow-green fluorescence were isolated. Three compounds could be isolated from these bands. Two isolated compounds (PI/1 and PI/3) from *P. incarnata* and three from SH1 extracts were characterized by mass spectrometry.

6.3.2.2 Structural characterization of the isolated flavonoids

The structural elucidation of the flavonoids isolated from the leaf extracts of *P. incarnata* and SH1 was attempted by EI and FAB-MS (see spectra in Appendix 7.2). FAB spectral data are summarized in Table 6.6.

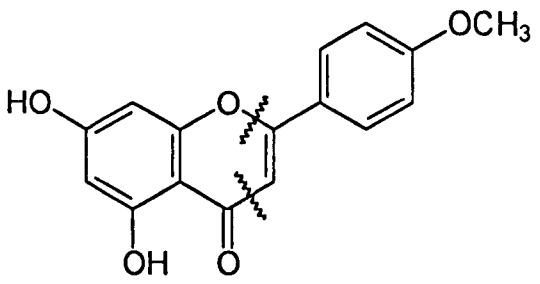
Table 6.6: FAB-MS spectral data for the isolated flavonoids

Compound	m/z[1] (%)[2]
PI/1	365 (26), 279 (55), 237 (54), 217 (15), 181 (76), 147 (27), 131 (100), 91 (84)
PI/3	181 (100), 279 (0.2), 237 (31), 149 (45), 117 (15), 107 (42), 91 (65), 73 (68)
SH1/2B	469 (15), 338 (32), 239 (16), 217 (22), 181 (14), 149 (36), 131 (77), 91 (100)
SH1/3A	279 (13), 239 (25), 217 (68), 181 (48), 131 (77), 91 (100)
SH1/3B	391 (2), 338 (29), 257 (1), 217 (20), 197 (4), 181 (22), 167 (3), 150 (3)

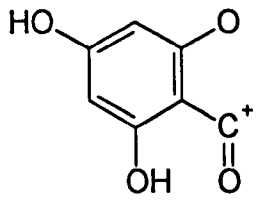
[1] m/z values are the expression of the mass of the ion divided by its charge; [2] Percentage values given in parentheses indicate the intensities of peaks relative to the base peak.

Based on the mass spectral fragmentation of $[M + H]^+$ ions of the isolated compounds some structures were proposed using the flavone acacetin (Fig. 6.2) as a model. This compound has a molecular mass of 284 and breaks down into two fragments m/z 152 and 132 corresponding to the two fragments **1a** and **1b** respectively.

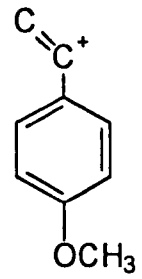
Fig. 6.2: Fragmentation pattern of acacetin



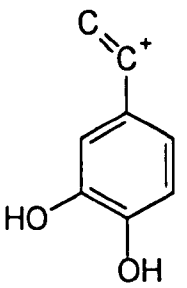
1 Acacetin (m/z 284)



1a m/z 152

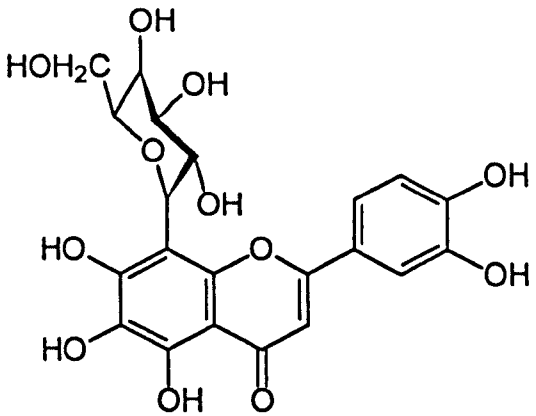


1b m/z 132



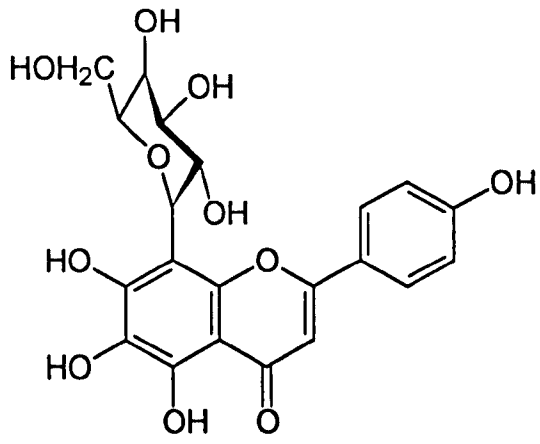
1c m/z 133

From the EI-MS spectrum of the compound PI/1 (Appendix 7.2.1) isolated from *P. incarnata* the molecular ion may be that at m/z 430 although there is also a peak at m/z 503 but no peak above m/z 365 or 338 can be seen in the FAB-MS spectrum (Appendix 7.2.2). The latter shows peaks at m/z 91, 131, 149, 181, 217, 237 and 279. The peak at m/z 131 could be due to an ion derived from **1c** (Fig. 6.2) corresponding to the loss of two hydrogens. The peak corresponding to m/z 152 in **1** (Fig. 6.2) may be m/z 181 in PI/1 with the carbon attached to the A-ring from the sugar moiety at C-8 of the aglycone. On this basis, the compound PI/1 might possibly be 5, 6, 7, 3′, 4′-pentahydroxyflavone 8-*C*-β-D-glucopyranoside (or 6-hydroxy orientin) represented by the structure **2,** which shows free hydroxyl groups at C-7, C-6, C-5, C-3′ and C-4′.



2 MW = 465

There was no obvious molecular ion peak in the FAB-MS spectrum (Appendix 7.2.3) of the compound PI/3 from leaf extracts of *P. incarnata*. The peaks at m/z 117 and 181 may originate as mentioned earlier in this Section (for the compound PI/1); this would indicate structure **3** below. The saccharide (glucose) is also attached directly to the aromatic A ring by a carbon-carbon bond as previously shown in the compound PI/1. The peak at m/z 117 corresponding to m/z 133 in the fragment **1c** is in agreement with the loss of oxygen (16 amu). Compound PI/3 may thus be 5, 6, 7, 4′-tetrahydroxyflavone 8-*C*-β-D-glucopyranoside.



3 MW = 431

In the case of compound SH1/2B, the ordinary mass spectrometric fragmentation (Appendix 7.2.4) showed a molecular ion peak at m/z 430 or 503, whilst the FAB-MS spectrum (Appendix 7.2.5) did not show anything above m/z 469. This compound (SH1/2B) could be constructed from the two parts as above, showing ions at m/z 338 and 131, which are portions corresponding to **1c** and the ion of m/z 181 plus a sugar. Therefore, it seems that compound SH1/2B is probably an isomer of **2**. For compound SH1/3A, the EI-MS (Appendix 7.2.6) showed a peak, perhaps a molecular ion, at m/z 428 or 503 but there was not much information from the FAB-MS spectrum (Appendix 7.2.7). Fragments that appeared at m/z 131, 181, 217 and others indicate that it is probably an isomer of **2** and/or SH1/2B. The EI-MS of the compound SH1/3B (Appendix 7.2.8) is possibly incomplete. The FAB-MS spectrum (Appendix 7.2.9) cuts off below m/z 150, but the peaks at m/z 181, 217 and 338 are very similar to most of the other compounds mentioned above.

6.3.3 HPLC profiles of leaf extracts of all lines of *Passiflora*

6.3.3.1 HPLC analysis of standards

The Rt (chromatograms not shown) of the standards are given in Table 6.7.

Table 6.7: Retention times of flavonoid standards[1]

Substance	Retention time (min)
Chlorogenic acid	6.55
Isoorientin	12.63
Isovitexin/vitexin mixture	15.72/18.76
Isovitexin	16.09
Orientin	16.29
Vitexin	19.14

[1] System solvent: 0.5% H_3PO_4 (Solvent A) and THF/IPA/MeCN [10:2:3, (v:v:v); Solvent B].

6.3.3.2 Qualitative fingerprint analysis of *P. edulis* and *P. incarnata*

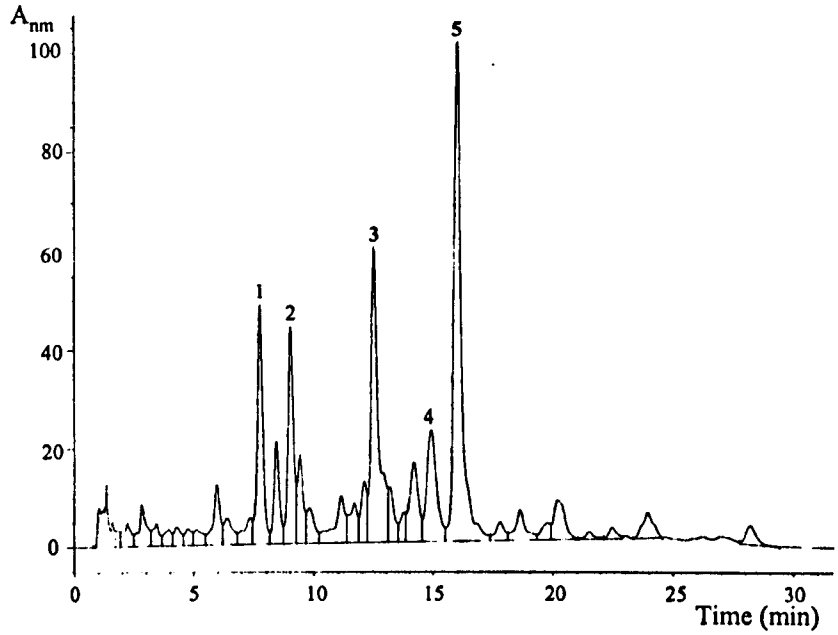
Five main peaks (1), (2), (3), (4) and (5) were observed in the chromatogram of *P. edulis* leaves (Fig. 6.3). Their respective Rt and UV-absorbance are given in Table 6.8. Peaks (1), (2) and (4) did not match with any of the standards used. The Rt of peak (3) was sligtly lower than that of the isoorientin standard (Table 6.7), but the UV spectrum of this peak (Fig. 6.4) was similar to that the standard (Fig. 6.4). On the basis of these results, peak (5), the major peak observed, had a Rt (Table 6.8) somewhat higher than that of the isovitexin mixture standard (Table 6.7). The selected UV spectrum of peak (5) gave three absorption bands with a shoulder (Table 6.8) and was in agreement with that of isovitexin. Thus, by comparison of the UV spectra of peak (5) (Fig. 6.5) and isovitexin standard (Fig. 6.5), this peak is possibly isovitexin.

Table 6.8: Retention times and maximum absorbance of peaks detected in the HPLC of leaf extracts of *P. edulis*

Peak number	Rt (min)	UV (λ_{max})[1]
1	7.73	215, 272, 334
2	9.01	215, 270, 338
3	12.49	208 sh, 268, 352
4	14.91	-
5	15.96	212 sh, 268, 339

[1] λ_{max} represents the wavelength (in nm) of maximum absorption detected by UV spectrometry and values are recorded only for selected peaks; sh means shoulder. The solvent was MeOH. Shading indicates the peaks that are believed to correspond to the isoorientin and isovitexin standards used.

Fig. 6.3: HPLC fingerprint chromatogram of leaf extracts of *P. edulis*



Peaks (1), (2) and (4) are unknown; (3) isoorientin; (5) isovitexin.

Fig. 6.4: Comparison of peak (3) and isoorientin standard UV spectra

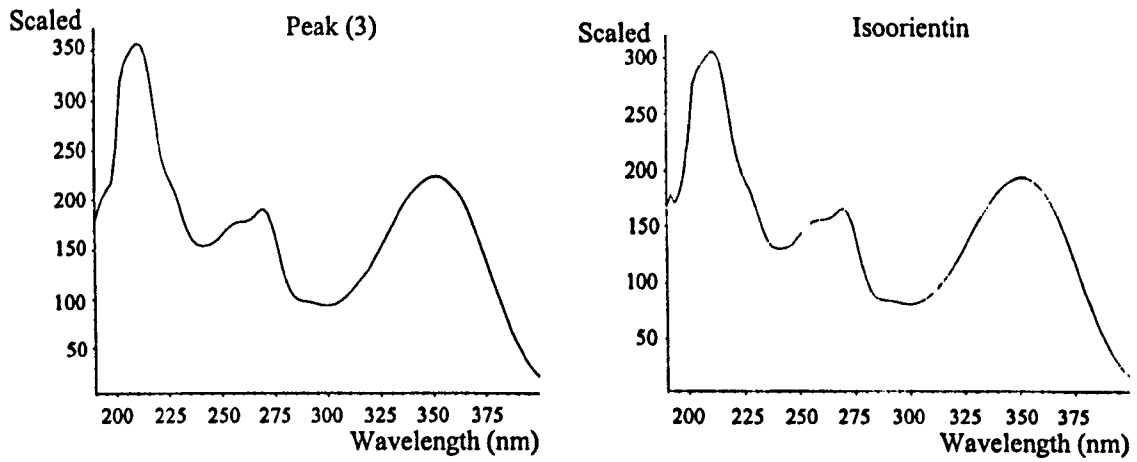
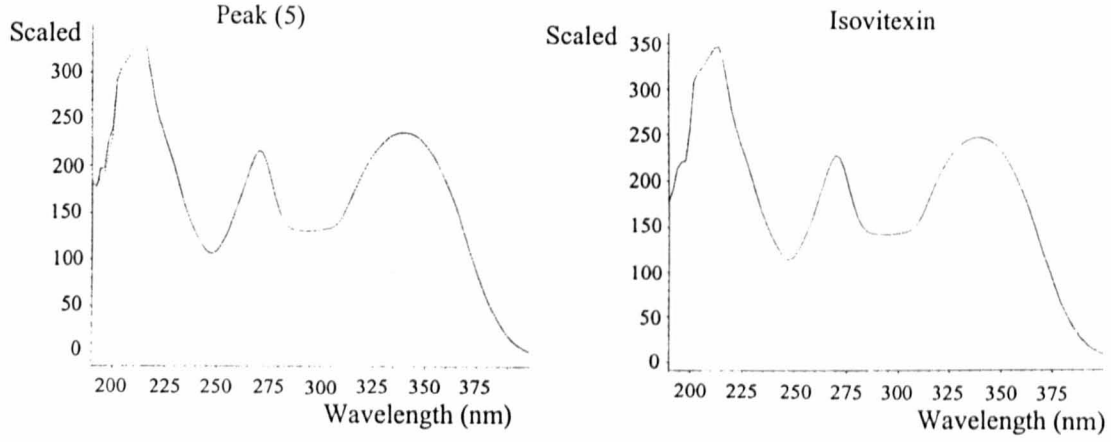


Fig. 6.5: Comparison of peak (5) and isovitexin standard UV spectra



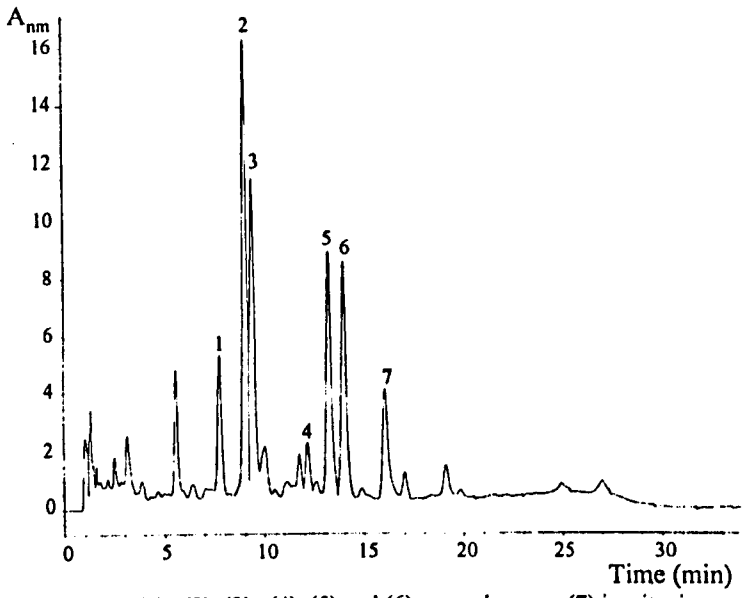
In the chromatogram of leaf extracts of *P. incarnata* (Fig. 6.6), six main peaks were detected and their Rt and UV-absorbance are shown in Table 6.9. Peaks (1) and (2) are similar to those of *P. edulis* (Table 6.8). Peaks (2) and (3) are very similar based on their UV spectra. They appear to be isovitexin type. Peaks (4), (5) and (6) are also unknown. Peak (7) is possibly isovitexin based on its UV spectrum (Fig. 6.6), even though the Rt of this peak (Table 6.9) was slightly lower than that of the isovitexin standard (Table 6.7).

