Production of diospyrin by *Euclea natalensis* seedlings and *in vitro* cultures

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Declaration

The experimental work described in this report was carried out in the Department of Molecular and Cell Biology, University of Cape Town, from February 2003 to July 2005, under the supervision of Prof. J.M. Farrant and Dr B. Hamman.

The results presented here are the original, unaided work of the author. Where use has been made of the work of others it is duly acknowledged in the text.

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Abstract

The mature roots of a medicinal tree, *Euclea natalensis*, contain a naphthoquinone, diospyrin, which has demonstrated powerful activity against *Mycobacterium tuberculosis*. Since continuous harvesting of mature roots is non-sustainable, alternative sources of diospyrin were investigated. This study focused on the potential use of *E. natalensis* seedlings, callus cultures and hairy root cultures as alternative sources of diospyrin. Chloroform extracts of *E. natalensis* seedling organs showed antimycobacterial activity against *Mycobacterium aurum*, and HPLC analysis of the extracts demonstrated a corresponding accumulation of diospyrin, mostly in the stem (0.23%, DW) and roots (0.17%, DW) of the seedlings. Using leaf material as explants, *E. natalensis* calli were successfully induced and subcultured on Murashige-Skoog (MS) (half-strength macronutrients) solid media supplemented with 2.5 mg l^{-1} 2,4-D, and on Fujii and Nito (FN) solid media supplemented with 5 mg l^{-1} adenine and 5 mg l^{-1} 2,4-D. Diospyrin was isolated from chloroform extracts of both sets of calli, and the chemical structure confirmed using MS and NMR spectral data. The amount of diospyrin accumulated was 5.2% (DW) in calli induced on MS media, and 1.2% (DW) in calli induced on FN media. In order to generate hairy root cultures of *E. natalensis*, the transformation capacity of five strains of *Agrobacterium rhizogenes* (ATCC 1724, ATCC 15834, ATCC 2659, ATCC 8196, and LBA 9402) were investigated, using *E. natalensis* seedlings and three different transformation protocols. All five strains successfully induced hairy root growth and all hairy roots were able to synthesize diospyrin (0.07 to 0.22%, DW). All transformed roots however, failed to continue to grow as hairy root cultures once separated from the explants. Since *E. natalensis* seedlings, calli and hairy roots all successfully accumulated diospyrin at levels

comparable to, or greater than, those found in mature roots (between 0.1 and 1%, DW), they would provide suitable and sustainable sources of diospyrin.

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Abbreviations

AS	Acetosyringone
6- BA	6- benzylaminopurine
bp	base pairs
cfu	colony forming units
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
ESI	lectron spray ionisation
HIV	human Immunodeficiency Virus
IPTG	isopropyl- β-D-thiogalactopyranoside
LB	luria Broth
MDR	multidrug resistant
MIC	minimum inhibitory concentration
MTT	3-(4, 5-dimethyl-2-thiazolyl (-2, 5-diphenyl-2H-tetrazolium bromide)
ORF	open reading frame
PVPP	polyvinylpolypyrrolidone
Rif	rifampicin
SEM	scanning electron microscopy
TB	tuberculosis
TBE	tris-borate-EDTA
TMS	tetramethylsilane
Tm	melting temperature
U	units
YEB	yeast extract broth
YMB	yeast maltose broth
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

General introduction

Tuberculosis (TB) has re-emerged as a deadly global health problem. Annually, three million deaths are caused by the disease, mostly in developing countries (WHO, 2003). Given the increasing number of multi-drug resistant (MDR) strains of *Mycobacterium tuberculosis*, the lack of new anti-TB drugs, the association between the Human Immunodeficiency Virus (HIV) and TB, as well as increasing social disparities on susceptibility to TB (van Helden, 2003), the impact of the disease is likely to increase unless strides are made towards understanding the nature of the disease, or towards discovering new compounds active against dormant bacilli, as well as against drug sensitive and drug resistant strains of the bacterium.

Euclea natalensis (A. DC.), a medicinal plant indigenous to Southern Africa (Pooley, 1993) has traditionally been used to cure symptoms associated with TB (Bryant, 1966; Pujol, 1990). An antimycobacterial compound, diospyrin, has been isolated from mature roots of the tree, and can inhibit even the growth of MDR strains *in vitro* (Lall and Meyer, 2001). The biological activity of diospyrin extends into other areas of research: it has anti-malarial, -cancer and -trypanosomiasis properties (Hazra *et al.*, 1994; Hazra *et al.*, 1995; Yardley *et al.*, 1996), and the compound is considered a 'lead compound' for the development of drugs against these diseases.

As with most medicinal plants, exploitation of *E. natalensis* mature roots either traditionally, or as a source of diospyrin, is destructive and therefore unsustainable (Mander, 1998). This project therefore hoped to address the potential threat facing *E. natalensis* by investigating a few sustainable means of harvesting diospyrin. Sustainable

sources that were explored were *E. natalensis* seedlings and plant cell cultures using *E. natalensis* as sources of diospyrin.

The first chapter of this thesis is devoted to the documented literature supporting the objectives of this project. In it, the use and importance of medicinal plants in drug research, as well as the pathology of TB is discussed, followed by a brief discussion on the possible mechanisms of toxicity employed by diospyrin against *M. tuberculosis*. The chapter also discusses the advances made in biotechnology which allowed us to explore plant cell cultures as superior sources of secondary metabolites traditionally extracted from whole plants. The underlying theory and applications of two types of cell culture, namely, callus and hairy root cultures is also discussed. In the second chapter, *E. natalensis* seedlings and callus cultures are explored as sources of antimycobacterial activity and of diospyrin. The distribution of diospyrin in the seedlings is analysed, and the effect of tissue culture media on the quantities of diospyrin accumulated, as well as on the secondary metabolite profile of calli, is determined. The third chapter discusses the factors influencing hairy root formation upon transformation with *Agrobacterium rhizogenes*, the conditions adopted in this study in order to successfully induce hairy root growth on *E. natalensis* explants, and finally, the levels of diospyrin accumulated in the hairy roots. The last chapter summarises the main findings, and discusses the sustainability of the various alternative sources of diospyrin explored in this study. Recommendations likely to improve the use of calli and hairy roots as sources of diospyrin are made.

Chapter One

Literature review

1.1 *Euclea natalensis* and the use of medicinal plants

Historically, various communities have used plants as sources of medicinal treatment. In 2002, 75% of the world's population was estimated to use medicinal plants (Rao and Ravishankar, 2002) mainly for primary health-care (Masood, 1997). The medicinal use of such plants stems from their ability to synthesize complex secondary metabolites such as phenolics, alkaloids, terpenes and steroids. For the most part, these metabolites generally protect plants from pathogens and competing plants via their anti-bacterial, -fungal, -germinative and –viral properties (Harborne, 1999). It is these diverse biological properties which have laid a foundation for the medicinal use of these plants traditionally, and in the pharmaceutical industry, where plant-derived secondary metabolites account for 25% of the compounds used (Payne *et al.*, 1991).

Euclea natalensis (A. DC.) is one of many plant species reputed to have medicinal properties (Hutchings *et al.*, 1996). The small dicotyledonous tree belongs to the family Ebenaceae and is naturally distributed along the eastern seaboard of Southern Africa, extending inland up to Botswana (Pooley, 1993). In South Africa, 75% of the population uses traditional medicines (Mander, 1998) and among the plethora of plants utilised, extracts of *E. natalensis* are used to alleviate complications such as bronchitis, skin infections and, most importantly, symptoms associated with tuberculosis (TB) (Bryant, 1966; Pujol, 1990).

1.2 Tuberculosis

Tuberculosis is one of the leading causes of death from infectious diseases. One-third of the world's population is estimated to be infected with the etiological agent, *Mycobacterium tuberculosis*, and five to ten percent of these are expected to develop the disease or become infectious during their lifetime (WHO, 2005). Annually, over three million deaths are attributable to TB, with 90% of these occurring in developing countries (Bloom and Murray, 1992; WHO, 2003). Globally, and especially in sub-Saharan countries, the increased incidence of TB has largely been due to the prevalence of the Human Immunodeficiency Virus (HIV) (Corbert *et al.*, 2003).

M. tuberculosis (*Mycobacteriaceae*) is a Gram-positive, aerobic, catalase positive and acid-fast bacterium. It is also non-sporing and rod shaped. Their cell walls contain unusually high levels of lipids - a possible consequence of 30% of the genome being involved in lipid metabolism or synthesis (Prescott *et al.*, 1996a; Cole *et al.*, 1998).

An infection with *M. tuberculosis* begins primarily with inhalation of infectious bacilli. Within the lungs, alveolar macrophages engulf the bacteria and initiate a localised hypersensitivity response which attracts mononuclear cells to form a granuloma. The bacteria are contained within the granuloma, and while this prevails the host may be asymptomatic and non-infectious. A weakening of the host's immune system usually induces caseation of the granuloma and release of viable bacilli to new infection sites in the body. This may then be followed by active disease, with various organs and the central nervous system being affected (Russel, 2001; Frieden *et al.*, 2003).

All bacteria, including *M. tuberculosis*, occasionally acquire genomic mutations which may change the nature of the bacterium, affecting, for example, its virulence (Dyes *et al.*, 1999). The pathology of TB, difficulty of treatment and the inappropriate use of anti-TB drugs has resulted in the selection of chromosomal mutations that enable *M. tuberculosis* to resist the effects of many drugs (Bloom and Murray, 1992; Snider and Roper, 1994). The emergence of multidrug-resistant TB (MDR-TB) (defined as unaffected at least by rifampicin and isoniazid) and the lack of new first-line anti-TB drugs has escalated the threat of TB and initiated various strategies to counter the problem (Harvard Medical School, 1999; Warner and Mizrahi, 2004). Different approaches are currently used in the search for novel drugs. The unravelling of the *M. tuberculosis* genome (Cole *et al.*, 1998) has presented potential drug targets which can be validated using existing genetic technologies (Warner and Mizrahi, 2004). Most importantly, the search for novel antimycobacterial compounds is also making use of the possible reservoir of anti-TB compounds existing in those plants reported to have medicinal properties. As already mentioned, one such plant is *E. natalensis*.