Table 6.9: Retention times and maximum absorbance of peaks detected in the HPLC of leaf extracts of *P. incarnata*

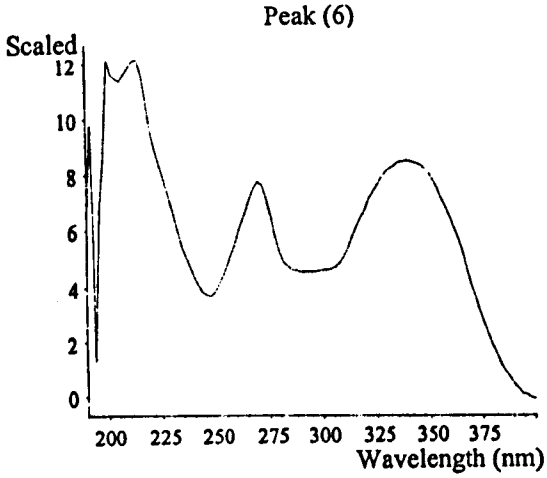
Peak number	Rt (min)	UV (λ_{max})[1]
1	7.77	203, 213, 269, 335
2	9.04	202 sh, 215, 270, 337
3	9.43	202, 215, 270, 336
4	12.15	212 sh, 214 sh, 270 sh, 271 sh, 337
5	13.24	204 sh, 215, 270, 337
6	13.98	204 sh, 213, 270, 337
7	16.04	203, 213 sh, 268, 340

[1] λ_{max} represents the wavelength (in nm) of maximum absorption detected by UV spectrometry and values are recorded only for selected peaks; sh means shoulder. The solvent was MeOH. Shading indicates the peak that is believed to correspond to the isovitexin standard used.

Fig. 6.6: HPLC fingerprint chromatogram of leaf extracts of *P. incarnata* and UV spectrum of peak (6)



Peaks (1), (2), (3), (4) (5) and (6) are unknown; (7) isovitexin.



6.3.3.3 Qualitative fingerprint analysis of *Passiflora* somatic hybrids

The four somatic hybrids studied show some similarities to each other in their chromatographic patterns. The separation of peaks by HPLC with the aforementioned solvents led to nine main peaks of increasing polarity (Fig. 6.7). Peaks with Rt values similar to the peaks (1), (2), (3), (6) and (7) (Table 6.10) were previously found in both parents (Tables 6.8 and 6.9). On the other hand, peaks (4) and (5) with Rt about 10.54 and 11.04 respectively were present in the somatic hybrids (Table 6.10) but not in either parent. Peak (6) with a Rt of 12.60 is possibly isoorientin, even though its Rt was slightly lower than of that of the isoorientin standard (Table 6.7). The UV spectrum of peak (6) (Fig. 6.8) as determined by the diode array detector agreed with the wavelengths and relative heights of absorbance maxima of the spectrum for pure isoorientin. Both spectra were superimposable. Isoorientin is also possibly detected in the chromatograms of SH2, SH3 and SH4 even though in the SH4 it had a Rt slightly higher (Table 6.10) than of that of the reference standard (Table 6.7). Peak (8) appeared as the main peak in the chromatograms of all somatic hybrids with Rt ranging from 14.03 to 14.08 (Table 6.10). It is of interest to note that a peak with Rt in the region of 14.00 and designated peak (4) was found in one of the parents, *P. edulis* (Table 6.8). In SH2, peak (8) is twice the size of peak (5) of *P. incarnata* (Fig. 6.6). Peak (8) did not correspond with any of the standards used. The wavelength values of the peaks (7) and (8) in SH1 are presented in Table 6.10 but their UV spectra (spectra not shown) are not very characteristic. In the chromatogram of SH1, peak (9) was identified as isovitexin by its Rt and UV spectrum. From the UV spectrum of this peak (Fig. 6.8) it may be seen to be a mixture of isovitexin with a not fully resolved component. When the same peak was observed in the chromatographic profiles of SH2, SH3 and SH4, it had Rt values somewhat higher than of that of the isovitexin standard (Table 6.7). Nevertheless, it is possible that isovitexin is also present in these somatic hybrids.

Fig. 6.7: HPLC fingerprint chromatograms of leaf extracts of SH1, SH2, SH3 and SH4

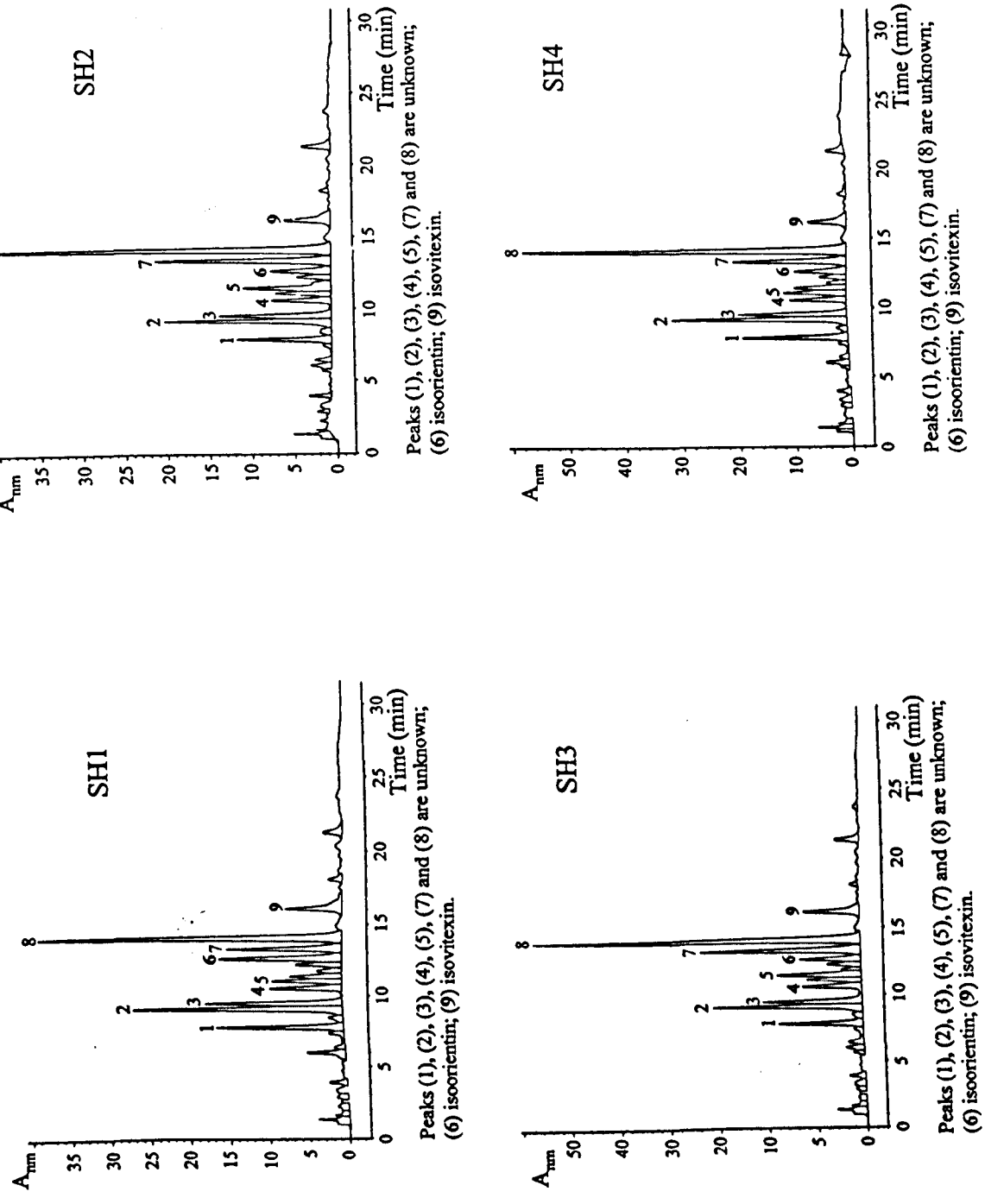
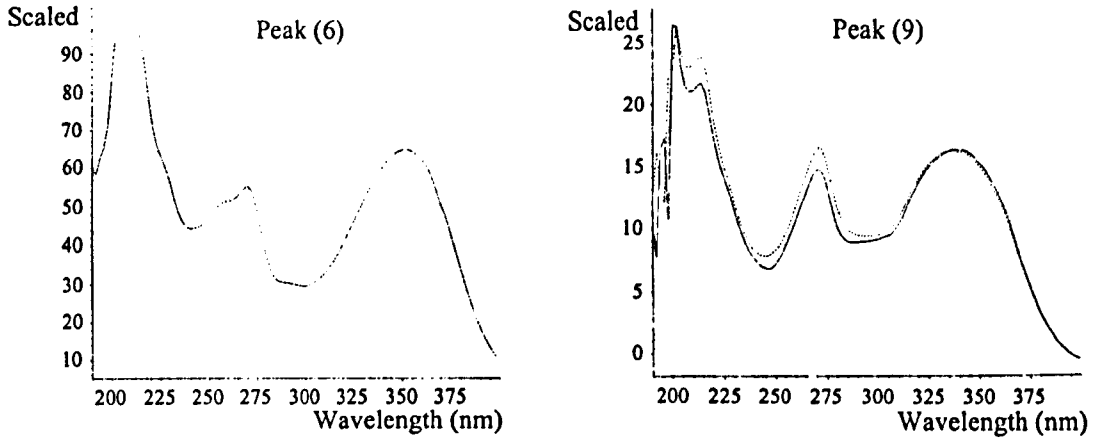


Table 6.10: Retention times and maximum absorbance of peaks detected in the HPLC of leaf extracts of somatic hybrids

Plant accession	Peak number	Rt (min)	UV (λ_{max})[1]
SH1	1	7.79	204 sh, 215, 219, 334
	2	9.09	206, 217 sh, 270, 337
	3	9.49	204, 217 sh, 276, 334
	4	10.54	205, 212 sh, 268 sh, 352
	5	11.04	204, 212 sh, 242 sh, 268, 347
	6	12.60	207 sh, 212, 270, 347
	7	13.29	203 sh, 214, 219, 269, 338
	8	14.03	203, 217, 270, 337
	9	16.09	203 sh, 212, 269, 328
SH2	1	7.81	-
	2	9.10	-
	3	9.49	-
	4	10.55	-
	5	11.43	-
	6	12.61	204 sh, 209 sh, 268, 353
	7	13.30	-
	8	14.04	-
	9	16.10	203, 213 sh, 268 sh, 337
SH3	1	7.82	-
	2	9.11	-
	3	9.51	-
	4	10.57	-
	5	11.44	-
	6	12.63	203, 204 sh, 268 sh, 352
	7	13.32	-
	8	14.07	-
	9	16.12	204 sh, 213 sh, 267 sh, 337
SH4	1	7.83	-
	2	9.12	-
	3	9.52	-
	4	10.58	-
	5	11.08	-
	6	12.65	204, 211 sh, 254 sh, 354
	7	13.34	-
	8	14.08	-
	9	16.13	204 sh, 214 sh, 269 sh, 337

[1] λ_{max} represents the wavelength (in nm) of maximum absorption detected by UV spectrometry and values are only recorded for selected peaks; sh means shoulder. The solvent was MeOH. Shading indicates the peaks that are believed to correspond to the isoorientin and isovitexin standards used.

Fig. 6.8: UV spectra of peaks (6) and (9) from the HPLC profile of SH1



6.4 Discussion

Consumption of the aerial parts of *P. edulis* and *P. incarnata* is well known to provide phytomedicinal value. Flavonoids are claimed to be the secondary metabolites responsible for some of the sedative properties of both *Passiflora* species. Of the parts of *P. incarnata* studied, the leaves have been reported to be the site of greatest flavonoid accumulation (Menghini and Mancini, 1988).

In this study, the novel somatic hybrids [*P. incarnata* (+) *P. edulis*] and the parental species were analysed biochemically by TLC, PLC and HPLC procedures. TLC methods are generally used for analysis and identification of flavonoids in *Passiflora*. After development, the flavonoids on the TLC plates are detected by their original colour, R_f, natural fluorescence, or as zones which are coloured after chemical reaction with an appropriate reagent. The majority of detection procedures used for flavonoids involve the spraying of dried TLC plates with a chromogenic reagent capable of forming coloured products. The preferred detection reagents have been those yielding sensitive colour reactions with several flavonoids and the reagent most used has been NP/PEG (Pothier, 1996). Flavonoid detection is completed by inspecting the chromatoplates, after spraying with NP/PEG, under UV light. This is a very sensitive procedure specially when UV fluorescent silica is used (Markham and Bloor, 1998). In the present study, leaf extracts of the somatic hybrids revealed similar flavonoid banding patterns on TLC plates precoated with a layer of UV fluorescent silica. The six orange fluorescent bands visualised in all somatic hybrids were strong evidence of the presence of phenolic compounds. Some of these zones could not be identified with any of the flavonoid standards used, although detection of isoorientin in the leaves of the somatic hybrids and *P. edulis* indicated *C*-glycosylflavone inheritance by the somatic hybrids. These findings may suggest that the gene(s) related to isoorientin synthesis in *P. edulis* was transferred and expressed in the somatic hybrids. It is interesting to note that isoorientin was not found in *P.*

incarnata (see Results, Section 6.3.3.2). According to the literature (Meier, 1995a), *P. incarnata* contains mainly *C*-glycosylflavones based on apigenin-8-*C*-glycoside (vitexin) and luteolin-8-*C*-glycoside (orientin). In this study, only vitexin was clearly detected in *P. incarnata*. A band corresponding to vitexin was also identified in the somatic hybrids but with different intensities. In terms of vitexin content, SH1 appears to be more closely related to *P. incarnata* [see Results, Section 6.3.1.1] than the other somatic hybrids. On this basis, the presence of vitexin in the somatic hybrids could provide information about its inheritance. Biochemically, the somatic hybrids exhibited *C*-glycosylflavonoids type intermediate between those of the two parental species. Vitexin and/or isovitexin are probably present in *P. edulis*. However, no conclusion can be drawn at this stage, since both *C*-glycosylflavones were masked by other bands.

The HPLC method used in the present study gave informative chromatographic data about leaf extracts of *P. edulis*, *P. incarnata* and their somatic hybrids. The optimum separation of peaks was obtained using a reverse-phase column, linear gradient and a diode array detector. Reverse-phase HPLC is a common analytical technique used for the separation of flavonoids (aglycones and glycosides) in many medicinal plants as for example *P. incarnata* (Rehwald *et al.*, 1994a) and *Crataegus monogyna* (Rehwald *et al.*, 1994b). Because the HPLC system was coupled with a diode array detector this procedure provided UV/visible absorption spectrum for each peak and also allowed for the detection of minor flavonoids. The ultraviolet-visible absorption spectrometry is mainly used for the identification of flavonoid type and definition of the oxygenation pattern (Markham and Bloor, 1998). In the present study, the HPLC fingerprint chromatograms of leaf extracts of *P. edulis* and *P. incarnata* revealed a distinct pattern of flavonoids. The chromatograms obtained were complex so it could not be confirmed that all the peaks referred to flavonoids. Isoorientin and isovitexin were characterized in the extract of *P. edulis*. Based on the retention times of the major peaks in the chromatograms of the somatic hybrids, SH1 appeared to be more closely related to SH2 whilst SH3 was close to SH4. However, the fingerprint patterns of the HPLC separations of the flavonoids were similar in all somatic hybrids. The presence of isoorientin in the extract of SH1 was confirmed by the UV spectrum of peak 6 (from SH1) clearly associated with isoorientin. If an absorption UV spectrum was obtained from HPLC it may show some variations from those of the reference standards because it is measured in the HPLC solvent and not in the usual solvents, methanol or ethanol (Markham and Bloor, 1998). However, when two spectra absolutely cover each other, these spectra can be considered as spectroscopically identical (Szepesi and Nyiredy, 1996). Thus, isoorientin was identified in the leaf extracts of SH1. This *C*-glycosylflavone may also

be present, although in low concentrations, in SH2, SH3 and SH4. Isovitexin was identified in the extract of SH1 and also probably in the other three somatic hybrids.

Due the small amounts of compounds isolated from leaf extracts of *P. incarnata* and the somatic hybrid SH1, they were characterized only by FAB-MS. These compounds were mono-*C*-glycosidic flavonoids (mono-*C*-glycosylflavones type) which are known to have a strong linkage between the saccharidic and aglycone moieties (Li, 1994b). The saccharide is attached directly to the aromatic ring at the 8-position of the aglycone by a carbon-carbon bond which is acid resistant. It has been demonstrated that the 8-*C*-carbohydrate moiety is less susceptible to fragmentation than the 6-*C*-substituent (Becchi and Fraisse, 1989). In the present study, the structures proposed for the isolated compounds could not be more precisely determined based on the FAB-MS data alone. Even though lack of structural information is typical when only microgram quantities of unknown compounds are available, mass spectrometry has been reported to be the only method suitable for structure elucidation of such flavonoids (Li, 1994b). Since 1981, FAB-MS, a soft ionization technique, has been used due the resistance of the *C*-glycosylflavonoids to hydrolysis. This technique allows for analysis of flavonoids without derivatization by producing ions directly from the liquid or solid state. The combination of FAB and collisionally activated dissociation (CAD), and also tandem mass spectrometry has been applied to the characterization and differentiation of 6-*C* and 8-*C*-glycosidic flavonoids (Li *et al.*, 1992), *O*-diglycosyl, *O*-*C*-diglycosyl and di-*C*-glycosil flavonoids (Li and Claeys, 1994a). Care has to be taken in the interpretation of the MS spectra in isolation, and additional information from a combination of other spectroscopic methods including UV, ^{1}H NMR and ^{13}C NMR is necessary in order to provide more complete data for the structural characterization of flavonoid glycosides. It is worth mentioning that NMR techniques require relatively large amounts of samples (in the order of milligrams) so larger quantities of compounds would have to be isolated from extracts of *P. incarnata* and SH1 for future structure elucidation studies.

Prior to the present study, none of the novel *Passiflora* somatic hybrids analysed had received scientific investigation in the context of flavonoids. Thus, these first phytochemical studies are relevant because they provide information about patterns of inheritance and synthesis of flavonoids within the four somatic hybrids. It seems that the somatic hybrids has inherited the tendency to produce phenolic compounds from *P. incarnata*. The *C*-glycosylflavone isoorientin found in the somatic hybrids could only have been inherited from *P. edulis*. In addition, the present studies have identified flavonoids which could be useful as biochemical markers and may also assist in the confirmation of somatic hybridity and will thus complement conventional phenotypic and molecular approaches.

Chapter Seven

General Discussion

The present studies have contributed to the extent of knowledge regarding the tissue culture and phytochemistry of *Podophyllum hexandrum*, *Diphylleia cymosa*, *Passiflora edulis* and *Passiflora incarnata* as well as their somatic hybrids.

There are clear reasons to preserve and to increase *P. hexandrum* and *D. cymosa* germplasms. Both species are sources of the aryltetralin lactone lignan podophyllotoxin, currently used as the starting material for the semisynthesis of the antitumour drugs etoposide and teniposide. Cancer causes about 500,000 deaths per year in the United States alone [Duke, in Principe (1991)], and is expected to become the leading cause of death in the United States by the turn of the century (Pezzutto, 1996). Therefore, the search for new drugs to treat several types of cancer still continues. As the demand of podophyllotoxin has increased with the clinical use of etoposide and teniposide, the search for alternative sources of this precursor remains a key area of research.

The leaf flavonoids of *Passiflora* species have received attention possibly due to their sedative activity. *C*-glycosylflavones are the major flavonoids identified in *Passiflora* (Meier, 1995b). Studies based on the flavonoid composition of the aerial parts of *P. incarnata* have attracted much research in Europe, where this species is cultivated and traditionally used as a herbal remedy. Although the variability of the flavonoid composition in this species has been continuously investigated, some of the results are somewhat controversial. In contrast, the fingerprint of the flavonoids in *P. edulis*, a tropical species, has not received, to date, so much research attention.

Flavonoids, besides their pharmacological activity, have a key role as indicators of natural hybridization in species of *Arnica*, *Parthenium* and *Populus* (Bohm, 1998) and may also be useful for the confirmation of somatic hybridization. For determination of the flavonoid profiles of hybrids, it is first necessary to identify the flavonoid pattern of the parents (Bohm, 1998). However, compounds that are not commonly present in the parental species are commonly found in the hybrids. In this context, more research needs to be done focussed on the control of flavonoid biosynthesis in order to answer many questions relating to their synthesis.

7.1 Concluding remarks and suggestions for future work

7.1.1 Tissue culture studies of *P. hexandrum*

To date, the seasonal availability of *P. hexandrum* has limited the tissue culture studies of this species. In the present study, axenic cultures were successfully established on full-strength Murashige and Skoog (1962)-based medium lacking growth regulators, giving rise to phenotypically normal plants (Chapter 2). Although callus induction from root explants was slow, such tissues became embryogenic after successive subcultures. Somatic embryos were obtained during culture in the dark. Within the subfamily *Podophylloideae*, somatic embryogenesis may be dependent upon dark growth conditions. Indeed, such darkness was also required for somatic embryogenesis in *D. pleiantha* (Chuang and Chang, 1987) and *D. cymosa* (Chapter 3), other members of the same subfamily. Embryogenic cell cultures of *P. hexandrum* were also obtained under the same conditions. In rice, it has been reported that the morphogenic potential of embryogenic cell cultures declines with prolonged subculture (Abe and Futsuhara, 1991). Thus, if embryogenic cell cultures of *P. hexandrum* synthesize lignans, it would be interesting to cryopreserve such cultures. These cultures may represent an alternative source of antitumour lignan production.

The present study suggested that the lipo-oligosaccharide produced by *Rhizobium* sp. NGR234, stimulated rooting of *in vitro* plantlets obtained from germinated somatic embryos. Whilst the data obtained was limited, this finding may help to open up new opportunities for *in vitro* mass production of *P. hexandrum*. Previous studies with this species have shown that shoot propagation can be achieved, but this is limited by lack of root development (Arumugan and Bhojwani, 1990). The novel approach used in the present study could be a means to overcome the aforementioned problem. Therefore, a more detailed study would be necessary in order to explore the effects of such Nod factors on root stimulation in tissue cultures of *Podophyllum*.

Plant regeneration from root explants of *P. hexandrum* cultured in liquid medium was via organogenesis. The inability to maintain tissue culture-derived plants once transferred to compost represents a limitation in the protocol developed and hence it will be essential to improve the acclimation process.

This study demonstrated that *P. hexandrum* remained highly recalcitrant to *Agrobacterium*-mediated transformation (Chapter 2) even though many strains including the supervirulent *A. rhizogenes* strain R1601 and *A. tumefaciens* strain 1065 were used. It was speculated that the cytotoxicity of the aryltetralin lignans present in *P. hexandrum* may have blocked bacterial infection. According to an early report (De Cleene and De Ley, 1976), *P. hexandrum* and other members of the *Berberidaceae* are not susceptible to infection with *A. tumefaciens* strain ChrIIb. Moreover, failure to

transform roots from axenic cultures of *P. hexandrum* with *A. rhizogenes* strain LBA 9402 had already been reported (Oostdam *et al.*, 1993). The use of other strains, or biolistic gene delivery, may be an alternative method to transform *P. hexandrum*. Biolistic gene delivery has been successfully employed for direct gene transfer into plants, including monocotyledonous species as well as woody and ornamental plants previously difficult to transform (Marchant *et al.*, 1998). In future studies, naringenin could also be added to the culture medium. The inclusion of this dihydroflavone in the co-cultivation medium has been reported to induce transient GUS expression with recalcitrant species of *Passiflora* (Cancino *et al.*, 1999). It would be interesting to investigate whether the approaches mentioned above could offer new possibilities for the genetic manipulation of *P. hexandrum*.

Rhizomes and roots of *P. hexandrum* are still valuable sources of aryltetralin lactone lignans. Within the lignans isolated and characterized in the current study (Chapter 4), podophyllotoxin and 4′-demethylpodophyllotoxin were used as authentic standards in the HPLC analysis of lignans in leaves and tissue culture material of *D. cymosa* (Chapter 5).

7.1.2 Tissue culture studies and HPLC analysis of lignans in *D. cymosa*

The tissue culture protocols (Chapter 3) are the first reported studies on *D. cymosa*. These studies demonstrated that *D. cymosa* was not recalcitrant to cell culture technology. Although it is important to undertake more studies in order to improve the results obtained, they could be useful as a baseline for tissue culture studies of other species of *Diphylleia* (*D. grayi* and *D. sinensis*) since there are no published reports concerning these species. The roots of *D. grayi* have been reported to contain the highest amount of podophyllotoxone within this group of plants and are a good source of podophyllotoxin (Broomhead and Dewick, 1990).

The use of non-ionic surfactants was not ultimately a good choice for substantially promoting biomass production even though some increases were obtained. However, it would be interesting to investigate, in the future, whether non-ionic surfactants have any effects on the lignan content of callus derived from leaf and petiole explants.

Although cryopreserved cells without cryoprotectants and unfrozen cells were partially dehydrated, they still underwent, post-thaw, mitotic division which were reflected by an increase in biomass even though it was not statistically significant. Petiole-derived calli contained podophyllotoxin (Chapter 5). Thus, further studies on cryopreservation of this species may offer an alternative preservation system for maintaining these important lignan-producing cell lines. In addition, dormant buds could be cryopreserved (frozen), with or without cryoprotectant, or just stored in

liquid nitrogen. After that, apical meristems could be aseptically excised from this material and cultured on Murashige and Skoog (1962)-based culture medium. This could lead to an increase in the availability of plant germplasms for tissue culture and phytochemical studies, since the limited amount of plants was a constant problem during the present work as previously mentioned (Chapter 3, Section 3.4).

The HPLC protocol used for the analysis of lignans in leaves and tissue culture material of *D. cymosa* was successful (Chapter 5). Enzymic hydrolysis of lignan glycoside performed prior to HPLC analysis led to the separation of the aglycone and the sugar moieties. The samples of whole leaves, petiole and leaf-derived calli and cell suspensions cultures containing free aglycones could be evaluated by a sensitive and rapid HPLC. This study demonstrated the presence of podophyllotoxin in the samples of petiole-derived calli. Although the content appeared low, this was a promising result because it indicated that there was a biosynthetic capacity in tissue cultures *D. cymosa* to synthesize podophyllotoxin *in vitro*. Future work should investigate whether optimisation of the tissue culture protocol (Chapter 3) will increase this lignan-production. It has been reported that the production of podophyllotoxin in leaf-derived calli of *Juniperus chinensis* increased either with the inclusion of chito-oligosaccharides (or Nod factors), as elicitors, or by the addition of phenylalanine, a biogenetic precursor of podophyllotoxin, to callus cultures (Muranaka *et al.*, 1998). It will be, therefore, worthwhile to see if treatments with such elicitors, individually or together and, with biosynthetic precursors can lead to the establishment of high-podophyllotoxin cell lines of *D. cymosa*.

7.1.3 Flavonoids in the study of *Passiflora* somatic hybrids

In the current study it was shown that using the classical TLC procedure (Chapter 6) it was possible to identify *C*-glycosylflavonoids from leaf extracts of the somatic hybrids [*P. incarnata* (+) *P. edulis*] as well as of their parental species. TLC is an effective technique possibly due to its simplicity, versatility, and low cost. In addition, the spots remain compact, without spreading, as occurs on paper chromatography. It was useful for the separation, qualitative analysis and semiquantitative analysis of *Passiflora* flavonoids before their analysis with more sophisticated instruments. In this study, the bulk of flavonoids from *Passiflora* leaves was extracted with pre-warmed methanol. Flavonoids are vacuolar components in *Passiflora* species though they are more likely to occur as glycosides bound to sugar molecule(s). Thus, a polar solvent had to be used in order to provide an efficient extraction. All the somatic hybrids showed a high degree of similarity in the flavonoid profiles by TLC. Unfortunately, due to the limited number of flavonoid standards available, it was not possible to identify some of the coloured zones in the present

study. However, identification of isoorientin in the four somatic hybrids suggested that isoorientin production was probably inherited from *P. edulis*, since it was absent from the profile of *P. incarnata*. Vitexin was also identified in the somatic hybrids. It has not been possible to prove, so far, the presence of vitexin in the profile of *P. edulis*. Thus it is suggested that the *P. incarnata* genome was responsible for the inheritance of isovitexin in the somatic hybrids.

One of the problems with the flavonoid investigation of *Passiflora* species was that many of the compounds were not commercially available as standards. Thus, it was necessary to carry out the isolation and identification of some of these compounds of interest, which was time consuming and expensive. In addition, artefacts can be inadvertently introduced during such separation and isolation procedures which can lead to false results. Therefore, with the increasing interest in medicinal plant extracts and plant-derived compounds, the application of combined techniques such as TLC-HPLC, TLC-MS and others has played an important role in their analysis.

HPLC studies were performed in order to confirm and to improve the results obtained by the TLC assays of *Passiflora* extracts. HPLC separations are more rapid than classical methods and provide high resolution and sensitivity. In the present studies, the reverse-phase HPLC system coupled with a diode array UV detection proved to be efficient in determining the pattern of occurrence of *C*-glycosylflavones in *Passiflora* parental species, as well as their somatic hybrids. Qualitative analysis of flavonoids was facilitated by the diode array detector that allowed the recording of characteristic absorption peaks of emerging components. From an identification point of view, leaf extracts of *Passiflora* species were complex mixtures of flavonoids. Although this did not indicate that all peaks on the HPLC chromatograms obtained corresponded to flavonoids, it did suggest that compounds with similar polarity were present in the extracts. The parental species revealed distinct flavonoid profiles as previously demonstrated by TLC chromatograms. Isovitexin was detected in *P. edulis* and *P. incarnata* whilst isoorientin was found in the former. The somatic hybrids were similar in their *C*-glycosylflavone patterns. Analysis of the hybrids, using HPLC, indicated the presence of isoorientin and isovitexin in all the somatic hybrids. This suggested that *P. incarnata*, indeed, supplied this part of the genome in the somatic hybrids, since their chromatograms revealed many peaks with retention times close to those of this parental species. However, the somatic hybrids have also been shown to contain isoorientin which was clearly detected only in the profile of *P. edulis*. These results showed that the somatic hybrids reflect input from the parental species suggesting that they both have contributed genetic material. The glycosylation process appeared not to be altered by somatic hybridization. In the chromatograms of all the

somatic hybrids, there were unknown peaks separated upon reverse-phase HPLC analysis that were not seen in either parent. This may have an explanation based on some change in the control of flavonoid biosynthesis caused by the integration of two distinct genomes. Additionally, these peaks did not correspond to any of the flavonoid standards used. Thus, a more detailed chemical characterization involving mass spectrometry and NMR data would be certainly necessary in order to identify flavonoids for which reference standards are not available. Additionally, it will be useful for further characterization to run the somatic hybrids samples on LC-MS. This process should provide molecular weight information of the unknown peaks through their mass spectra; and specific fragments could be obtained by complementary MS-MS. The results of these combined techniques would clearly improve phytochemical knowledge of such *Passiflora* somatic hybrids.

It would be interesting to undertake further analysis on the quantification of isoorientin and isovitexin in the somatic hybrids as well as the parents. These data would indicate the content of both compounds in the somatic hybrids when compared with their parental species. Future work with the somatic hybrids and/or their sexual offspring could also be included in an evaluation of different batches of dried leaves taken at different development stages for their flavonoid content. It has been reported that accumulation of flavonoids commences at the seedling state and increases during vegetative growth in the field (Menghini and Mancini, 1988). For flavonoid quantification, leaves post harvesting should be frozen in liquid nitrogen (Markham and Bloor, 1998). This information would be useful for timing the harvesting of leaves in order to obtain the highest contents of total flavonoids. Based on this information and its comparison with the parental species data, it would be possible to recommend (or not) leaves of the somatic hybrids for medicinal use.