1.3 Antimycobacterial properties of *E. natalensis*

The major chemical constituents of *E. natalensis* responsible for the medicinal properties are naphthoquinones (Khan *et al.*, 1978; Hazra *et al.*, 1984). One of these, diospyrin, was isolated from extracts of mature *E. natalensis* roots and was able to inhibit the growth of a drug-sensitive strain of *M. tuberculosis* and six MDR strains. All the strains were inhibited at a minimal inhibitory concentration (MIC) of 100 $\mu g\ ml^{-1}$ (Lall and Meyer, 2001). An aminoacetate derivative of diospyrin was also subsequently found to display enhanced activity against sensitive and MDR strains of *M. tuberculosis* (Lall *et al.*, 2003).

In addition to anti-TB activity, diospyrin has been documented to display activity against other micro-organisms as well. To address the toxicity of the compound towards normal cells in Swiss mice, Hazra *et al.* (2005) encapsulated diospyrin in liposomal vesicles which reduced the toxicity to those cells, and increased activity towards the Ehrlich ascites tumours borne by the mice. As a consequence of the activity displayed by the molecule, diospyrin is considered to be a potential 'lead compound' from which drugs against malaria, cancer, leishmaniasis and trypanosomiasis may be designed (Hazra *et al.*, 1995; Yardley *et al.*, 1996; Ray *et al.*, 1998).

The mechanism with which diospyrin, or its derivatives, inhibit the growth of *M. tuberculosis* is not known. Quinones, however, of which naphthoquinones are a subclass, are thought to induce their toxicity towards microorganisms via two possible mechanisms: In the first of the mechanisms Medentsev and Akimenko (1998) believe that much of the toxicity of naphthoquinones is due to their inherent redox potentials. They suggest that naphthoquinones undergo a quinone "redox cycle" which may be initiated by electron carriers such as reduced glutathione, or catalysed by flavine enzymes. The ensuing one-electron reduction changes the naphthoquinone into a semiquinone. This free radical intermediate may then disrupt nucleic acids or, in the presence of molecular oxygen, form superoxide or hydroxyl radicals which can destroy biological membranes and various proteins (Wefers and Sies, 1983; Smith *et al.*, 1985). The second mechanism, proposed by Seung *et al.* (1998), suggests that quinones are activated intracellularly by alkylation and then covalently bind targets such as DNA and proteins, consequently disrupting their functions. Alternatively, Tran *et al.* (2004) investigated the toxicity of several quinones against *M. tuberculosis* H37a and other Mycobacteria and, without elaborating further,

suggested that the toxicity may potentially be mediated by new and uncharacterised mechanism unlike those mentioned above.

1.3.1 Physical and chemical properties of diospyrin

Diospyrin (a dimer of 7-methyljuglone) is an orange-red compound with a molecular weight of 374 daltons (Figure 1.1). Typically of naphthoquinones, it has three spectral maxima in ethanol at 223, 254 and 438 nm (Thomson, 1971).

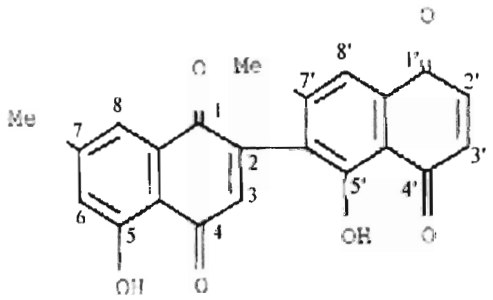

Figure 1.1 Chemical structure of diospyrin. (2, 6-bis (5-hydroxyl-7-methyl-1, 4-naphthoquinone). $C_{22}H1_4O_6$

The physical measurements include;

a) Electron ionisation mass spectroscopy (EI-MS) (70 eV): *m/z* (%): 374 (100) [M^+], 359 (16), 357 (11), 356 (12), 328 (10), 187 (9), 163 (8), 135 (10), 134 (10), 106 (13), 99 (10);

b) ^{1}H nuclear magnetic resonance (NMR) (500 MHz, $CDCl_3$): δ = 2.31 (s, 3 H, 7' – Me), 2.46 (s, 3 H, 7-Me), 6.90 (s, 1 H, 3-H), 6.96 (s, 2 H, 2'-H, 3'-H), 7.13 (s, 1 H, 6-H), 7.51 (s, 1 H, 8-H), 7.57 (s, 1 H, 8'-H), 11.88 (s, 1 H, 5-OH), 12.14 (s, 1 H, 5'-OH) (Yoshida and Mori, 2000).

1.4 Bio-prospecting of secondary metabolites from medicinal plants

With the demand for traditionally used plants being so great, species such as *Siphonochilus aethiopicus* and *Warburgia salutaris* for example, are nearly extinct outside their protected regions. As with many medicinal plants, the conservation status of *E. natalensis* is not known, but these plants are continually harvested from the wild, with little management or cultivation occurring, and it is the mature roots mostly, that are harvested (Mander, 1998; Lall and Meyer, 1999). The demand for medicinal plants is expected to increase at the expense of existing wild plant stocks. The most likely effect of over harvesting and depletion of plant species will be a decline in biodiversity within the country (Mander *et al.*, 1997). This will have obvious implications to pharmaceutical or academic research as the majority of plant species have yet to be chemically characterised and analysed for new products (Cox and Balick, 1994). In spite of the increasing ability to chemically synthesise many plant products, there are still many that are too complex to produce synthetically (Pezzuto, 1995). The biodiversity among the plant population remains therefore our most potential source of novel pharmaceuticals.

1.5 Advances in biotechnology and the search for sources of secondary metabolites

Often the use of medicinal plants for research or pharmaceutical purposes is beset with practical difficulties. Trees such as *Taxus brevifolia* for example, from which taxol (anticancer drug) is extracted may require up to decades of cultivation prior to harvesting. Other plants may be difficult to cultivate outside their normal ecosystems, and even if cultivated in their natural biotopes, they may be subject to environmental and political

factors (Dicosmo and Misawa, 1995; Masood, 1997). Recent advances in biotechnology have addressed this by making it possible to use plant cell, tissue and organ cultures as alternative sources of the desired plant metabolites. In contrast to harvesting from intact plants, these sources offer significant advantages in terms of sustainability. Not only can cultures for, example, be used as sources of metabolites traditionally extracted from intact mature plants, they can also be maintained in defined *in vitro* systems, ensuring that product quality and quantity is uniform. Most importantly though, these cultures can be used as model systems with which to study the metabolic pathways leading to the synthesis of particular metabolites, or the effect of external factors on selected metabolites (Dörnenburg and Knorr, 1995).

1.6 Callus cultures as sources of medicinal compounds

The development of plant cells is largely regulated by endogenous phytohormones, most notably cytokinins which stimulate shoot growth as well as lateral bud formation, and auxins which induce root formation. With the exact combination and concentration of exogenous phytohormones *in vitro*, differentiated plant cells can be induced to dedifferentiate and grow as callus cultures (Thain and Hickman, 1996). As these are totipotent, they retain the biosynthetic capabilities of the parent plant (Rao and Ravishankar, 2002). They are also particularly susceptible to genetic changes (Ravishankar and Venkataraman, 1993). Consequently, cell lines are usually established only after ensuring that the calli have stabilised. Since markers for genetic stability are not available the uniformity of growth parameters over time are generally used as an indication of cell line stability. Since genetic shifts have an effect on the biosynthetic characteristics of a

culture (Bourgaud *et al.*, 2001), stability is important to establish. Once stabilised, calli are able to synthesize metabolites characteristic of the intact plant and this property is successfully used to induce cell cultures to accumulate metabolites with medicinal value, often at levels greater than those found in the parent plant (Fujita, 1988; Park *et al.*, 1992). Since the biosynthetic potential of cell cultures has the potential to differ from that of the intact plant (DiCosmo and Towers, 1984) various factors have been used to maintain, or even improve, the yield of medicinally important metabolites of a particular culture. For instance, factors such as the medium composition (e.g. nutrients, nitrate levels and phytohormones) and culture conditions (e.g., oxygen levels, pH, and temperature) improve the recovery of metabolites from certain cell cultures. Increases in metabolite levels have also been obtained using abiotic and biotic elicitors that are able to induce physiological responses affiliated with the production of phytoalexins for example (reviewed in Rao and Ravishankar, 2002). In instances where levels the of metabolites in a culture are low, a lack of specialised storage structures for the metabolites is sometimes the cause, however artificial storage compartments can be used in order to enhance the levels of metabolites previously found only in low quantities (Berlin *et al.*, 1984; Strobel *et al.*, 1991). Although it is possible that calli can potentially experience changes in their biosynthetic abilities when compared to the explants, the simplicity with which they can be established, and their ability to produce the same metabolites as those found in intact plants, make them valuable alternatives to harvesting medicinal metabolites from plants in the wild.

1.7 Hairy root cultures as sources of secondary metabolites

Plants often co-exist with various micro-organisms in relationships that are sometimes mutually beneficial. A number of bacteria have exploited their intimate proximity to plants

and evolved to express various degrees of pathogenicity. The soil bacterium *Agrobacterium rhizogenes* is a member of the Rhizobiaceae family which also includes *A. rubi*, *A radiobacter* and *A tumefaciens*. All *Agrobacterium* species are generally rod-shaped, Gram-negative aerobic bacteria (Prescott *et al.*, 1996b) with circumtrichetally-orientated flagella (Tanaka, 1985). *A. rhizogenes* is generally pathogenic towards dicotyledonous plants (Tepfer, 1990), with infection resulting in the initiation of hairy roots that are capable of synthesising amino opines. As only the *Agrobacterium* can metabolise opines they gain a metabolic advantage over other phytopathogens which may be infecting the plant. In the absence of infection though, many of the *Agrobacterium* species are able to use various sugars or sugar alcohols (Sonoki *et al.*, 1978) as sources of carbon, and arginine or ornithine as a source of nitrogen (Cunin *et al.*, 1986).

1.7.1 *Agrobacterium rhizogenes* root-inducing plasmid

The ability of *A. rhizogenes* to induce hairy root growth in the site of infection is due to the presence of a root-inducing plasmid (pRi). The plasmid's transfer DNA (T-DNA), which is transferred to the host genome, dictates changes that result in hairy root induction (Chilton *et al.*, 1982). The genes essential for the bacterium's pathogenic abilities are encoded in the plasmid and these include the virulence (*vir*), root locus (*rol*) and opine metabolic genes (Figure 1.2). The particular opine metabolised by the bacterium classifies the Ri plasmids as agropine, cucumopine, mannopine or mikimopine-type (van de Velde *et al.*, 2003).