Besides the identification of the flavonoid patterns by TLC and HPLC, two compounds were isolated from the leaf extracts of *P. incarnata* and SH1, although this work was not the main objective of the present studies. The postulated structures of both compounds were based only on FAB-MS data. In flavonoid-containing extracts, separation of components is necessary in order to obtain a detailed structure elucidation as well as to evaluate single flavonoids activity (Raffaelli *et al.*, 1997). Therefore, repeating the isolation protocols used in the present study should provide more quantities of the isolated compounds that could be used for other spectroscopic methods to confirm their postulated structures.

7.2 Perspectives for applications of *Podophyllum*, *Diphylleia* and *Passiflora*

There is no doubt that medicinal species will always have an important role in the life of humans. Genetic engineering, which has already been applied to medicinal species for the manipulation of their secondary metabolites (Verpoorte *et al.*, 1998), might have implications for the development of novel therapeutic agents. It may be possible through manipulation of the plant cell biosynthetic pathways to give rise to plants/cell lines with high production of 4′-demethyl compounds particularly 4′-demethylpodophyllotoxin for the synthesis of etoposide and teniposide. It is known that the commercial synthesis of these anticancer drugs involves low yield reactions in order to convert podophyllotoxin into 4′-demethylepipodophylltoxin. This chemical process may be more efficient and economical if 4′-demethylpodophyllotoxin could be used as a starting compound.

On the other hand, the inhabitants of developing countries, such as Brazil, are continuously facing social and economic problems. Such situations can contribute to increases in the consumption of more tranquilizer(s) drugs. However, it is not possible for the majority of the population to afford orthodox medicines. Therefore, there will remain, for a long time, a place for the traditional medicinal use of *Passiflora* species. In Brazilian popular medicine, there is a particular interest in *Passiflora edulis* due to its use as a mild tranquilizer (Matos, 1991). Further studies with the somatic hybrids will be important for at least two reasons. If their total flavonoid content is ultimately shown to be higher than of that of the parental species, it could lead to the development of a low-cost commercially available phytopreparation. On the other hand, studies focussed on the unknown compounds revealed by TLC and HPLC in the somatic hybrids, could identify novel *C*-glycosylflavonoids in *Passiflora*.

Appendices

Appendix 1: Sterilisation of media, equipment and media preparation

1.1 By steam

All media were sterilised in an autoclave for 20 min at 121°C (118 Kpa nominal steam pressure). The equipment and glassware were sterilised by autoclaving or in a hot air oven for 2 h at 160°C.

1.2 Filtration

Thermolabile substances such as growth regulators, surfactants and aminoacids were filter-sterilised by passage through a Sartorius single-use filter unit (0.20 μm pore size, Sartorius Instrument Ltd., Belmont, Surrey, UK).

All instruments used in the tissue culture experiments were sterilised by dipping in methcol (industrial methylated spirits) and flaming prior to use.

1.3 Aseptic technique

In vitro experiments such as innoculation and subculture were carried out aseptically in a laminar air-flow cabinet (Slee, London, UK).

1.4 Composition of methcol solution

EtOH	95.0% (v:v)
CH_3OH	5.0% (v:v)

1.5 Composition of Domestos bleach[1]

$NaOCl_3$ commercial grade	10.5% (w:v)
Na_2CO_3	0.3% (w:v)
NaCl	10.0% (w:v)
NaOH	0.5% (w:v)

[1] Lever Industrial Ltd., Runcorn, UK; Patented thickner and softener added to 100%.

1.6 Media preparation

MS-based media were prepared using commercially-available formulations (Flow Laboratories, Irvine, Scotland) supplemented with sucrose, growth regulators and agar (Sigma Chemical Co., St. Louis, USA). The pH of the media was adjusted to 5.7 or 5.8 or as required using 1.0M NaOH or 1.0M HCl prior the sterilisation. Thermolabile substances after filter-sterilisation were added to warm autoclaved medium (45 - 50°C).

Appendix 2: Media composition

2.1 Media used in tissue culture experiments

MS medium (Murashige and Skoog, 1962)

Component	Concentration (mg l^{-1})	Component	Concentration (mg l^{-1})
$CaCl_2$	440.0	$ZnSO_4.7H_2O$	8.6
NH_4NO_3	1650.0	Na_2EDTA	37.25
KNO_3	1900.0	2,4-D	2.5
KI	0.83	Glycine	2.0
$CoCl_2.6\ H_2O$	0.025	Myo-inositol	100.0
KH_2PO_4	170.0	Nicotinic acid	0.5
H_3BO_3	6.2	Pyridoxine HCl	0.5
$Na_2MoO_4.2H_2O$	0.25	Thiamine HCl	0.1
$MgSO_4$	370.0	Sucrose	30000
$MnSO_4$	22.3	Agar	8000.0
$CuSO_4.5H_2O$	0.025	pH 5.8	

Half-strength MS medium

The components of MS medium (salts and organics) were used at half-strength with MS powder diluted as required with reverse-osmosic water.

MS6 medium

The nitrates of MS medium were used at half-strength with the vitamins of Nitsch and Nitsch medium and 6% (w:v) sucrose.

MSP media

Picloram was added to normal strength MS medium at the following concentrations:

Media code	Concentration (mg l^{-1})
MSP2	4.8
MSP3	7.2
MSP4	9.6
MSP5	12.1
MSP6	14.5

MSZ medium

Zeatin was added to normal strength MS medium at the concentration shown:

Medium code	Concentration (mg l^{-1})
MSZ	1.0

B5 medium (Gamborg *et al.*, 1968)

Component	Concentration (mg l^{-1})	Component	Concentration (mg l^{-1})
KNO_3	2500.0	$CoCl_2.6H_2O$	0.025
$CaCl_2.2H_2O$	113.24	$FeSO_4.7H_2O$	27.85
$MgSO_4.7H_2O$	122.09	Na_2EDTA	37.25
$(NH_4)_2SO_4$	134.0	Inositol	100.0
$NaH_2PO_4.2H_2O$	130.5	Pyridoxine HCl	1.0
KI	0.75	Thiamine HCl	10.0
H_3BO_3	3.0	Nicotinic acid	1.0
$MgSO_4$	10.0	Kinetin	0.1
$ZnSO_4.7H_2O$	2.0	2,4-D	0.1 - 1.0
$Na_2MoO_4.2H_2O$	0.25	Sucrose	2000.0
$CuSO_4.5H_2O$	0.025		

BGS medium

The following additions were made to normal strength MS medium:

Component	Concentration (mg l^{-1})
IAA	0.00875
Kinetin	0.03
Folic acid	0.001

Nitsch medium (Nitsch and Nitsch, 1969)

The Nitsch and Nitsch basal sal mixture was used at full-strength and the powder diluted with reverse-osmosic water. This was prepared with macro and micronutrients lacking the vitamins.

Component	Concentration (mg l^{-1})	Component	Concentration (mg l^{-1})
NH_4NO_3	720.0	$MgSO_4$	90.27
H_3BO_3	10.0	$MnSO_4.H_2O$	18.94
$CaCl_2$	166.0	$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025	KNO_3	950.0
FeNaEDTA	36.7	KH_2PO_4	68.0
$ZnSO_4.7H_2O$	10.0		

N3 and N6 media

The components of Nitsch and Nitsch medium were used at the same concentrations with 0.7% (w:v) agar and supplemented with sucrose [0.3% (w:v) for N3 medium] and [0.6% (w:v) for N6 medium].

UM medium

The following additions were made to normal strength MS medium:

Component	Concentration (mg l^{-1})	Component	Concentration (mg l^{-1})
Nicotinic acid	4.5	2,4-D	2.0
Pyridoxine HCL	9.5	Kinetin	0.25
Thiamine HCL	9.9	Sucrose	3000.0
Casein hydrolysate	2000.0	pH 5.8	

Vitamins of Nitsch and Nitsch medium

The Nitsch vitamin mixture was used at full-strength and the powder diluted with reverse-osmosic water.

Component	Concentration (mg l^{-1})
Biotin	0.05
Folic acid	0.5
Glycine	2.0
Myo-inositol	100.0
Nicotinic acid	5.0
Pyridoxine HCL	0.5
Thiamine HCL	0.5

2.2 Media used for culturing *Agrobacterium* species

APM medium (Morgan *et al.*, 1987)

The APM medium was made up in reverse-osmosic water.

Component	Concentration (mg l^{-1})	Component	Concentration (mg l^{-1})
Yeast extract	5000.0	$(NH_4)_2SO_4$	2000.0
Casaminoacids	500.0	NaCl	5000.0
Mannitol	8000.0	pH 6.6	

LB medium[1]

The LB medium was made up in reverse-osmosic water.

Component	Concentration (mg l^{-1})	Component	Concentration (mg l^{-1})
Yeast extract	5000.0	Agar (for semi-solid)	15000.0
Tryptone	10000.0	pH 7.0	
NaCl	5000.0		

[1] After Sambrook *et al.* (1989).

YBM medium (Ooms *et al.*, 1985)

The YBM medium was made up in reverse-osmosic water.

Component	Concentration (mg l^{-1})	Component	Concentration (mg l^{-1})
Yeast extract	400.0	$MgSO_4 . 7H_2O$	100.0
Mannitol	1000.0	KH_2PO_4	200.0
NaCl	100.0	pH 7.0	

Appendix 3: Assessment of cell viability with FDA

$$\text{Cell viabilty (\%)} = \frac{\text{number of fluorescing cells}}{\text{total number of cells}} \times 100$$

Appendix 4: Scanning and enhancing images of chromatograms and spectra

HPLC chromatograms and UV spectra were scanned at 300 dpi by an scanner (Model Gemini D-16, UMAX Data Systems Inc., Hsinchu, Taiwan) and transferred to an IBM-compatible PC (Viglen, Pentium II, 233MMX) for image enhancement. Images were saved in JPEG format which were manipulated using the commercial software Photoshop® 2.5.1 LE (Adobe Systems Inc., Mountain View, CA, USA). After removing excess information of the chromatograms/spectra the images were converted into files using Power Point (Microsoft).

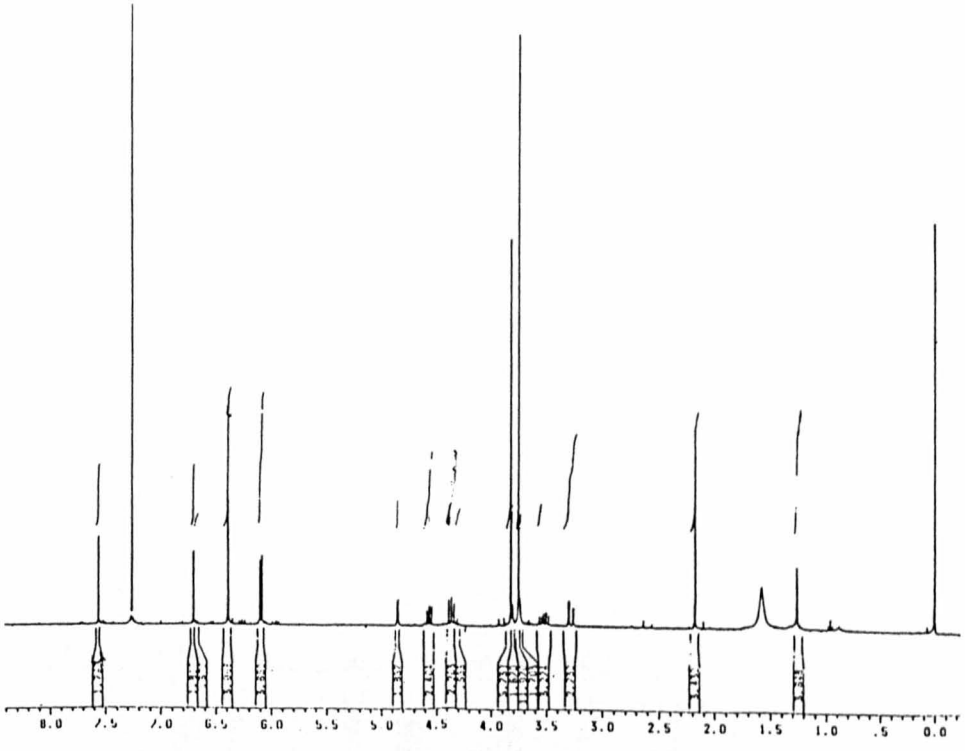
Appendix 5: Retention factor in TLC (Fried and Sherma, 1996)

$$\text{Retention factor } (R_f) = \frac{\text{distance between the center of spot and the start line}}{\text{distance of the mobile phase front from the start line}}$$

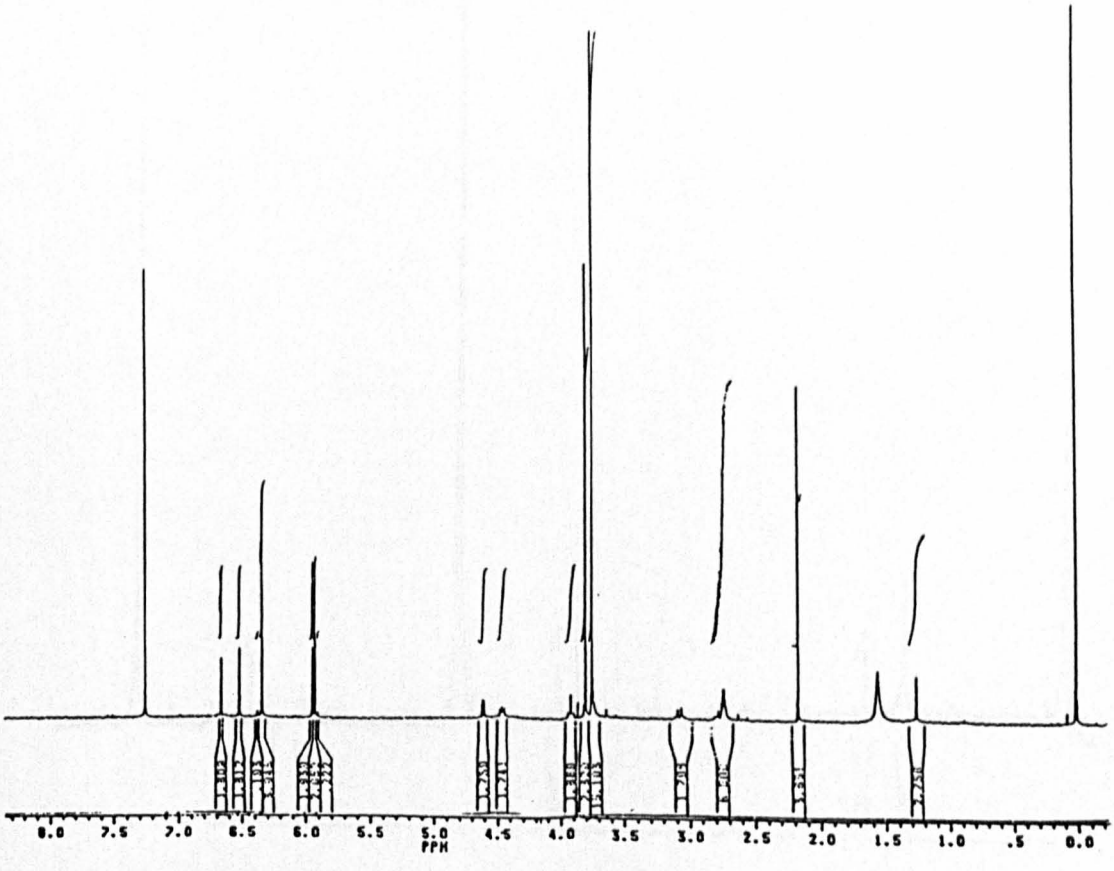
Appendix 6: Proton nuclear magnetic resonance spectrometry for lignans

^{1}H NMR data of the lignans isolated from rhizomes/roots of *P. hexandrum* and *P. peltatum* were summarized in Tables 4.3 and 4.4 (Chapter 4, Section 4.3.2.3).