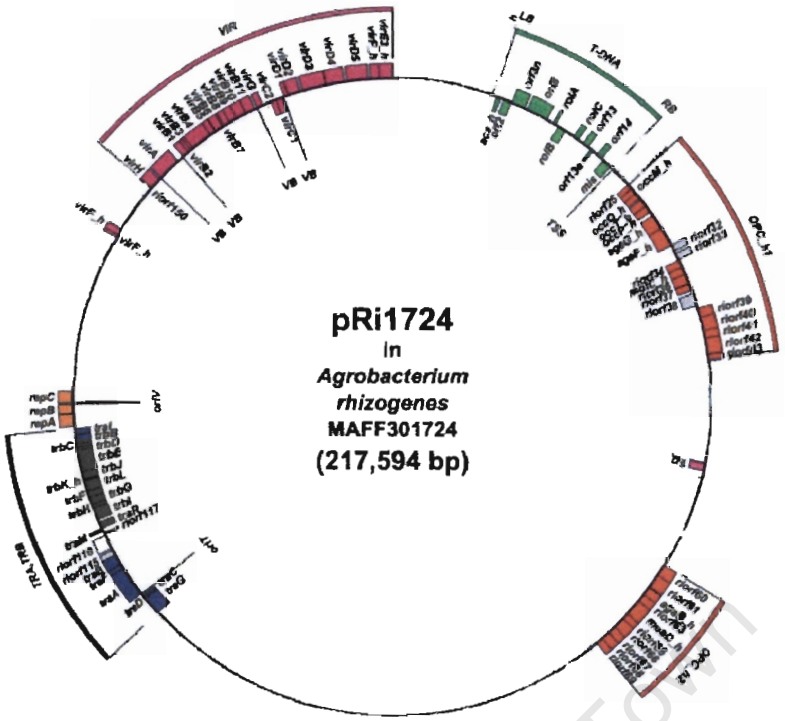

Figure 1.2 Partial map of pRi1724 in *Agrobacterium rhizogenes* MAFF301724. Genes or open reading frames (ORF) within the T-DNA, virulence, putative opine catabolism and bacterial conjugation (*tra* and *trb*) regions represented in green, pink, red, and blue, respectively. The genes for vegetative DNA replication (*rep*) are represented in orange. The initiation sites for circular DNA replication (*ori*V), and gene transfer by conjugation (*ori*T) are also indicated. (Adapted from Moriguchi *et al.*, 2001).

1.7.2 Summary of T-DNA transfer into host plants

The molecular events that initiate the transfer of *A. rhizogenes*' Ri T-DNA into the host genome are much like those which occur during transformation with Ti T-DNA from *A. tumefaciens* (van de Velde *et al.*, 2003). The molecular events known to occur during an infection with *A. tumefaciens* are used to explain the events which possibly occur during an infection with *A. rhizogenes* (Figure 1.3):

Upon incurring mechanical damage, plant cells release several compounds as exudates, some of which are phenolic compounds such as acetosyringone, and sugars that initiate the expression of virulence genes by activating the membrane-spanning protein VirA (Cangelosi *et al.*, 1990; Shimoda *et al.*, 1990). This activation leads to autophosporylation of VirA and subsequent transfer of the phosphate to VirG. These two proteins work as a two-component system responsible for transcriptional regulation of the rest of the *vir* genes (Stachel and Zambryski, 1986). Once synthesized, VirD1 identifies and attaches itself to the T-DNA borders (25-bp repeats on either side of the T-DNA) and directs VirD2 towards this region in order for it nick one strand of the plasmid (Scheiffele *et al.*, 1995). The ensuing unidirectional DNA replication and nicking of the other T-DNA border releases a single strand (ss) copy of the T-DNA (T-strand), to which VirD2 binds covalently on the 5' end, whereas VirE2 molecules cover the entire length of the T-strand, protecting it from nucleolytic degradation (Baron and Zambryski, 1996; Rossi *et al.*, 1996). Upon formation of this T-complex, the nuclear localisation signals on VirD2 and VirE2 direct the nucleoprotein towards the host plant cell (Zupan *et al.*, 2000).

Meanwhile, another set of proteins assemble to form the T-complex transporter, through which the T-complex is channelled out of the bacterium (Figure 1.4). It is thought that initially VirB1, with its possible glycosidase activity, alters the peptidoglycan layer of the *Agrobacterium* so that VirB2 and VirB5 can congregate on the outer membrane of the bacterium so as to initiate the synthesis of the T-pilus (Fullner, 1998).

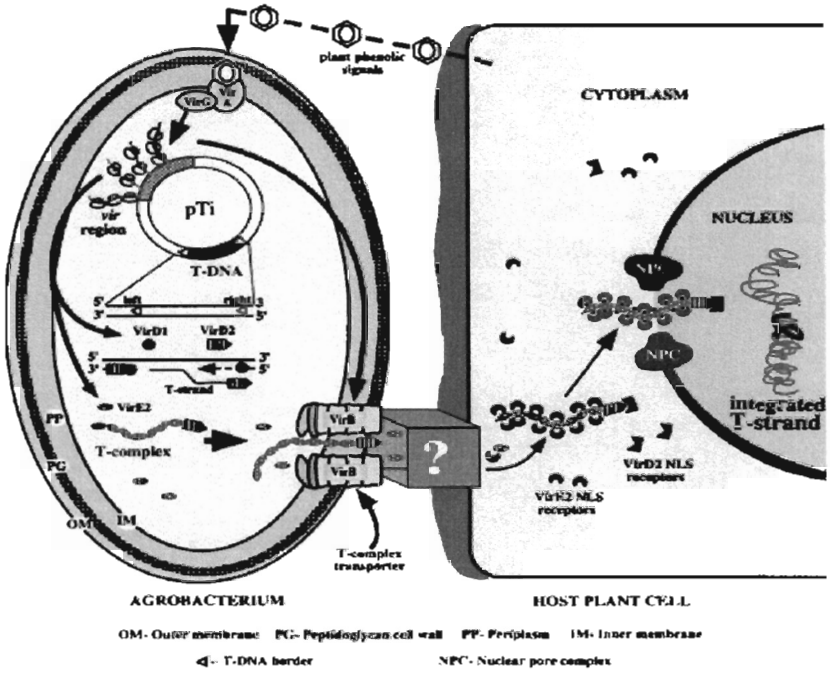

Figure 1.3 Overview of the events which probably occur during transformation with *A.rhizogenes'* T-DNA. See text for details. Figure from Zupan *et al.* (2000).

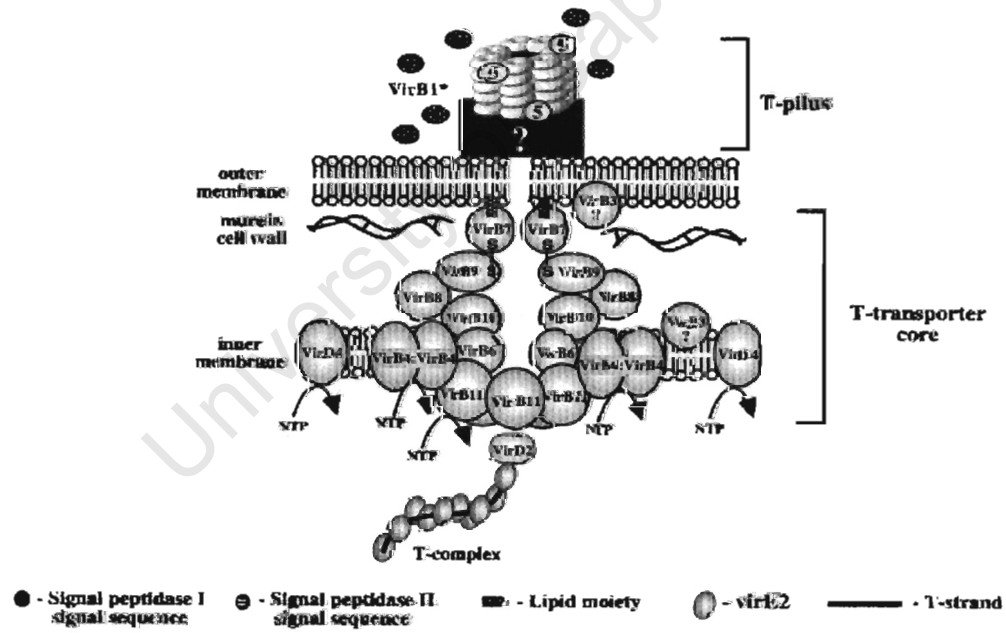

Figure 1.4 Possible arrangement of the T-transporter and T-pilus in *A. rhizogenes*. The T- complex is transported through the core and pillus towards the host cell. Figure adapted from Zupan *et al.* (2000).

This is essential as the pilus extends towards the host cell, thus creating a channel through which bacterial conjugation may result in DNA transfer from the bacterium to the host. Concurrently, the T-complex transporter is constructed on the inner membrane using VirD4 and 11 membrane or periplasm-associated protein encoded by the *vir*B operon (Christie and Vogel, 2000; Zupan *et al.*, 2000).

The exact mechanism by which the T-DNA integrates into the host genome is not known, yet it is agreed that host plant factors, in addition to VirD2 and VirE2, may play a role in the process (Tzfira *et al.*, 2004). Taking various integration models into consideration Tzfira *et al.* (2004) have proposed that during translocation to the host nucleus, the 3' end of the ss T-DNA is degraded slightly whereas the 5' end is protected by VirD2. Once in the nucleus the ss T-DNA changes to a double stranded (ds) intermediate that integrates into the plant genome most probably via a non-homologous recombination pathway, and possibly to a lesser degree via a homologous recombination pathway (Bundock *et al.*, 1995; Bundock and Hooykaas, 1996; Risseuw *et al.*, 1996).