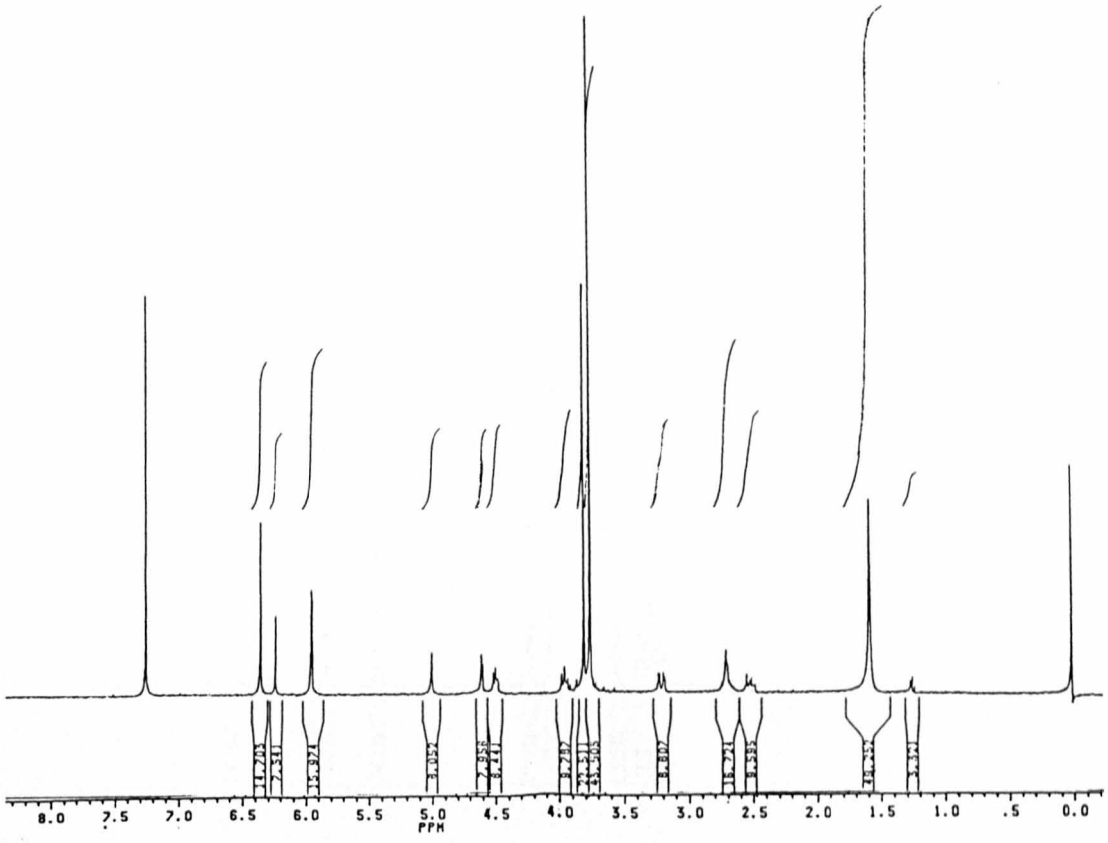
Appendix 6.1.1: ^{1}H NMR spectrum of podophyllotoxone



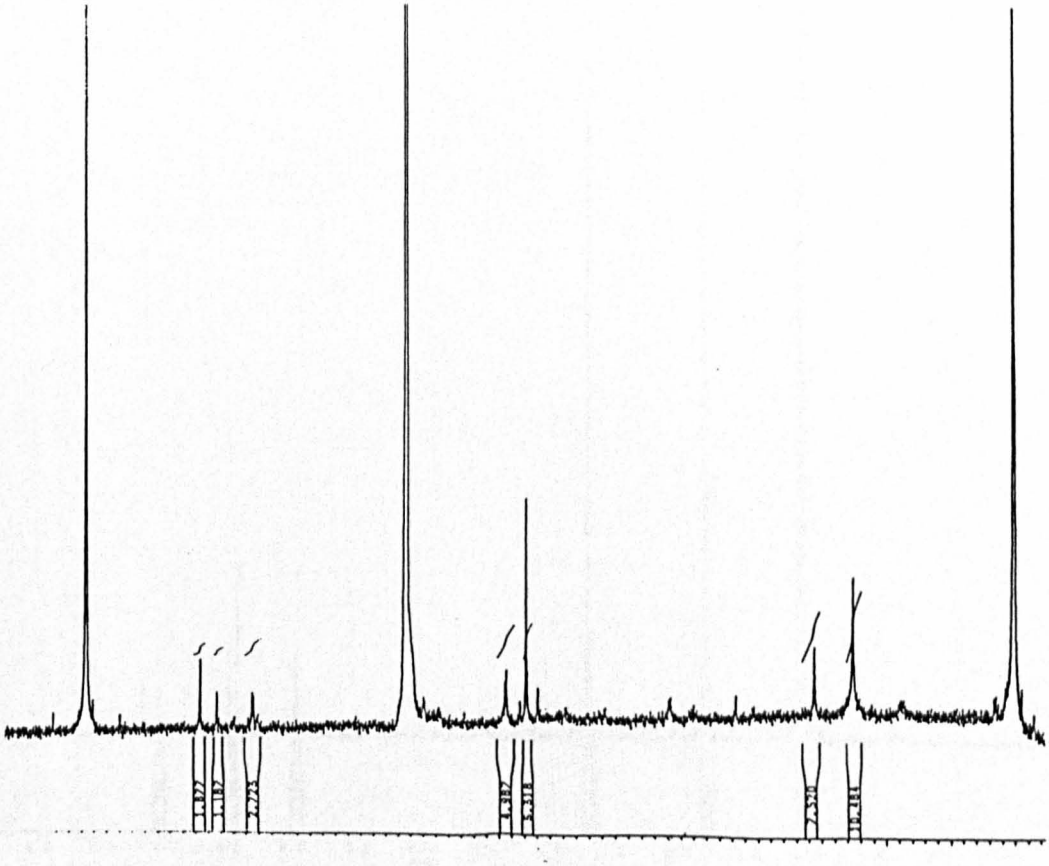
Appendix 6.1.2: ^{1}H NMR spectrum of desoxypodophyllotoxin



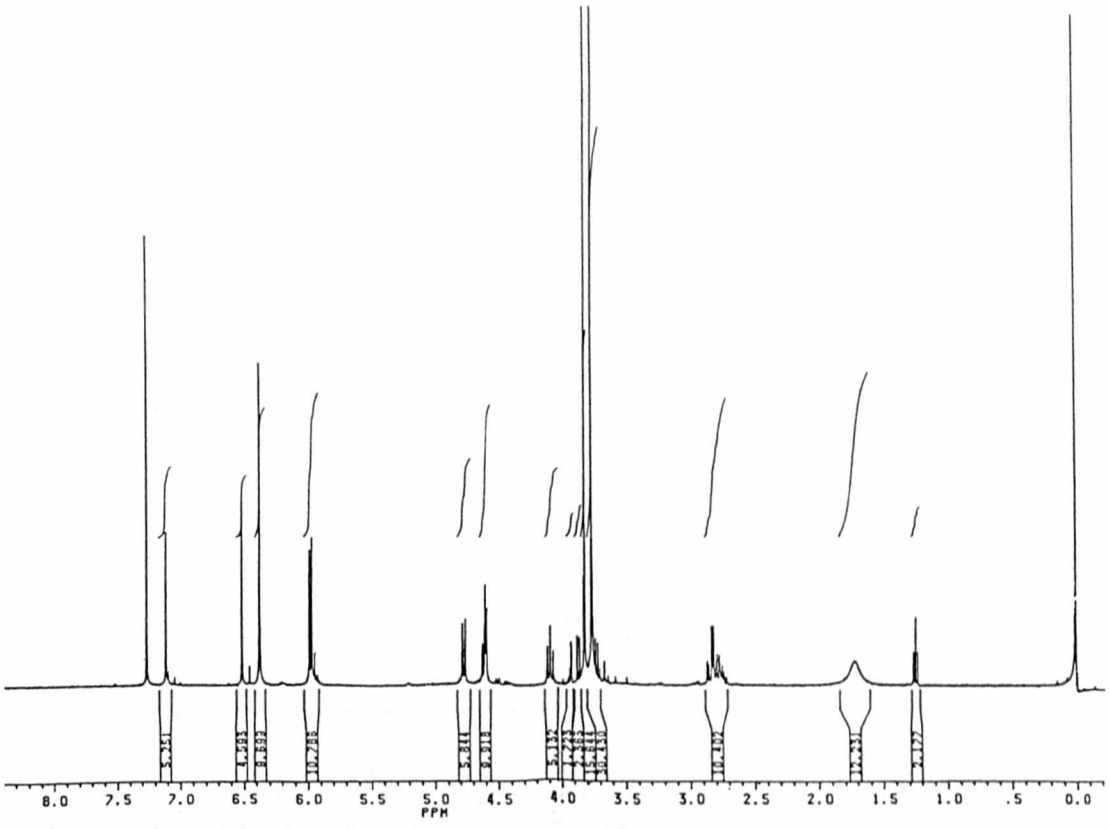
Appendix 6.1.3: ^{1}H NMR spectrum of β-peltatin



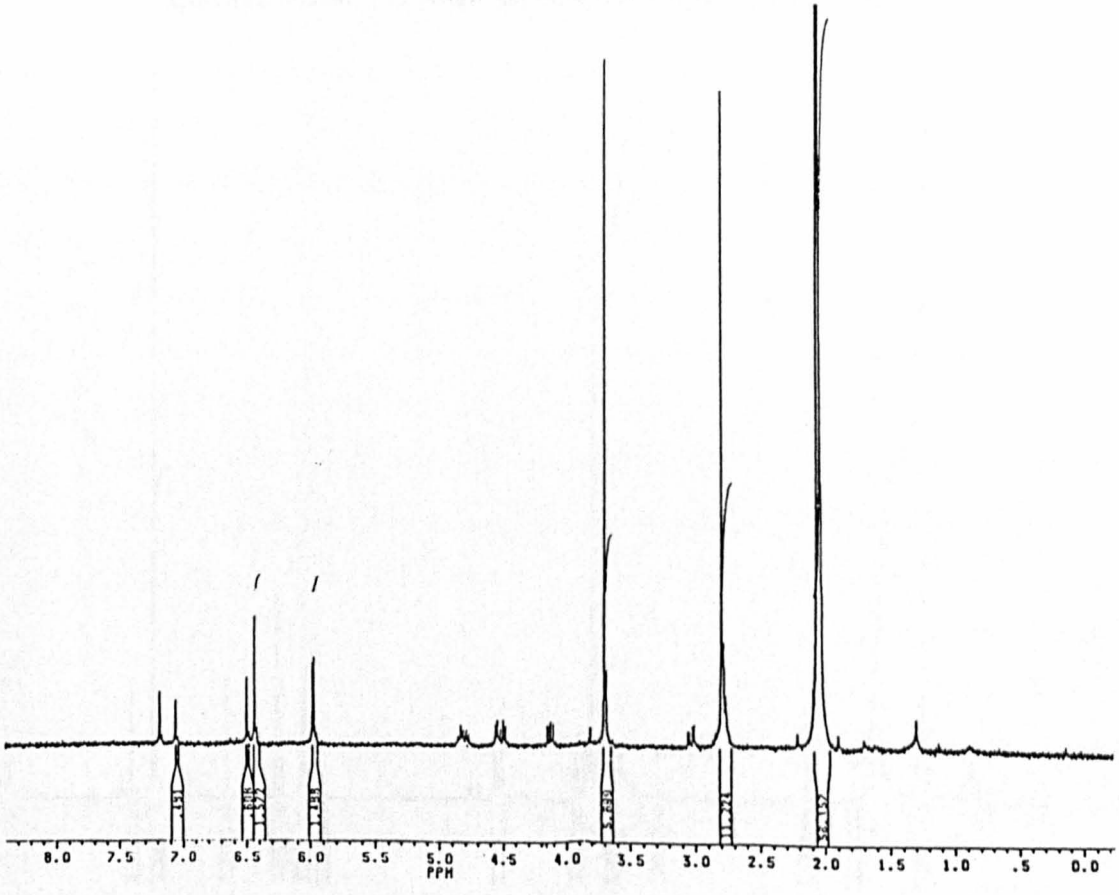
Appendix 6.1.4: ^{1}H NMR spectrum of α-peltatin



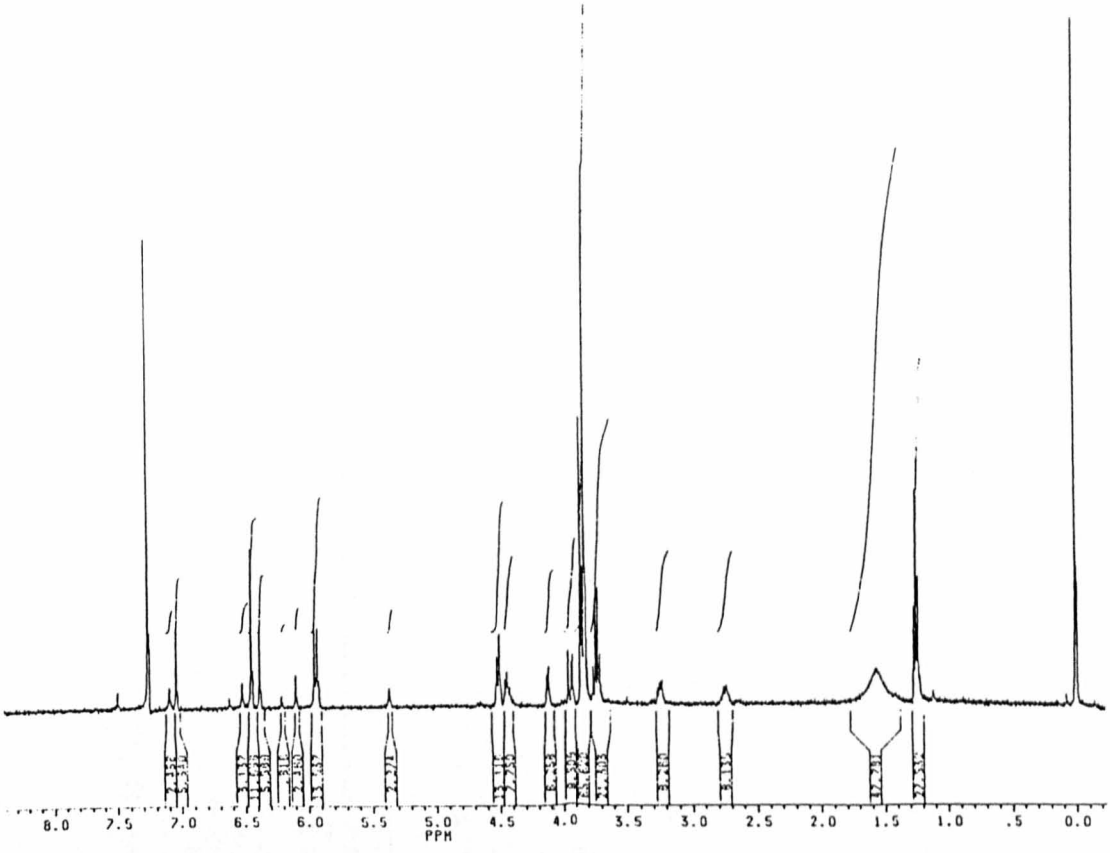
Appendix 6.1.5: ^{1}H NMR spectrum of podophyllotoxin



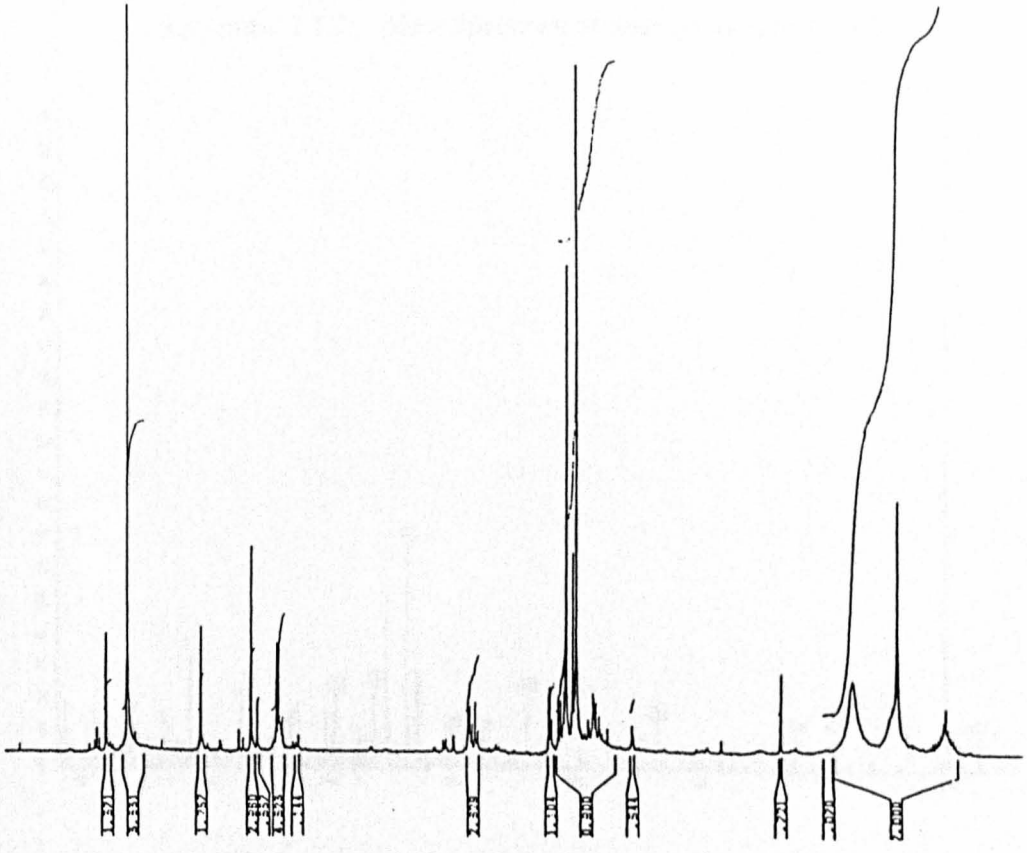
Appendix 6.1.6: ^{1}H NMR spectrum of 4′-demethylpodophyllotoxin



Appendix 6.1.7: ^{1}H NMR spectrum of picropodophyllin



Appendix 6.1.8: ^{1}H NMR spectrum of isopicropodophyllone

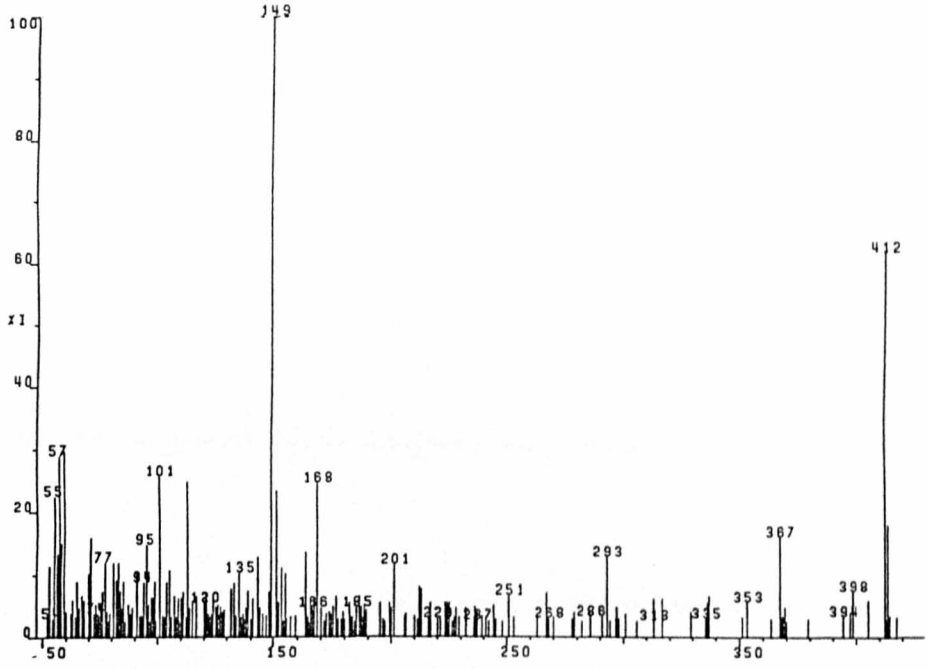


Appendix 7: Mass spectrometry

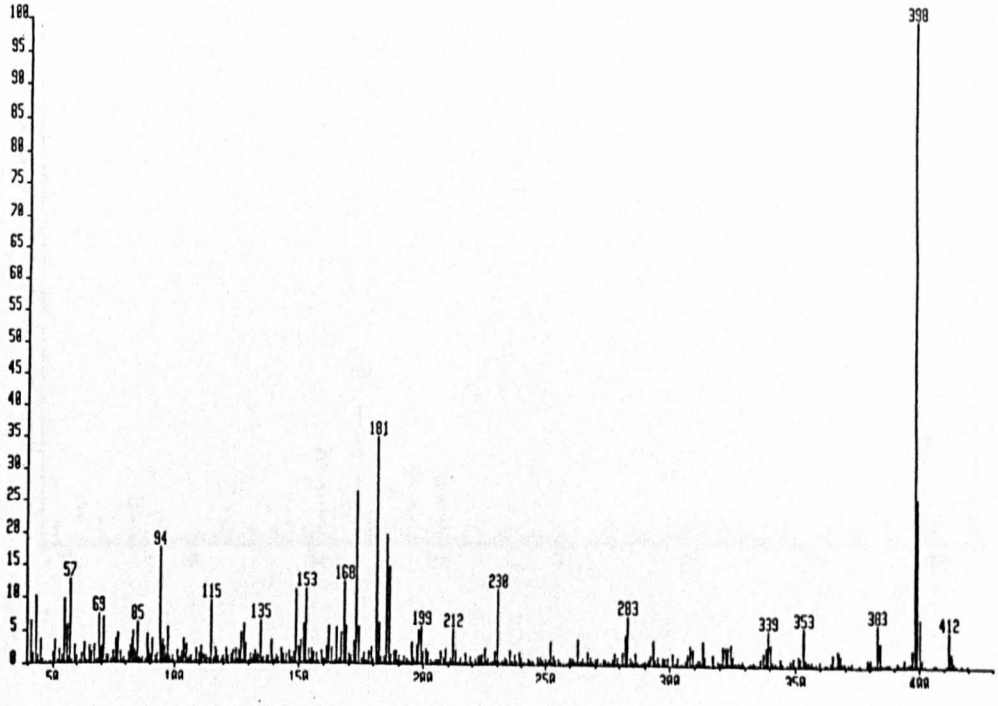
7.1 Electron impact spectra for lignans

MS data of the lignans isolated from rhizomes/roots of *P. hexandrum* and *P. peltatum* were summarized in Table 4.5 (Chapter 4, Section 4.3.2.4).

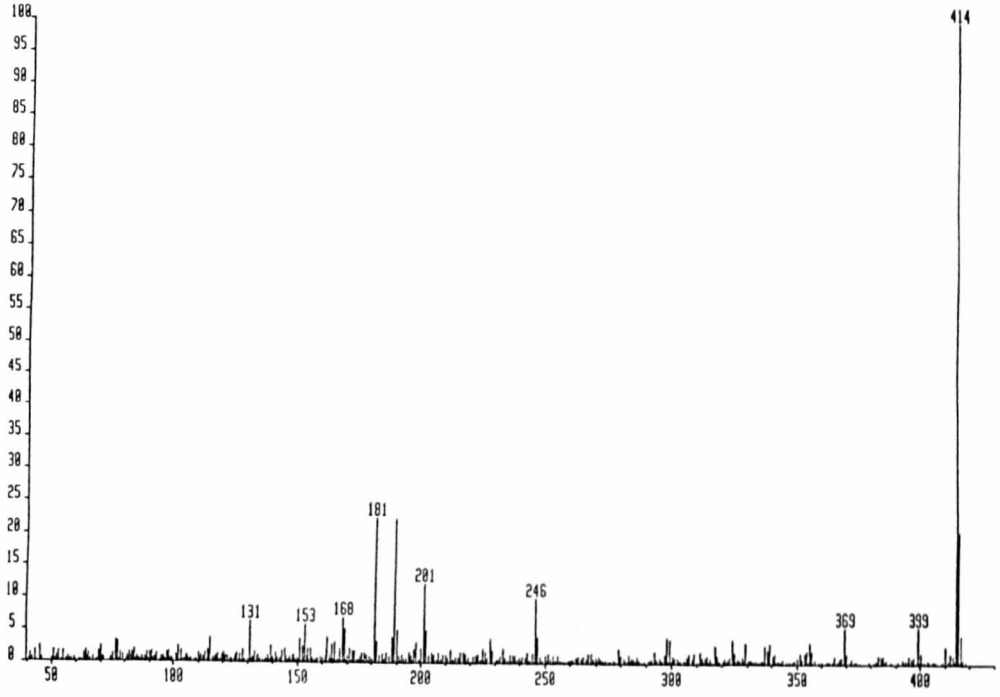
Appendix 7.1.1: Mass spectrum of podophyllotoxone



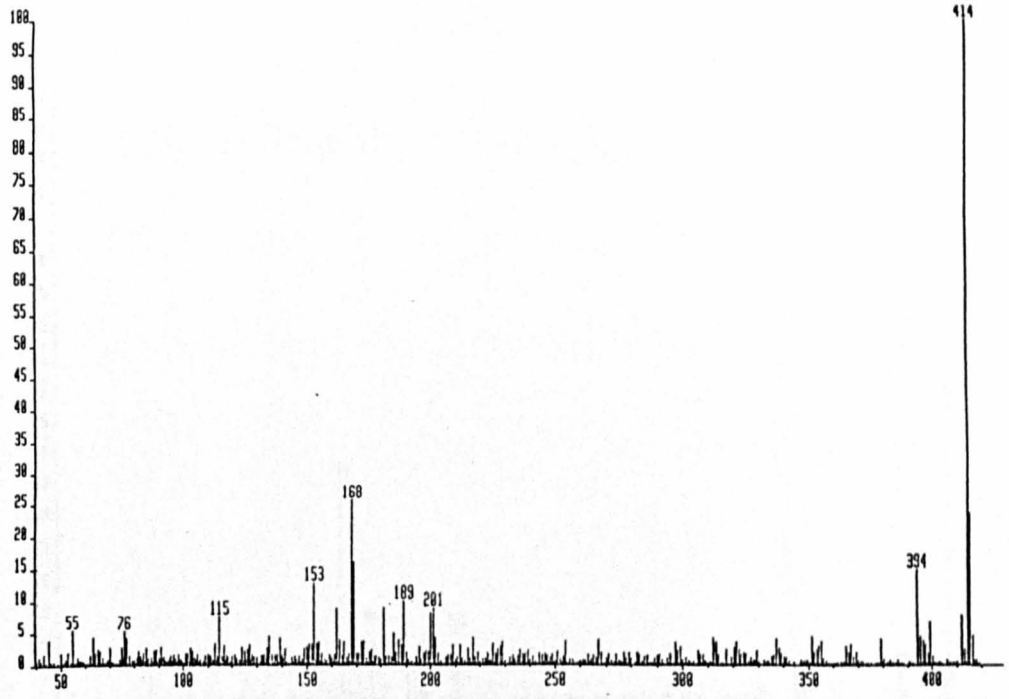
Appendix 7.1.2: Mass spectrum of desoxypodophyllotoxin



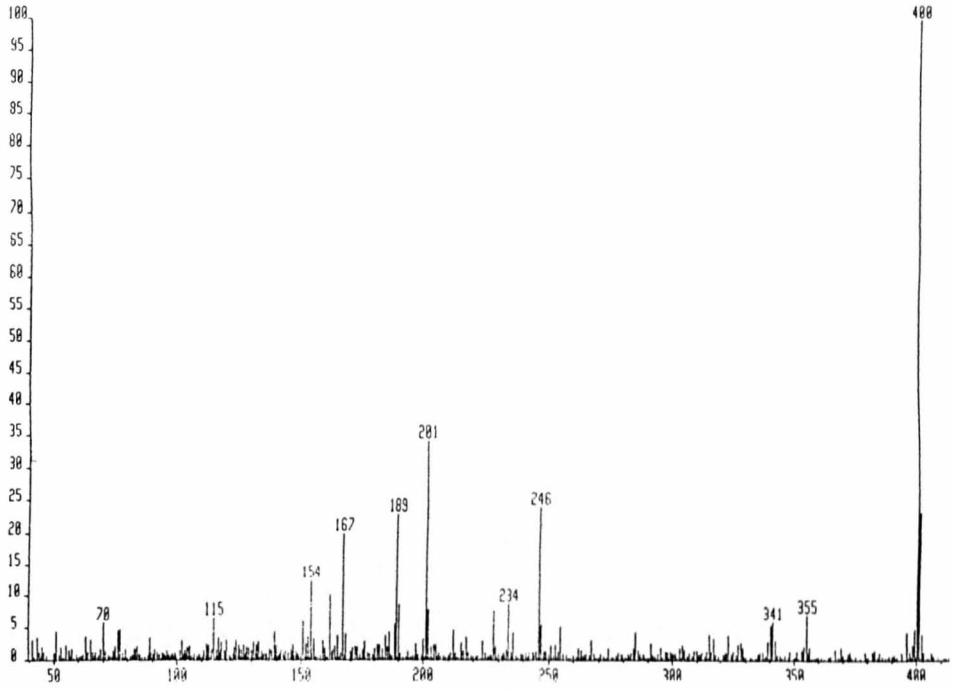
Appendix 7.1.3: Mass spectrum of β-peltatin



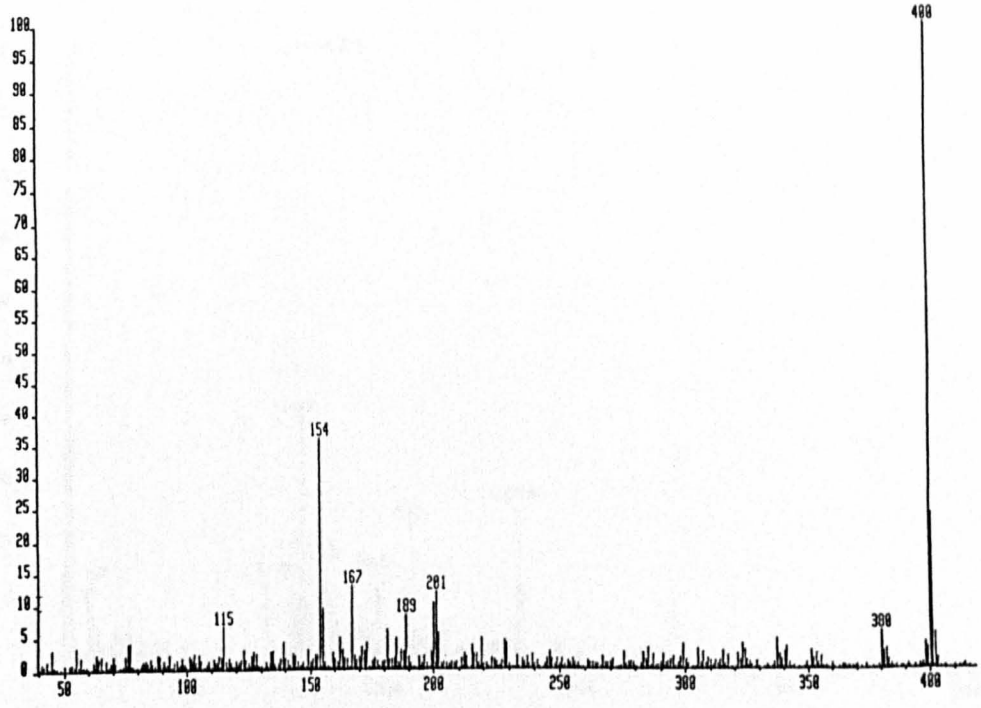
Appendix 7.1.4: Mass spectrum of podophyllotoxin