1.7.3 *A.rhizogenes* Ri T-DNA and the function of *rol* genes

The expression of Ri T-DNA genes in the host plant induces the "hairy root syndrome", an outcome unlike that which occurs after transformation with *A. tumefaciens*. The reason for this response lies in the structural differences and the genes encoded by the two different types of T-DNAs. Agropine strains such as HRI have Ri T-DNAs which are fragmented into a right T-DNA (T_R-DNA) and left T-DNA (T_L-DNA) region, with the former encoding auxin and opine biosynthetic genes, and sharing some homology to similar genes in T-DNA of *A. tumefaciens*, and the latter encoding the *rol* genes (van der Salm *et al.*, 1996). Cucumopine, mannopine and mikimopine-type strains such as ATCC 1724 only have the

T_L-DNA segment (Koplow *et al.*, 1984), a segment that is both unique to *A. rhizogenes* and contains opine biosynthesis genes, and the *rol*ABCD genes (ORFs 10, 11, 12 and 15, respectively) which are responsible for the "hairy root syndrome" (White *et al.*, 1985; van der Salm *et al.*, 1996).

Although an exact function has not yet been attributed to the rolA protein, McClure *et al.* (1989) showed that the *rol*A promoter has an upstream domain possibly regulated by auxin. The r*ol*B gene is known to encode a β-glucosidade that possibly plays a role in the conversion of inactive indole- β-glucosides to active auxin (Estruch *et al.*, 1991). Although this has not been observed *in vivo*, *rol*B cloned into *Medicago sativa* and apple rootstock M26 induced an auxin-like response (Frugis *et al.*, 1995; White *et al.*, 1985). In *Escherichia coli*, r*ol*C codes for a cytokinin- β-glucosidase that converts cytokinin- β-glucosides into free cytokinin (Estruch *et al.*, 1991). In spite of this, it is believed that *rol*C does not necessarily hydrolyse the cytokinin glucosides *in planta*, but may in fact alter the metabolism of cytokinins directly, or cause such an effect as a results of its own expression in the host (Faiss *et al.*, 1996; Nilsson *et al.*, 1996). Unlike the *rol*ABC genes, *rol*D is thought to play a role in root elongation rather than initiation (White *et al.*, 1985), possibly through the activity of the encoded ornithine cyclodeaminase (OCD) which converts ornithine to proline. As high levels of proline have been found to accumulate in growing maize primary roots (Verslues and Sharp, 1999), Trovato *et al.* (2001) have proposed that increases in proline levels as a result of OCD activity, may alter the synthesis of hydoxyproline-rich glycoproteins, some of which are involved in control of cellular division and extension (Varner and Lin, 1989). They also proposed that since high levels of a polyamine, putrecine, are known to reduce root growth (Masgrau *et al.*, 1997), OCD may

enhance root growth by decreasing the levels of ornithine, which is a precursor of the polyamine pool.

Although the exact mechanism by which *rol*ABCD genes exert their effects *in planta* is not yet fully understood, it is clear that these genes cause changes in the levels of, and sensitivity to, phytohormones in the host cells. Changes would typically include an outgrowth of adventitious roots (Ricker *et al.*, 1930), but phenotypes such as plant dwarfing have also been observed (Handa *et al.*, 1995).

1.7.4 Plant hairy root cultures

Several properties unique to hairy roots cultures have contributed to the growing interest in them. Among these properties are greater genetic and biosynthetic stability, as well as growth rates occasionally superior to those of undifferentiated cells (Signs and Flores, 1990; Maldonado-Mendoza *et al.*, 1993; Lipp Joao and Brown, 1994). Hairy root cultures are also easier and cheaper to maintain as they can grow indefinitely on hormone-free culture media (Giri and Narasu, 2000). Consequently, hairy roots have been used as model systems with which to study either root metabolism (Shanks and Morgan, 1999) or their potential use in phytoremediation (Bhadra *et al.*, 1999). Of greater interest however, has been their potential as a source of valuable secondary metabolites.

1.7.5 Hairy root cultures as sources of medicinal compounds

Hairy roots are able to synthesize metabolites characteristic of the infected plant (Signs and Flores, 1990). Although this has also been reported for plant cell and organ cultures, the poor biosynthetic stability of the former, slow growth rate of both, and the requirement of media supplemented with phytohormones, has supported the use of hairy root cultures as

superior sources of plant metabolites (Giri and Narasu, 2000; Rao and Ravishankar, 2002). The production of metabolites parallels growth, unlike the case in plant cell cultures (Bourgaud *et al.*, 2001), and hairy roots have also become especially useful as sources of metabolites with pharmacological properties. Several hairy root cultures, some of which are listed in Table 1, have already been used extensively as sources of compounds with medicinal properties.

Table 1.1 Pharmacologically active metabolites produced in hairy root cultures

Plant	**Metabolite**	**Function**	**References**
Drosera rotundifolia L.	Juglone	Antifungal	Seigler (1998)
Papaver somniferum L.	morphine	Analgesic	Park and Facchini (2000)
Lithospermum erythrorhizon	Shikonin	Antitimour	Brigham *et al.* (1999)

Although the synthesis of metabolites is genetically controlled, the manipulation of several external factors has been used to improve the yield of particular, sought-after compounds. Using an empirical approach, abiotic factors such as incubation temperature, light, and culture medium composition were found to have an effect on the hairy root growth rate and the synthesis of particular metabolites (Rhodes *et al.*, 1994; Nussbbaumer *et al.*, 1998).

The ability of hairy roots to respond to pathogenic attack by secreting protective metabolites into the rhizosphere has also been exploited in attempts to improve the recovery of specific products from the growth medium itself, as opposed to from the roots. Both abiotic and biotic elicitors have been used to stimulate this defensive response. One well documented example occurs in the hairy roots of *L. erythrorhizon*, where a small

group of antibacterial naphthoquinones known as shikonins, accumulated during unstressed conditions in the root hairs and root border cells, but upon stimulation with a fungal elicitor, increased significantly and were secreted into the growth medium by the root hairs and epidermal cells (Brigham *et al.*, 1999).

Plant metabolic engineering has also been used in attempts at improving the synthesis of secondary metabolites with medicinal properties. With a growing number of metabolic pathways being elucidated, single genes have been used to alter metabolic pathways, or preferentially change certain secondary metabolites within hairy root cultures (Giri and Narasu, 2000). One such example is the increased conversion of hyoscyamine into the more pharmacologically active compound scopolamine in *Atropa belladonna* hairy roots transformed with the hyoscyamine 6β-hydroxylase gene from *Hyoscyamus niger* (Yun *et al.*, 1992). Alternatively, in cases where several pathways may originate from one compound, the metabolic flux has been geared and directed towards the synthesis of a particular product, as was shown during the synthesis of berberine in *Coptis* cells (Sato *et al.*, 2001). Although this was not performed in hairy root cultures as such, it is speculated that such findings may be used to improve the synthesis of pharmaceutical compounds in hairy root cultures.

From the evidence available, it ought to be possible to generate hairy root cultures using *E. natalensis* explants. As a source of medicinal compounds, the consensus is that these cultures are a better *in vitro* system than plant cell cultures. Hairy root cultures of *E. natalensis* take on a greater significance since there are a number of factors that can be explored and manipulated so as to possibly enhance the synthesis of diospyrin, and therefore provide an alternative, sustainable source of this very important compound.

Aims and objectives

There is clearly a need for sustainable sources of diospyrin, sources that are an alternative to the harvesting of mature roots of *Euclea natalensis*. The aim of this study, therefore, was to: a) explore *E. natalensis* seedlings and calli for potential antimycobacterial properties, and diospyrin production, and b) attempt to generate hairy roots, and explore their diospyrin-producing potential if successfully generated.

Chapter Two

Screening of *Euclea natalensis* seedlings and calli for antimycobacterial activity, and for accumulation of diospyrin.

2.1 Introduction

The reappearance of tuberculosis (TB) and the growing incidence of multi-drug resistant tuberculosis (MDR-TB) due to the failure of patients to comply with treatment, or because the incorrect treatment regimen is prescribed, has prompted the South African government to identify the disease as a national health priority (WHO Report, 2002; WHO Report, 2004). In South Africa, 1.5% of new TB cases are MDR, and globally the country is ranked 9th with respect to new cases of TB. Much of the local rise in TB is attributed to the increased incidence of Human Immunodeficiency Virus (HIV), and as a result, 60% of TB patients are co-infected with HIV (WHO Report, 2004). Due to the high mortality rate of TB patients co-infected with HIV, coupled with an increase in infections resulting from sensitive and MDR strains, the annual global estimate of three million deaths resulting from TB is most likely to increase (Corbett *et al.*, 2003; WHO, 2003).

Historically, Southern Africans have used medicinal plants to cure or alleviate the symptoms associated with various diseases, and 75% of the population continues to rely heavily on such remedies (Mander, 1998). One such medicinal plant used to treat the symptoms associated with TB is *Euclea natalensis* (A. DC.), and the source of successful antimycobacterial activity has been attributed to diospyrin, a naphthoquinone isolated from

the mature roots of the tree. In addition to being toxic towards sensitive and MDR strains of *Mycobacterium tuberculosis* (Lall and Meyer, 2001), anti-malarial and anti-cancer properties have also been attributed to the activity of diospyrin (Hazra *et al.*, 1994; Hazra *et al.*, 1995). The levels of diospyrin in mature roots vary seasonally and can range between 0.1 to 1%, on a dry mass basis (van der Kooy, pers. comm., 2005). Although it is theoretically possible to produce the compound synthetically, the method is complex (Yoshida and Mori, 2000). The method of choice for obtaining diospyrin remains harvesting either the root or bark of the tree, an approach that is clearly destructive and unsustainable.

E. natalensis grows easily from seed (Pooley, 1993), but it is not known whether the seedlings themselves possess the same antimycobacterial activity as the mature roots, and so whether they could serve as a potential, sustainable, source of diospyrin. To date, a method for successfully inducing callus growth on *E. natalensis* explants has still not been reported, and the use of *E. natalensis* plant cell cultures as a sustainable source of diospyrin has also not been explored. Such cultures have been documented to accumulate secondary metabolites characteristic of the intact plant, and often in much greater quantities (Fujita, 1988; Park *et al.*, 1992). They are also amenable to manipulations designed to enhance the levels of secondary metabolites (Dörnenburg and Knorr, 1995), and so offer an attractive potential source of diospyrin worthy of exploring.

To accommodate the interest and demand for diospyrin, the aim of this study was to assay all *E. natalensis* seedling organs for antimycobacterial activity, and if present, to determine whether any of the antimycobacterial activity can be attributed to an accumulation of diospyrin. The study also aimed to develop a method for production of calli using various

explants of *E. natalensis*, and if successfully generated, explore the diospyrin-producing capacity of the calli.

2.2 Materials and methods

2.2.1 Plant material and extract preparation

Seeds of *E. natalensis* obtained from Silverhill Seeds (Cape Town) were placed in potting soil (mixed with peat and vermiculite) and germinated in the dark at 30 °C, and then allowed to grow further in a greenhouse for 5 months. The explants used to initiate callus cultures were obtained from three to five month old seedlings. Root, stem and leaf material were selected from healthy seedlings and sterilised as follow: all explants were sequentially surface sterilised in 70% ethanol, 0.3% mercuric chloride (w/v), 3.05% commercial bleach solution containing 0.65% (v/v) Tween-20 (George and Sherrington, 1984), and then rinsed in sterile distilled water. Leaf material was placed in each sterilant for three minutes, whereas six minutes were used for stem and root explants. All sterilisation and calli maintenance procedures were performed aseptically under laminar air flow.

2.2.2 Establishment of *in vitro* calli

Attempts to establish *E. natalensis* calli used media as formulated by Murashige and Skoog (MS) (Murashige and Skoog, 1962), Yokoyama and Takeuchi (YT) (Yokoyama and Takeuchi, 1976) and Fujii and Nito (FN) (Fujii and Nito, 1972) (the latter two amended with 50 g l^{-1} sucrose). Each medium was modified so as to contain the phytohormones listed in Table 2.1. In spite of the pronounced association of 2,4- dichlorophenoxyacetic acid (2,4-D) with inhibition of secondary metabolite synthesis (Rao and Ravishankar,

2002), the growth regulator was included as it induces dedifferentiation (DiCosmo and Misawa, 1995), and in cultures of *Macuna pruriens* (Brain, 1976) and *Oxalis linearis* (Meyer and van Staden, 1995) it promotes production of anthocyanins and L-DOPA, respectively.

Once sterilised, the explants were transferred onto each callus-inducing medium and incubated in the dark at 25 °C, with regular subculturing (George and Sherrington, 1984) every second week. Once calli were visible, they were severed from the explants and transferred to a similar, fresh medium for two to three months. Thereafter, the calli growing in a particular medium were pooled, frozen overnight at -80 °C and freeze-dried (Labotec Dry-O-Vac), until assayed quantitatively for diospyrin content.

Table 2.1 Calli induction media. Each culture medium, except MS, contained various combinations of plant growth regulators (2, 4-D; KIN, kinetin; NAA, naphthylacetic acid).

Culture medium	Phytohormone used	Phytohormone variation (mg l^{-1})					
FN	Adenine	5	5	5	1	2	5
	2, 4-D	0	1	5	5	4	8
YT	NAA	3	0.1	3	0		
	KIN	0.1	3	0	0.1		
MS	2, 4-D	2.5					

2.2.3 Preparation of calli and seedling chloroform extracts

Three month-old brown callus tissue cultured on MS and FN media were separately homogenized and macerated in 50 ml $CHCl_3$ (3X) at ambient temperature. These

chloroform extracts were then filtered (Advantec filter paper) and partially purified (3X) by fractionating each chloroform extract with water. The chloroform portions were then collected and concentrated *in vacuo* at 40 °C to yield dry chloroform extracts of calli cultured on MS and FN medium, which were subsequently assayed for diospyrin accumulation. The leaves, stems and roots of 13 four month-old seedlings were separated, frozen in liquid nitrogen and freeze-dried. Thereafter, a chloroform extract of these organs was also prepared as already discussed.

2.2.4 Bacterial preparation

A culture of *Mycobacterium aurum* was initiated and maintained by regular transfer and incubation at 37 °C in fresh 2 x Yeast Tryptone broth (2YT) (Miller, 1972). Prior to using the cultures in the various assays, a Gram-stain (Prescott *et al.*, 1996c) was used to ascertain the absence of contaminating bacteria.

2.2.5 Disk diffusion assay

A paper disk imbued with sample extract was placed on a plate of agar inoculated with the test organism. The contents of the extract then diffused radially away from the centre of the disk creating a concentration gradient that is strongest closest to the disk. During incubation growth of the test organism was inhibited away from the centre of the disk to a point where the concentration of the extract was no longer inhibitory to the organism. This created a ring of no bacterial growth around the disk, the length of which reflected the sensitivity of the organism towards the extract, and how easily the extract diffused across the agar (Prescott *et al.*, 1996d).

A culture of *M. aurum* was grown overnight in 2YT at 37 °C to a density of 0.5 (A_{600nm}) (DU-64 Spectrophotometer, Beckman). This culture (120 µl) was then used to inoculate 6 ml of molten 2YT medium solidified with 0.7% (w/v) bacteriological agar (Merck). The sloppy medium was overlaid and allowed to set over 2YT solidified with 1.5% (w/v) bacteriological agar. Sterile filter-paper disks of approximately 6 mm in diameter (Whattman) were impregnated with 10 µl of 20 mg ml^{-1} of each seedling extract, dried and placed on the plates. The plates were then incubated at 37 °C for five days, after which a measurement of the zones of inhibition (difference between the inhibition diameter and the paper diameter divided by two) was made. Filter-paper disks imbued with 2 µg ml^{-1} rifampicin and disks imbued with evaporated acetone were used as controls. For each extract and control tested, the assay was performed three times, and from each replicate an average diameter of inhibition and standard deviation was obtained.

2.2.6 Bioautographic assay

This assay relied on the interaction of living cells with a tetrazolium salt. Initially, the constituents of the sample extract were separated on a TLC plate according to their partition coefficience values, and a thin film of the test organism was sprayed over the plate. The compounds that are toxic to the test organism were viewed after addition of the tetrazolium salt, which in the absence of viable reducing enzymes, remains colourless. In the presence of living cells the salt was converted into a purple formazan crystal.

For this assay the constituents of each seedling extract were separated on Silica gel F_{254} TLC plates (Merck) using chloroform-hexane (1:1). In order to assign the antimycobacterial activity to the presence of diospyrin, a modified version of the bioautographic TLC assay developed by Lund and Lyon (1975) was used. In this case, a

freshly grown 5 ml suspension of *M. aurum* (A_{600} 0.5) was sprayed over a developed TLC plate (7 x 8 cm) and incubated overnight at 37 °C under humid conditions. Subsequently, a 5 ml solution of 1.5 mg ml^{-1} 3-(4, 5-dimethyl-2- thiazolyl (- 2, 5- diphenyl-2H- tetrazolium bromide (MTT) was sprayed onto the plates which were then incubated at 37 °C for 30 minutes before assessing the bioautograms. Over three replicates were used to localise the antimycobacterial activity on the bioautograms.

2.2.7 Quantitative analysis of diospyrin in seedlings and calli extracts

Diospyrin concentration was determined in each of the various seedling organs, and calli grown on MS and FN media using a reverse phase Luna C_{18} HPLC column (150 x 4.6 mm; particle size 5 µm), after calibrating the column with 0.01 to 5 µg of authentic diospyrin. The seedling and callus-chloroform extracts were dissolved to 1 - 2 mg ml^{-1} in acetonitrile, of which 10 µl was injected into the column. The samples were then eluted with acetonitrile:water:methanol (12.5:6.5:1) at a flow rate of 1 ml min^{-1} and detected at 254, 325 and 430 nm (Thermoquest UV6000LP PDA).

2.2.8 Purification, isolation and characterisation of diospyrin

The chloroform extracts from calli grown on MS and FN media were pooled, and purified further by eluting through Silica gel 60 columns (hexane-ethyl acetate 5:1). The chromatographic fractions containing diospyrin were identified based on sample co-migration with authentic diospyrin on Silica gel F_{254} TLC plates (hexane-ethyl acetate 5:1), combined and concentrated to dryness under vacuum. Purification was completed at room temperature (RT) by repeated crystallisation of diospyrin upon addition of 2 ml of methanol to the dry chloroform extract. Diospyrin crystals were collected on a Pasteur pipette plugged with cotton wool, and then eluted by dissolving the crystals in chloroform.

The identity of the purified compound was confirmed as diospyrin using mass measurements obtained on an electron spray ionisation mass spectrometer (ESI-MS) (Waters API Q-TOF Ultima) set on negative mode. Prior to this analysis, the purified chloroform extract was dissolved in acetonitrile and analysed using a Waters Alliance 2690 Gradient HPLC system by injecting 10 µl into a 30 X 2 mm Phenomenex Gemini 5 µm C_{18} reverse phase column. Acetonitrile and water were used as the mobile phase using conditions as follows: a linear gradient of acetonitrile:water (2:98%) to acetonitrile (100%) for 30 min, followed by 100% acetonitrile isocratically for 5 min, then a linear gradient of acetonitrile (100%) to acetonitrile:water (2:98%) for 5 min, and finally acetonitrile:water (2: 98%) isocratically for 5 min using a flow rate of 1 ml min^{-1}. The parameters of the MS were set to: capillary voltage 2.5Kv; cone voltage 35, desolvation temperature 350 °C; desolvation gas 400 l h^{-1}. The identity of the purified compound was also confirmed by dissolving it in deuterochloroform ($CDCl_3$) and measuring its ^{1}H-NMR data (Varian Mercury Plus) using 200 MHz, and tetramethylsilane (TMS) as an internal standard.