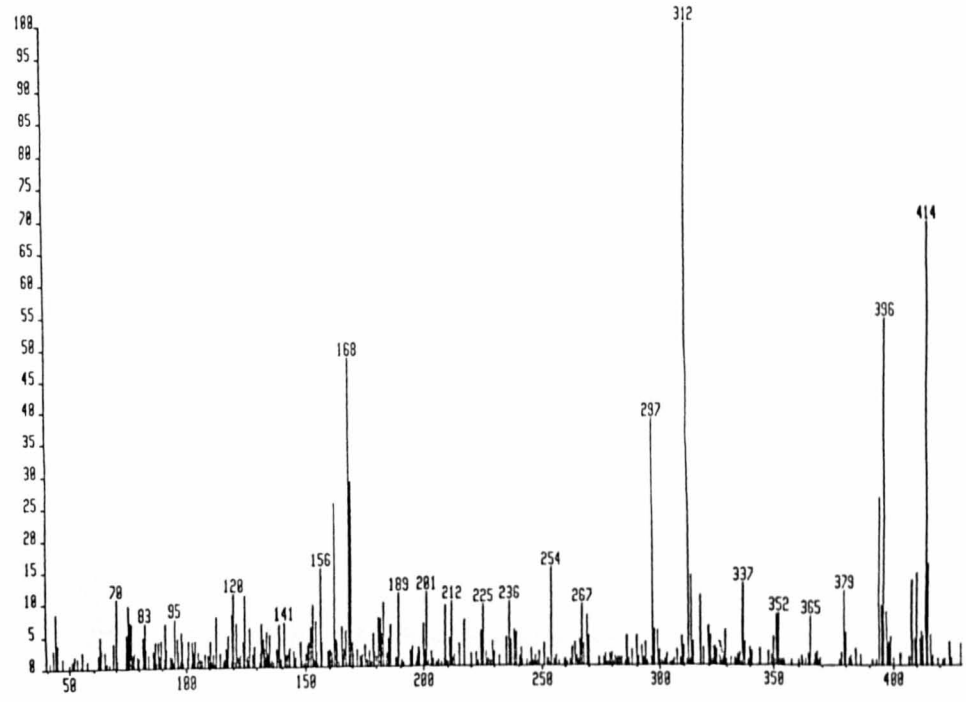
Appendix 7.1.5: Mass spectrum of α-peltatin



Appendix 7.1.6: Mass spectrum of 4′-demethylpodophyllotoxin



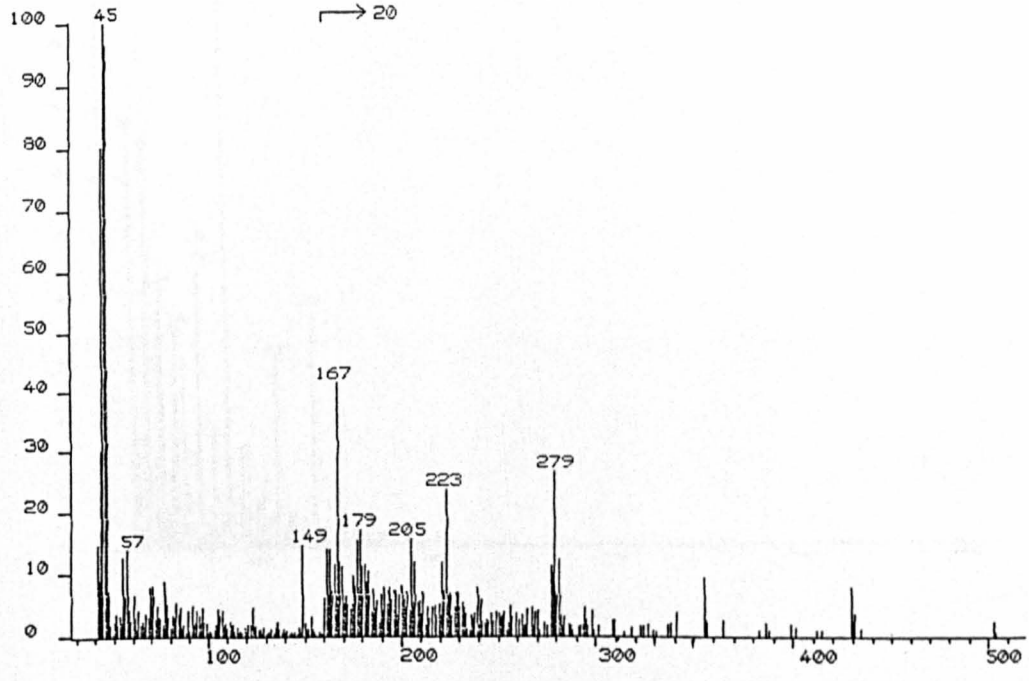
Appendix 7.1.7: Mass spectrum of picropodophyllin



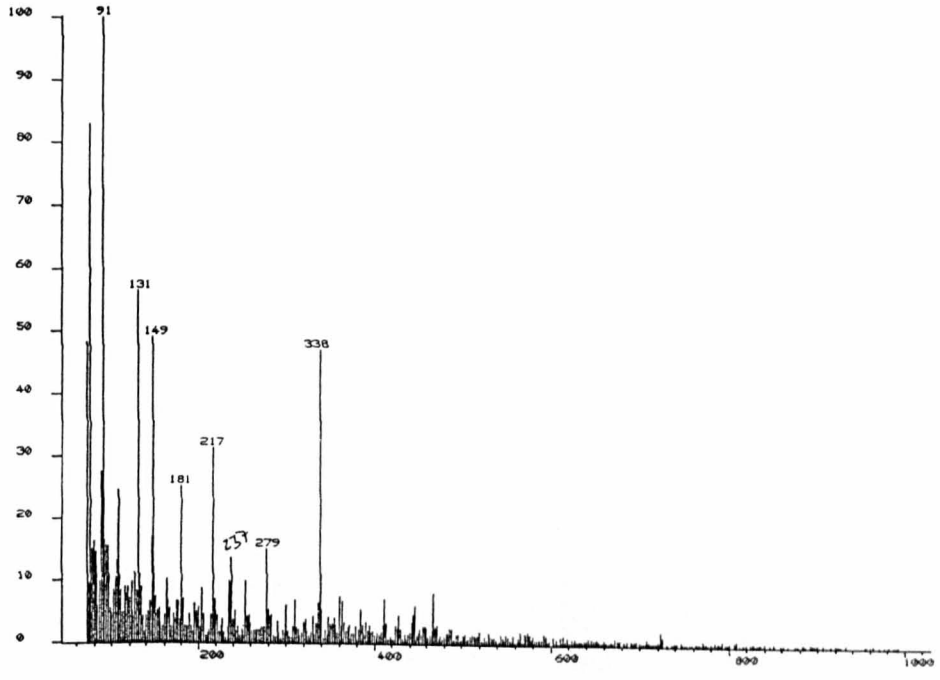
7.2 Electron impact and fast atom bombardment spectra for flavonoids

FAB data of the flavonoids isolated from leaves of *P. incarnata* and the somatic hybrid SH1 were summarized in Table 6.6 (Chapter 6, Section 6.3.2.2).

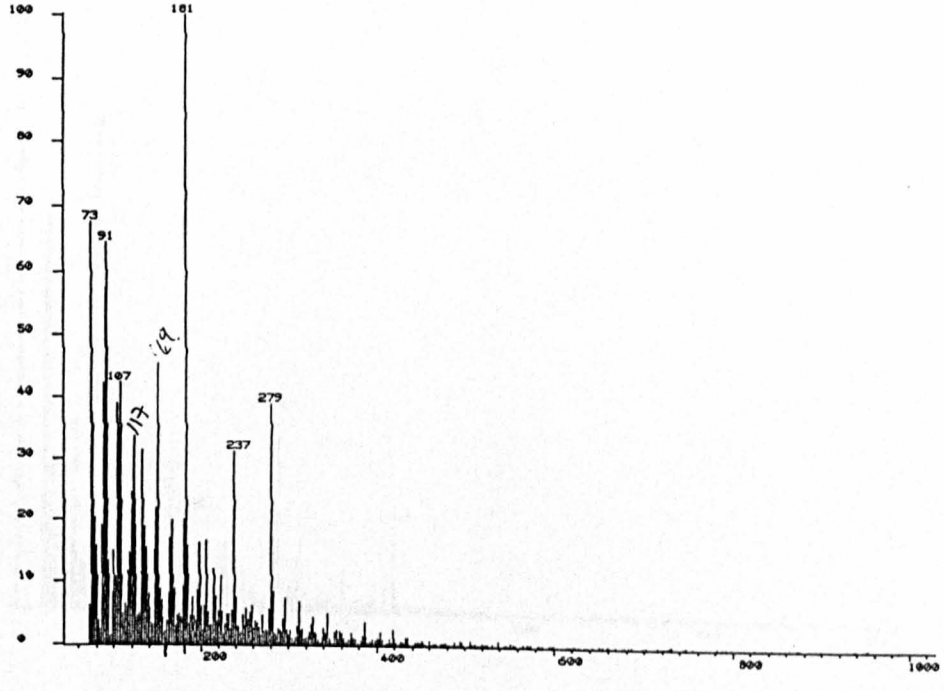
Appendix 7.2.1: EI spectrum of compound PI/1



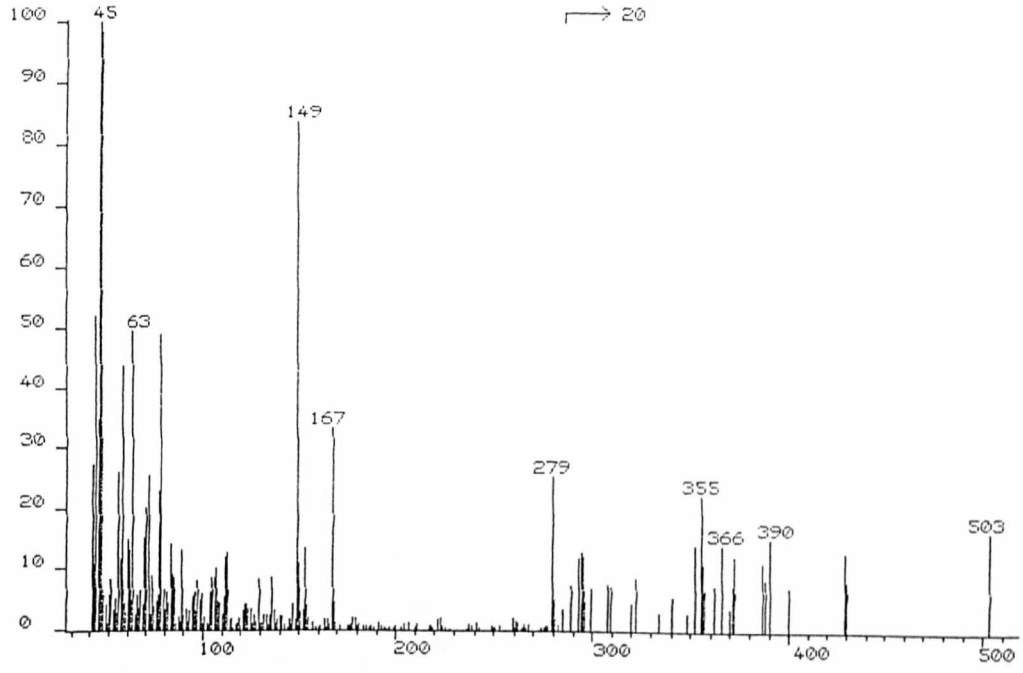
Appendix 7.2.2: FAB spectrum of compound PI/1



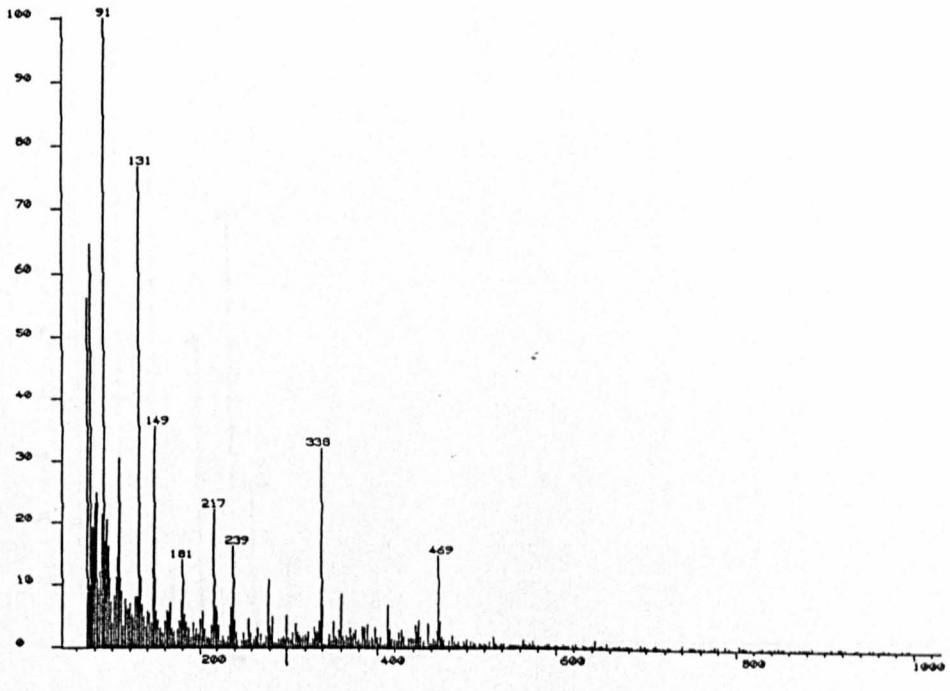
Appendix 7.2.3: FAB spectrum of compound PI/3



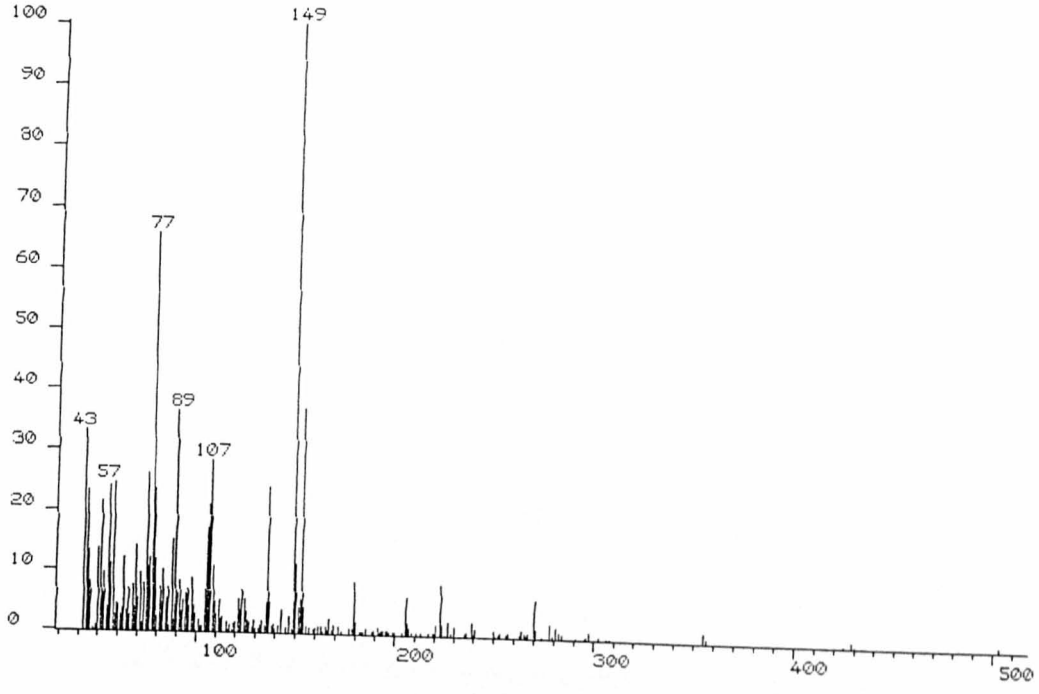
Appendix 7.2.4: EI spectrum of compound SH1/2B



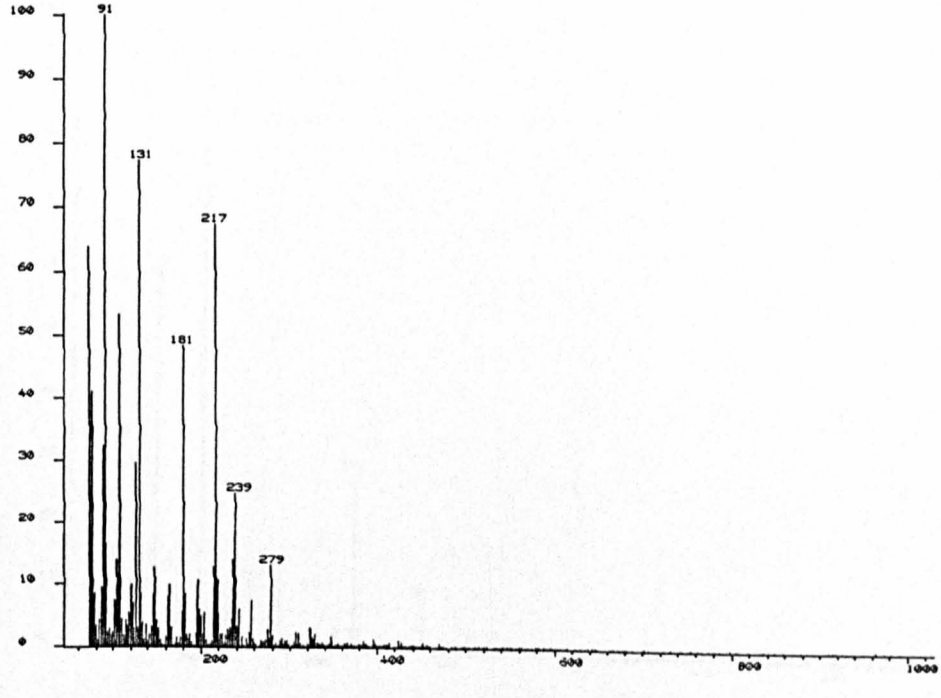
Appendix 7.2.5: FAB spectrum of compound SH1/2B