2.3 Results and discussion

2.3.1 Establishment of *in vitro* calli

Of the 22 media used, attempts to induce *E. natalensis* callus cultures were successful only when FN and MS media variations were used. Seedling stem and root explants were both able to produce calli on at least two types of FN media supplemented with 5 mg l^{-1} adenine and either 1 or 5 mg l^{-1} 2, 4-D. Due to the higher levels of microbial contamination associated with stem and root explants, no further attempts to generate calli were made using these as explants. Leaves only, were subsequently used, and calli were successfully

induced when FN media supplemented with 5 mg l^{-1} adenine and 5 mg l^{-1} 2, 4-D, as well as MS media containing 2.5 mg l^{-1} 2, 4-D were used (Figure 2.1). Calli emerged two and a half weeks after transfer onto MS media, but in FN media calli were only observed after four weeks. In addition, calli grown on MS grew faster and were also larger than those growing on FN media. All the calli cultured were initially translucent, but turned brown within three months of initiation, possibly as a result of metabolite accumulation, especially phenolics. This effect decreased slightly when the calli were subcultured more regularly. This is the first documented report of successful initiation of *E. natalensis* callus cultures.

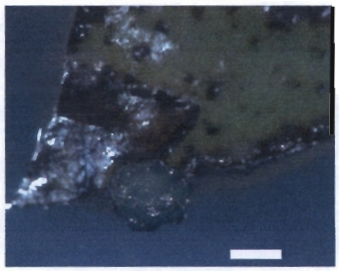

Figure 2.1 Calli induction on an *E. natalensis* leaf explant placed on MS media supplemented with 2.5 mg l^{-1} 2, 4-D. The bar represents 1 mm.

2.3.2 Disk diffusion assay

This assay is widely used to give a rapid indication of the overall antimicrobial activity of an extract towards an organism. Instead of *M. tuberculosis*, *M. aurum* was used as the test organism due to its faster growth rate, non-pathogenic nature, and because its drug susceptibility profile reflects that of *M. tuberculosis* (Chung *et al.*, 1995). The antimycobacterial activity of extracts of different parts of *E. natalensis* seedlings were determined five days after incubation, and directly compared to the activity of rifampicin at its minimum inhibitory concentration (MIC) against *M. tuberculosis* (Chung *et al.*, 1995). All extracts tested were able to inhibit the growth of *M. aurum*, although to different degrees (Figure 2.2). There were no statistical differences between the inhibitory effect of

the root extract, and that of rifampicin. Although the inhibitory effect of the stem and leaf extracts were lower than that of rifampicin, all three seedling extracts successfully demonstrated antimycobacterial activity against *M. aurum*.

This indicated that each of the extracts contained compounds that were probably active against *M. tuberculosis*. The obvious limitation with this assay lies in its inability to attribute the activity towards any particular compound. This means that although it was entirely possible that diospyrin may have been the cause of the activity, it could just as well have been due to any of the naphthoquinones, terpenoids, or triterpenoids commonly found in *Euclea* species, and known to be biologically active against several microorganisms (Watt and Breyer-Brandwijk, 1962; Khan *et al.*, 1978).

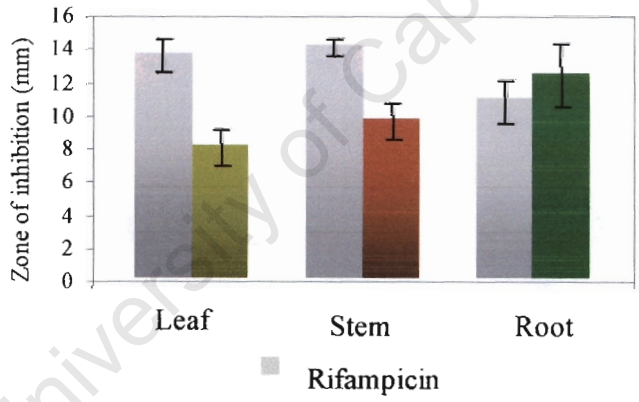

Figure 2.2 Inhibitory capacity of various parts of *E. natalensis* seedlings. Bars represent the diameter of the zone of inhibition around the discs used in the disc diffusion assay. The concentration of the seedling extracts and rifampicin control are 20 mg ml^{-1} and 2 µg ml^{-1}, respectively. Values represent the average of three replicates.

2.3.3 Bioautographic assay

The chromatographic separation of the molecular constituents of each seedling extract, followed by antimycobacterial tests on TLC plates, revealed the presence of a region of

activity against *M. aurum*. With an R_f value of 0.3, and under conditions as described by Lall and Meyer (2001), this activity was preliminarily confirmed as due to the presence of diospyrin in each of the extracts (Figure 2.3, A). Given that 390 µg of each extract was tested, the size of the zones of inhibition obtained on the plates suggests that there were higher levels of diospyrin in root extracts, followed by stem and then leaf extracts. These findings suggest that diospyrin is predominately accumulated in the stem and roots of the plant, as in mature trees (van der Vivjer and Gerritsma, 1974). In addition to diospyrin, other antimycobacterial compounds were found primarily in stem and root extracts (Figure 2.3, B) of a second cohort of seedlings, which probably explains the higher inhibitory capacity of these extracts observed during the disk diffusion assay.

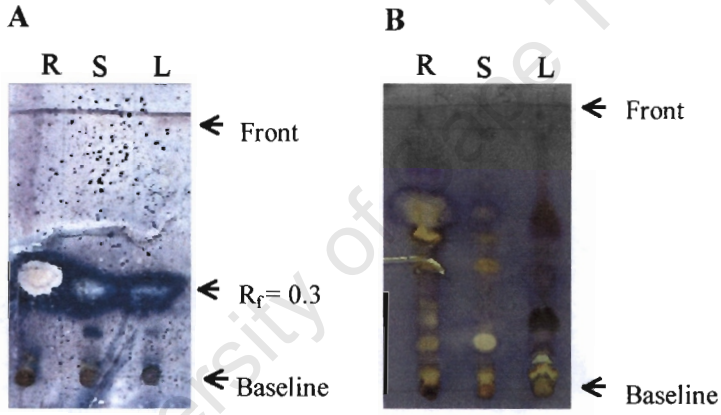

Figure 2.3 Silica gel 60 bioautograms of crude extracts of *E. natalensis* seedlings with regions of toxicity towards *M. aurum* visible as clear spots, and conversion of MTT into purple formazan crystals where bacterial viability is unaffected by the extract used. (A) Separation of compounds using chloroform-hexane (1:1), and inhibition of *M. aurum* growth attributed most likely to diospyrin. (B) Separation of compounds using chloroform and antimycobacterial compounds other than diospyrin in *E. natalensis* seedling extracts. R, root; S, stem; L, leaf.

2.3.4 Quantitative analysis of diospyrin in calli and *E. natalensis* seedling organs

The accumulation of diospyrin by the seedling organs and calli was confirmed using a reverse phase C_{18} HPLC column. Polar compounds present in the chloroform extract of the seedlings and calli were removed by partitioning repeatedly into water (3 x 80 ml volumes). In all the extracts analysed, HPLC analysis yielded a group of naphthoquinones, one of which was confirmed to be diospyrin by virtue of a comparison of retention time with that of an authentic sample (Figure 2.4). *E. natalensis* seedlings and calli were therefore clearly able to synthesize and accumulate diospyrin, with calli induced on MS (5.2%) and FN (1.2%) media doing so more successfully than any of the seedling organs (Table 2.2).

The amount of diospyrin in the different seedling organs falls within the range reported for mature roots (0.1 to 1%, DW) (van der Kooy, pers. comm., 2005). As environmental and seasonal variations are thought to influence the levels of diospyrin in mature roots, it is possible that such factors may also have had an impact on the levels of diospyrin found in the seedlings assayed. The distribution of diospyrin in the seedlings reflects that reported for mature trees, where the compound is predominantly found in the roots and bark, but little is found in leaves (van der Vijver and Gerritsma, 1974), suggesting therefore that the biosynthetic or accumulation potential of diospyrin in the seedlings is as it is in mature trees.

Greater levels of diospyrin accumulated in undifferentiated tissue compared to those amounts found either in the seedlings (Table 2.2), or reported for mature *E. natalensis* roots (0.1 to 1%, DW). This suggests that the synthesis of diospyrin is not dependent on the differentiated state, and in fact, the undifferentiated state may enhance the synthesis or the potential to accumulate the compound, possibly due to the absence of regulatory factors

associated with the differentiated state and responsible for regulating the levels of diospyrin within reported values.

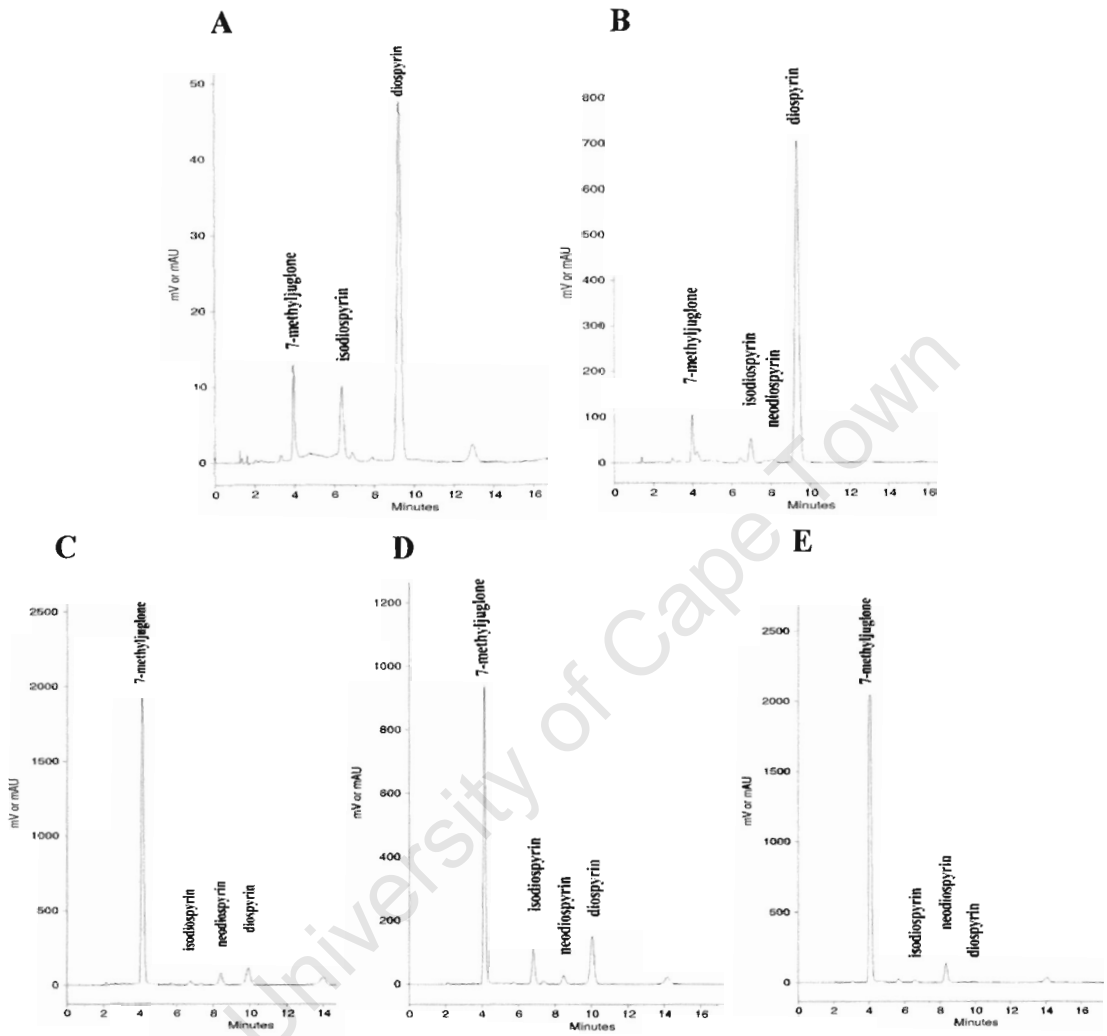

Figure 2.4 HPLC chromatograms of chloroform extracts of calli induced on FN (A) and MS (B) tissue culture medium, using *E. natalensis* seedling leaf explants; and chromatograms of root (C), stem (D), and leaf (E) chloroform extracts illustrating the presence of diospyrin and other naphthoquinones, which are labelled. Compounds were detected at 430 nm.

Table 2.2 Concentration of diospyrin and other naphthoquinones in four-month old *E. natalensis* seedling organs and three-month old calli. Levels of diospyrin in mature roots range between 0.1 and 1% (DW). The standard curves required to calculate the amounts of the other naphthoquinones were kindly provided by van der Kooy (2005). The percentages were calculated on a dry mass basis.

E. natalensis sample	Diospyrin	7-methyljuglone	Isodiospyrin	Neodiospyrin
	%			
Leaf	0.006	0.2	0.08	0.18
Stem	0.23	0.56	0.75	0.22
Roots	0.17	0.84	0.35	0.53
Calli (on FN media)	1.2	0.39	3.55	1.16
Calli (on MS media)	5.2	0.56	2.91	0.84

FN, Fujii and Nito; MS, Murashige and Skoog

Between the calli grown on MS and FN media there were also differences in the amount levels of diospyrin accumulated, with calli grown on MS accumulating four times more diospyrin than those grown in FN media (Table 2.2). Such variations were probably due to the effect of the tissue culture medium on the rate of calli growth, and on their ability to synthesize secondary metabolites. *E. natalensis* calli were observed to grow much faster on MS compared to FN media, which implies that calli grown on MS media would probably have entered a stationary growth phase earlier than calli grown on FN media. As is the case in suspension cultures, once within the stationary phase, cellular metabolism changes from primary metabolism, which occurs during the exponential growth phase, to synthesis of secondary metabolites (Bourgaud *et al.*, 2001). Since chloroform extracts were prepared using calli up to three months-old, the higher levels of diospyrin in calli grown on MS media may have been due to a greater number of these being in the stationary phase, in which case they would presumably have synthesized and accumulated more secondary

metabolites such as diospyrin. The nutrients and phytohormones incorporated into a culture medium also have an effect on the accumulation of secondary metabolites (Rao and Ravishankar, 2002). The effect of these is complex and dependent on the metabolite synthesized as well as the particular cell culture used; therefore an empirical approach is necessary to study the factors which contribute to enhanced levels of any particular secondary metabolite. Suffice to say however, that unlike FN, MS is a high salt media (Dixon, 1985), and the presence of higher levels of ammonium nitrogen in the medium may have resulted in higher levels of naphthoquinones in calli grown on MS, as is reported to occur in *Fusarium* (Baker *et al.*, 1981).

In *E. natalensis* calli and each of the seedling organs, diospyrin extractions revealed the presence of other naphthoquinones such as 7-methyljuglone, isodiospyrin and neodiospyrin, all of which are typically present in the family Ebenaceae (van der Vijver and Gerritsma, 1974; Wube *et al.*, 2005) (Figure 2.4, A, B, C, D). While the main focus of this study was the diospyrin production ability of seedlings and calli, the presence of these compounds in both sources is also important. Like diospyrin, isodiospyrin and neodiospyrin are dimers of 7-methyljuglone, the precursor (Thomson, 1971; Wube *et al.*, 2005).

The amount of each compound obtained in seedling organs and calli indicates that there is a complex relationship between the precursor and diospyrin, its isomer, isodiospyrin, and neodiospyrin. However, by analysing the amounts of 7-methyljuglone and diospyrin, it is apparent that there is a consistent inverse relationship between the two compounds. Although the amounts of diospyrin are inversely proportional to those of 7-methyljuglone, this relationship is not indicative of the amounts of diospyrin likely to be found in relation to the amounts of 7-methyljuglone either in the seedling organs or calli (calli grown on FN

media, in comparison to those grown on MS media, have less 7-methyljuglone, but also less diospyrin) (Table 2.2). The complexity in the relationship between 7-methyljuglone and diospyrin, isodiospyrin, and neodiospyrin may reflect the metabolic events which regulate the synthesis of these compounds, and may have been influenced by the synthesis of other dimers of 7-methyljuglone, for example, mamegakinone, bis-isodiospyrin, and elliptinone (Thomson, 1971; van der Vijver and Gerritsma, 1974), all of which are typically synthesized in Ebenaceae plants, but were not analysed in the seedlings and calli.

Isodiospyrin, 7-methyljuglone and neodiospyrin are also important because they display antimicrobial properties. The activity of these naphthoquinones against *M. tuberculosis* is not known, but isodiospyrin has antimycobacterial activity against *M. chelonae* (Adeniyi *et al.*, 2000), whereas 7-methyljuglone and neodiospryin have antibacterial and anticancer properties, respectively (Khan *et al.*, 1978; Wube *et al.*, 2005).

2.3.5 Purification and characterization of diospyrin

Purification of the chloroform extract derived from *E. natalensis* calli and submitted to HPLC analysis yielded a major peak (retention time of 14.38 min), presumed to be diospyrin (authentic diospyrin was not subjected to this HPLC analysis or the subsequent mass spectral analysis), and a few other compounds (Figure 2.5, A). The UV-VIS spectrum of the major peak exhibited absorption maxima (λ_{max} in acetonitrile) at 220 nm, 253.35 nm and 433.35 nm, which is typical of naphthoquinones (Harborne, 1998) (Figure 2.5, B).

Further analysis of this peak using ESI-MS (negative mode) revealed a $[M\text{-}H]^-$ ion at 373.47 (75%) *m/z*, and since diospyrin has a calculated molecular mass of 374 daltons, this indicated that the peak was that of diospyrin (Ravishankara *et al.* 2000). Another peak was also obtained at 374.47 (90%) *m/z*, possibly as a result of ionization of diospyrin molecules containing ^{13}C instead of ^{12}C (Figure 2.5, C).

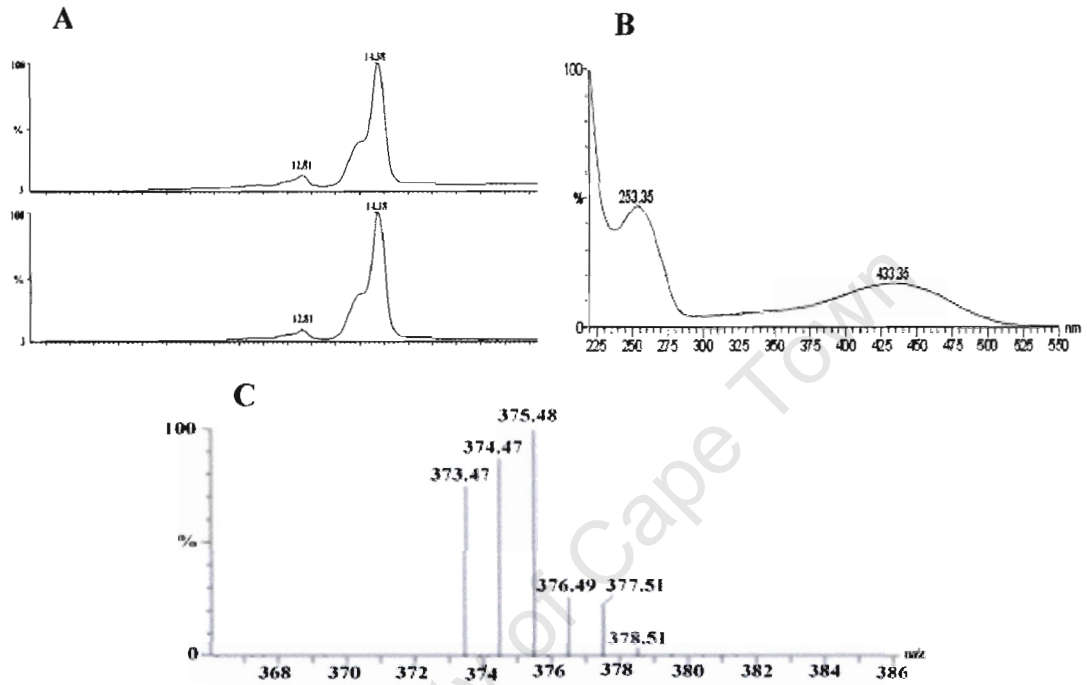

Figure 2.5 Physical measurements of the purified chloroform extract. (A) HPLC analysis of the chloroform extract showing presumably diospyrin (retention time of 14.38 min) as the major peak, in addition to other compounds also present (top chromatogram detected at 254 nm, bottom chromatogram detected at 438 nm). (B) Absorption spectra of the major peak in acetonitrile. (C) Electron spray ionization mass spectrometric (negative mode) analysis of the major peak.