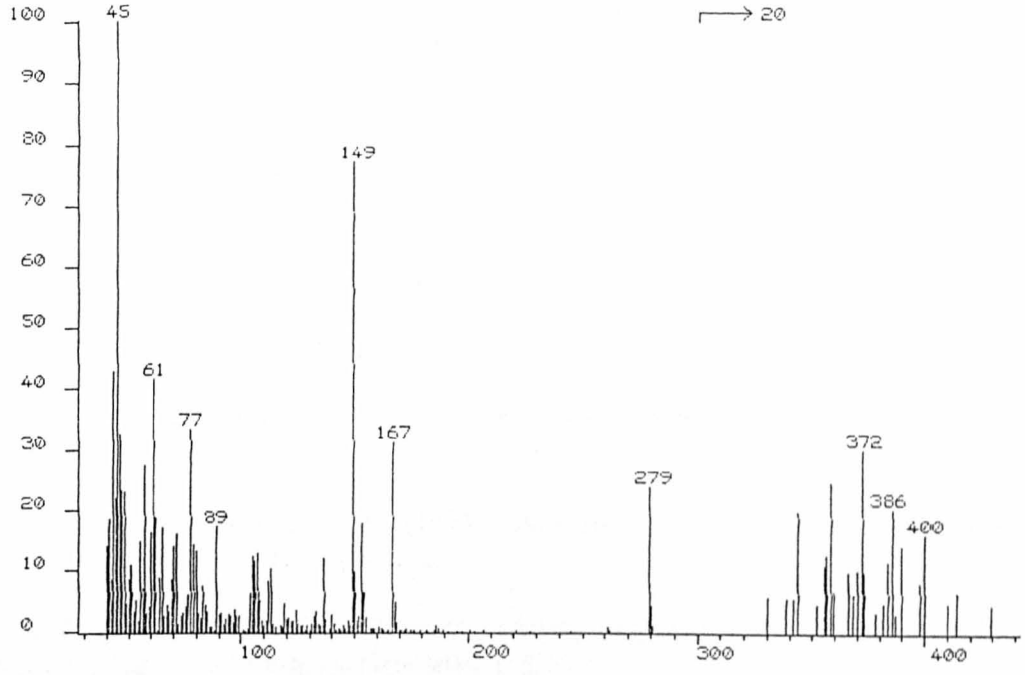
Appendix 7.2.6: EI spectrum of compound SH1/3A



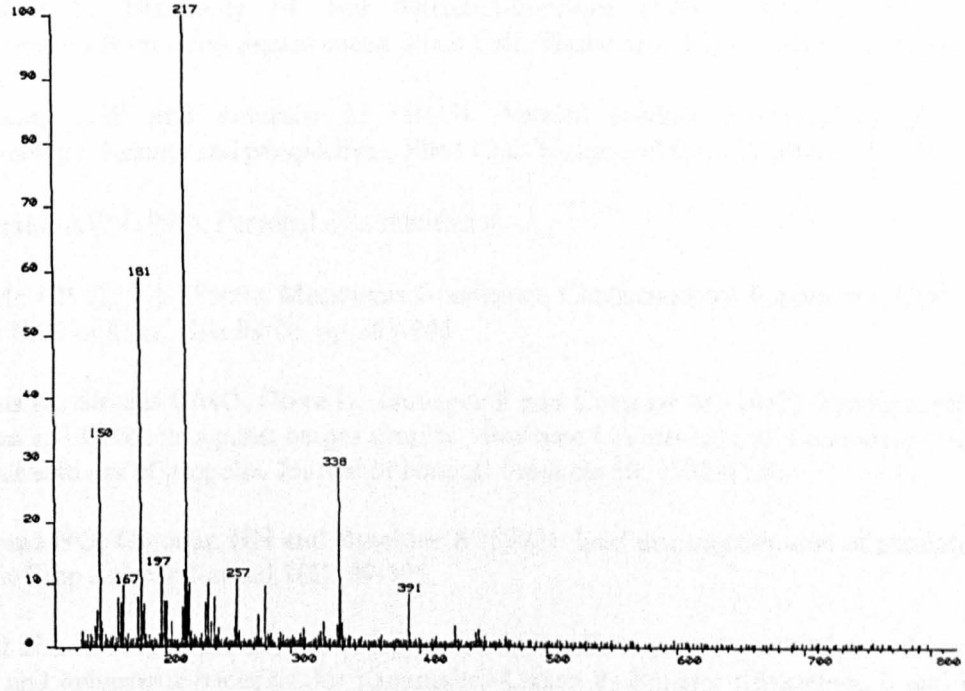
Appendix 7.2.7: FAB spectrum of compound SH1/3A



Appendix 7.2.8: EI spectrum of compound SH1/3B



Appendix 7.2.9: FAB spectrum of compound SH1/3B



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Publications

The abstracts listed below have been published during the course of the present studies:

Silva CG, Davey MR, Lowe KC, Power JB (1995). Flavonoid analyses in *Passiflora* somatic hybrids. Journal of Experimental Botany 46(suppl.), P7.25, Annual Meeting of the Society for Experimental Biology, St. Andrews University, Scotland. April 3-7, 1995.

Silva CG, Davey MR, Lowe KC, Power JB and Houghton PJ (1995). Flavonoids in somatic hybrids of *Passiflora*. In Nahrstedt A (ed.), Abstracts of Lectures and Poster Presentations, 43rd Annual Congress on Medicinal Plant Research (Secondary Products - Physiologically Active Compounds), *Abstract H69, Halle* (Saale), Germany. September 3-7, 1995. pp 69-70.

Silva CG, Power JB, Lowe KC, Power JB and Davey MR (1998). Plant regeneration from root explants of the medicinal plant *Podophyllum hexandrum* cultured in liquid medium. Journal of Experimental Botany 49(supp.), Abstract C2.08, Annual Meeting of the Society for Experimental Biology, University of York, UK. March 23-27, 1998. pp 88-89.

Silva CG, Davey MR, Power JB, Lowe KC, Konuklugil B, Alfermann AW (1999). Detection of antitumour lignans in tissue culture of *Diphylleia cymosa*. In: Book of Abstracts (2000 Years of Natural Products Research - Past, Present and Future), Poster 793, Amsterdam, The Netherlands. July 26-30, 1999.

Presented at the Annual Meeting of the Society for Experimental Biology, St. Andrews, Scotland. April 3-7, 1995.

Flavonoid analyses in *Passiflora* somatic hybrids. [P7.25]

C. G. Silva, M. R. Davey, K. C. Lowe and J. B. Power, Life Science, University of Nottingham; P. J. Houghton, Pharmacy, King's College, London.

Extracts from *Passiflora* species have widespread use in herbal medicine as sedatives, anxiolytics and antispasmodics. Somatic hybrids were obtained following electrofusion of leaf mesophyll protoplasts of *P. edulis* and *P. incarnata*. Methanolic extracts of leaves from such hybrids was analysed using TLC and HPLC. TLC was performed on silica gel GF-254 plates using ethyl acetate/ formic acid/glacial acetic acid/water (100: 11: 11: 27). After treatment with a 1% (v/v) methanolic solution of the aminoethyl ester of diphenylboric acid followed by 5% polyethylene glycol 4000 in ethanol (100%), the chromatograms showed typical fluorescent zones under UV (365 nm) indicating the presence of the flavonoids, isoorientin, isovitexin, vitexin and orientin. Isoorientin was present in *P. edulis*, the somatic hybrid and, to a lesser extent, in *P. incarnata*. Vitexin occurred in *P. incarnata* and the hybrid, but was undetectable in *P. edulis*. Trace quantities of orientin in all samples were confirmed by HPLC (5 µl sample; 1.5 ml min^{-1} flow rate) using an Apex 1 ODS reversed phase column eluted with either 2-propanol/tetrahydrofuran/water (5: 15: 85) or acetic acid/acetonitrile/water (10: 180: 820) as mobile phases and UV at 340 nm. Flavonoid analysis, combined with phenotypic, genotypic and molecular approaches, permits confirmation of somatic hybridity.

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Flavonoids in somatic hybrids of *Passiflora*

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Methanolic extracts of leaves of somatic hybrids generated by electrofusing leaf mesophyll protoplasts of *P. edulis* f. *flavicarpa* Degener and *P. incarnata* L. (Passifloracae) were analysed using TLC and HPLC. TLC was performed using silica gel GF-254 plates with ethyl acetate/formic acid/glacial acetic acid/water (100: 11: 11: 27), followed by treatment with a 1% (v/v) methanolic solution of the aminoethyl ester of diphenylboric acid preceding 5% (w/v) polyethylene glycol 4000 in ethanol (100%). Under U.V. (360 nm) the chromatograms showed typical fluorescent zones indicating the flavonoids, isoorientin, isovitexin, vitexin and orientin. HPLC (5 µl sample; 1.5 ml min^{-1} flow rate) using an Apex 1 ODS reversed phase column eluted with either 2-propanol/tetrahydrofuran/water (5: 15: 85), or acetic acid/acetonitrile/water (10: 180: 820), as mobile phases, confirmed that isoorientin was present in *P. edulis*, the somatic hybrid and, to a lesser extent, in *P. incarnata*. Vitexin occurred in *P. incarnata* and the hybrid, but was not detectable in *P. edulis*. Trace quantities of orientin occurred in all samples. Thus, flavonoid analyses, combined with phenotypic, genotypic and molecular assessments, permits confirmation of hybridity of novel plants produced through the fusion of somatic cell protoplasts.

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March 23-27, 1998.

C2.08 - Plant regeneration from root explants of the medicinal plant *Podophyllum hexandrum* cultured in liquid medium.

C.G. Silva, J.B. Power, K.C. Lowe and M.R. Davey, Biological Sciences, Nottingham.

Podophyllum hexandrum is a source of tumour-inhibitory lignans; its rhizomes and roots yielding podophyllotoxin as important commercial precursors for the semi-synthesis of anticancer drugs. Concern has been expressed about a shortage of rhizomes from *Podophyllum*, now a rare species. A protocol for the micropropagation of *P. hexandrum* var. Majus from roots has been developed, whereby seeds, pre-germinated on moist filter paper, were placed on Murashige and Skoog (MS) solid medium lacking growth regulators. Root explants from in vitro cultured seedlings and maintained in MS liquid medium containing different concentrations (0-3.0 mg l^{-1}) of (-naphthaleneacetic acid (NAA) were incubated on a rotary shaker at 25 ± 1°C in the dark. Regeneration was observed after 45 d in medium containing 0-1.0 mg l^{-1} of NAA. Plantlets were transferred to fresh MS medium without NAA and kept on a rotary shaker at 22 ± 1°C with a 16 h photoperiod (25 µMol m^{-2} sec^{-1}).

After 4 weeks plantlets were transferred to pots with compost and acclimatized in the culture room at 17 ± 2°C under a 16 h photoperiod (25 µMol m^{-2} sec^{-1}). Plants grew normally over a period of 6 months with no phenotypic abnormalities. This new approach provides a rapid procedure for generating plants. In addition, this system is an option for the in vitro conservation of *P. hexandrum*.

Detection of antitumour lignans in tissue cultures of *Diphylleia cymosa*

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Introduction

Diphylleia cymosa (Plate 1) is a perennial herbaceous species within the subfamily *Podophylloideae* (1) and is indigenous to Eastern North America (2). The American Cherokee Indians used an infusion of this medicinal species as a diuretic, an antiseptic and for treatment of smallpox (3). Only a few studies concerning the production of secondary metabolites in *D. cymosa* have been published. Lignans are the most important group of natural products, in terms of their pharmacological interest, reported in its leaves, stems and roots (4). No phytochemical work dealing with the analysis of lignan contents in tissue cultures of *D. cymosa* have been performed to date. Here, we report HPLC analysis for lignans in petiole-derived calli of *D. cymosa*.

Materials and Methods

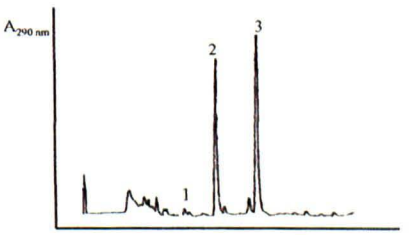
Tissue culture material: Petiole-derived calli (Plate 2) were cultured on MS medium (5) supplemented with 2,4-D and kinetin in the dark (22 ± 1°C).

Extraction and hydrolysis of lignan glycosides: Ten friable calli (6 months old) were stored overnight (-70°C) before lyophylization. Freeze-dried samples (200 mg d. wt.) were extracted by ultrasonication with 2.0 ml ethanol. Aglycones were liberated after enzymic hydrolysis with 1.0 mg β-D-glucosidase. After 1 h incubation (35°C), the reaction mixture was extracted with 12.0 ml ethanol, sonicated and centrifuged.

Standards: Podophyllotoxin (Ptox) and 4'-demethylpodophyllotoxin (4'-demethyl/Ptox) were isolated from *P. hexandrum* roots. Diphyllin was kindly provided by Dr RS Ward (Swansea, U.K.), 5-methoxypodophyllotoxin (5-MPT) by Dr W van Uden (Groningen, The Netherlands) and 4'-demethyldesoxypodophyllotoxin (4'-demethyldes/Ptox) by Professor A San Feliciano (Salamanca, Spain). The solutions were prepared at 0.05 mg ml^{-1} (w:v) in 1.0 ml methanol.

HPLC: Qualitative analyses were performed using an instrument from LDC Analytical (Gelnhausen, Germany). Filtered samples (10 µl) were injected onto a reverse-phase C-18 column (290 mm x 4.6 mm i.d.) packed with Nucleosil 100. The mobile phase consisted of water (100 %) and acetonitrile/orthophosphoric acid (15:85, v:v). The flow rate of the solvents was 0.8 ml min^{-1} with gradient elution. The peaks and their retention time (Rf) values were detected at 290 nm.

Fig.1: HPLC profile of petiole-derived friable calli



(1) Podophyllotoxin; (2) and (3) are unknown peaks.

Results

The HPLC pattern of petiole-derived friable calli (Fig. 1) showed a minor peak (1) [Rt = 6.60] which corresponded to the same Rt value and UV spectrum as that of the authentic podophyllotoxin standard. The two major peaks, (2) and (3), had a Rt of 8.60 and 11.24 respectively, but did not match with any of the standards. The Rt of 4'-demethyl/Ptox, 5-MPT, 4'-demethyldes/Ptox and diphyllin were 6.40, 8.89, 12.24 and 12.48, respectively.

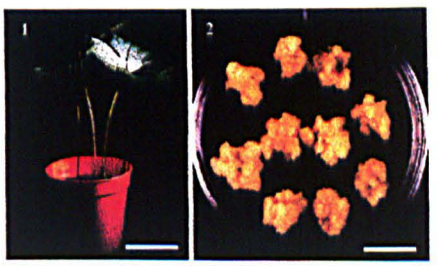


Plate 1. *D. cymosa* plant (5 years old) cultivated at the University of Nottingham. (Bar = 10.7 cm)

Plate 2. Pale yellow, friable calli from petiole explants cultured on MS agar medium with 2.0 mg l^{-1} 2,4-D and 0.25 mg l^{-1} kinetin in the dark. (Bar = 1.9 cm)

Conclusion

This is the first report of a phytochemical study of *D. cymosa* tissue cultures. HPLC, complemented by UV-visible spectrophotometry, allowed a rapid screening of lignans in petiole-derived friable calli. The peak corresponding to podophyllotoxin suggested that the lignan may be present at low concentrations in these undifferentiated cultures even when they grown in the presence of 2,4-D. Additional studies will enable confirmation of this observation.

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