Since the diospyrin crystallized from the chloroform extract was not fully purified, further crystallizations and ^1H NMR analysis were necessary to exclude the possibility that the $[M\text{-}H]^-$ ion at 373.47 *m/z* was due to possible traces of isodiospyrin or neodiospyrin, both of

which have the same molecular mass (374 daltons, $C_{22}H_{14}O_6$) and can potentially ionize to produce the 373.47 ion (Thompson, 1971; Wube *et al.*, 2005). However, the ^{1}H NMR spectrum (Figure 2.6) showed chemical shifts different from those caused by isodiospyrin (Fallas and Thomson, 1968) and neodiospyrin (Wube *et al.*, 2005), but similar to those already reported for diospyrin (Yoshida and Mori, 2000).

Thus the identity of the compound tentatively identified after HPLC analysis as diospyrin, was unequivocally confirmed as the important compound, diospyrin using MS and NMR spectral analysis.

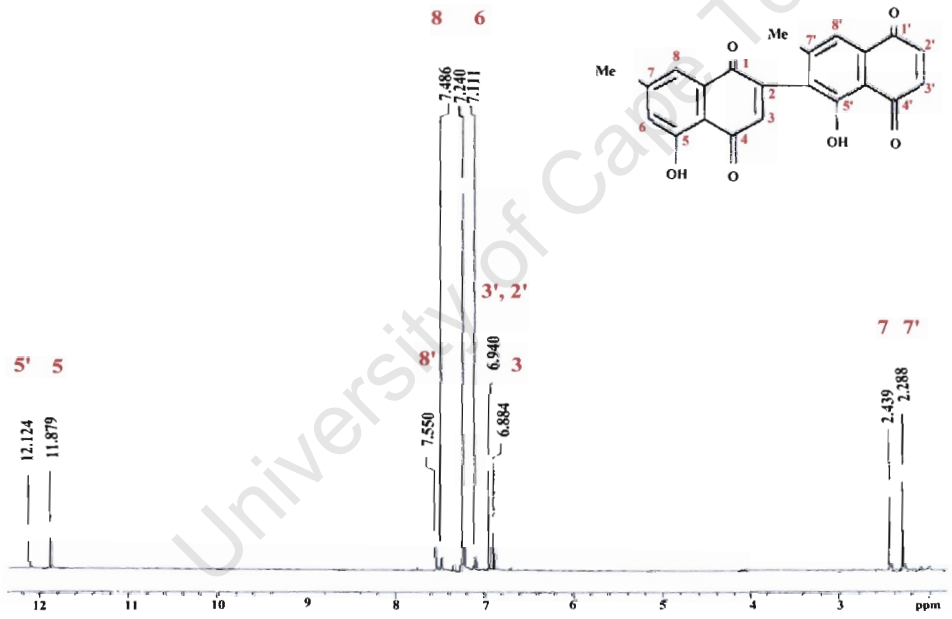

Figure 2.6 ^{1}H NMR spectral data of diospyrin isolated from calli generated on *E. natalensis* leaf explants (200 MHz, $CDCl_3$). The chemical shifts caused by each proton indicated in red on the molecule are indicated on the spectrum. The chemical shift caused by the solvent is δ 7.24. ppm, parts per million.

2.4 Conclusion

Chloroform extracts of all the various parts of *E. natalensis* seedlings showed activity against *M. aurum*, and the assumption was made that the antimycobacterial activity was due to the presence of diospyrin. This was confirmed when diospyrin was found to accumulate predominantly in the root and stem of the seedlings, along with other antimycobacterial compounds. Given the ease with which the seeds are able to germinate, and the ability of the seedlings to successfully synthesize and accumulate diospyrin and its precursor, 7-methyljuglone, they represent a potential source of the compound that is sustainable.

Using *E. natalensis* leaf explants, callus cultures were also successfully obtained on modified FN and MS tissue culture media. This is the first report of successful callus production using explants of *E. natalensis*. The calli were confirmed to accumulate diospyrin using HPLC, MS and ^{1}H NMR spectral data, at levels much greater than both those found in the seedlings, and those reported for mature roots. Part of this ability lies perhaps in the metabolic nature of undifferentiated *E. natalensis* cells, although tissue culture media formulation can be used to enhance the levels of diospyrin. With the ability to synthesize diospyrin, the enhanced synthesis capacity, and potential response to specific media formulations, *E. natalensis* calli also represent an additional potential sustainable source of diospyrin that is also sustainable.

Chapter Three

Agrobacterium rhizogenes-mediated transformation of *Euclea natalensis*, and diospyrin production.

3.1 Introduction

Hairy root cultures that are formed after transformation with *Agrobacterium rhizogenes* have emerged as a powerful alternative source of secondary metabolites with medicinal properties. These cultures are able to accumulate secondary metabolites that are characteristic of the intact plant, and are also able to secrete these important compounds into the surrounding growth medium (Brigham *et al.*, 1999; Giri and Narasu, 2000). When compared to undifferentiated plant cell cultures, hairy root cultures are biosynthetically and genetically more stable, and they have occasionally been reported to produce secondary metabolites in quantities greater than those in cell cultures, and intact roots (Giri and Narasu, 2000; Flem-Bonhomme *et al.*, 2004).

The hairy root syndrome is caused by the integration and expression of root loci (*rol*ABCD) encoded on the T-DNA of the bacterium's Ri plasmid (White *et al.*, 1985). The growth of adventitious roots, which are heavily branched and display altered geotropism (Chilton *et al.*, 1982) generally serve as confirmation that successful transformation events have taken place. The presence of opines, resulting from the expression of opine biosynthesis genes in the T-DNA, has also been used to confirm the transformation. Even

more conclusive results however, are obtained using molecular techniques such as Southern blot and Polymerase Chain Reaction.

An indigenous tree (*Euclea natalensis*) currently used to treat symptoms related to tuberculosis (TB) (Khan *et al.*, 1978) is faced with potential threat, since the medicinally active compound, diospyrin, is found in mature roots and the bark of the tree only (van der Vijver and Gerritsma, 1974; Lall and Meyer, 2001). Attention has therefore turned to alternative, sustainable sources of this important naphthoquinone. Success has already been achieved with *E. natalensis* seedlings and calli using *E. natalensis* leaf explants (Chapter two). Hairy roots however, and the potential they present for increased secondary metabolite production which they present, have yet to be explored as a sustainable, alternative source of diospyrin.

Attempts to infect *E. natalensis* or other members of the Ebenaceae family with *A. rhizogenes* have yet to be documented, but the prospect of successfully transforming *E. natalensis* with *A. rhizogenes* is supported by the fact that transformation of hundreds of dicotyledonous plants, in different genera and families, has been successful (Porter, 1991). In addition, De Cleene and De Ley (1981) suggested that plant families that accumulate phenolics are probably more susceptible to *A. rhizogenes*, and members of the Ebenaceae family are particularly known to accumulate phenolic compounds in the form of napthoquinones (Van der Vijver and Gerritsma, 1974; Mallavadhani *et al.*, 1997).

The aims of this chapter therefore were to establish hairy root cultures of *E. natalensis* using various seedling explants together with a number of strains of *A. rhizogenes*, and if established, to determine whether, and to what degree, diospyrin production was possible.

3.2 Materials and methods

3.2.1 Plant material and sterilisation

Seeds of *E. natalensis* were obtained from Silverhill Seeds and germinated as previously described. After the sterilisation procedure described below, seeds were germinated *in vitro* in jars containing 50 ml MS (Murashige and Skoog, 1962) medium solidified with 0.9% agar (Sigma), and incubated in the dark at 25 °C, until hypocotyls emerged. Thereafter, the seedlings were grown under constant light intensity of 70 μmol s^{-1} m^{-2} (Li-Cor light meter, Li-Cor, USA) at 25 °C.

To generate sterile plant material, the seeds to be germinated *in vitro* were sequentially placed in 70% ethanol, 0.3% (w/v) mercuric chloride, 3.05% commercial bleach solution containing 0.65% (v/v) Tween-20 for five minutes in each sterilant, and rinsed thoroughly in sterile distilled water. Six to eight month-old greenhouse-grown saplings were also utilised as explants. These were sterilised following the same procedure as outlined above, except that the time in each sterilant was reduced to three minutes. All sterilisation procedures were performed aseptically under laminar air flow.

Various parts of the seedlings were used for inoculation with *A. rhizogenes*. In all cases the youngest material available was used, due to their higher efficiency of hairy root formation (Senior *et al.*, 1995; Sanita di Toppi *et al.*, 1997). Axenic leaves, cotyledons, hypocotyls, stems and roots were obtained from four-month-old seedlings grown *in vitro*. Leaves only were used when greenhouse-grown saplings were utilised as explants. Immediately after sterilisation, saplings leaves were placed on MS agar plates and left overnight in the dark at

25 °C before being inoculated with *A. rhizogenes*. These explants were either used as whole leaves or leaf discs (approximately 8 x 8 mm), depending on the infection protocol used.

3.2.2 Bacterial strains and preparation

Five strains of *A. rhizogenes* were used in attempts to induce hairy roots on *E. natalensis* explants. Cultures of *A. rhizogenes* strains ATCC 1724, ATCC 15834, ATCC 2659 and ATCC 8196 were maintained at 27 °C in yeast-extract broth (YEB) agar plates (Sambrook, 1989) (with beef extract replaced by tryptone), whereas the strain LBA 9402 was maintained at the same temperature in yeast-extract mannitol broth (YMB) agar plates (Hooykaas, 1977). Both growth media were supplemented with 10 μg ml^{-1} rifampicin.

3.2.3 Bacterial growth curve

It was necessary to establish the population growth patterns of each bacterial strain before transformation. Single colony forming units (cfu) of *A. rhizogenes* strains ATCC 1724, ATCC 2659, ATCC 15834 and ATCC 8196 were selected from YEB agar plates (with 10 μg ml^{-1} of rifampicin) incubated at 27 °C and used to inoculate 5 ml of YEB broth supplemented with 5 μg ml^{-1} of rifampicin. The bacteria were grown overnight at 25 °C on a rotary shaker at 150 rpm, after which 250 μl of each culture were used to inoculate 50 ml of YEB supplemented with 5 μg ml^{-1} of rifampicin. These were incubated under the same conditions as before, while the microbial growth in each flask was monitored at 600 nm (DU-64 Spectrophotometer, Beckman). Triplicate readings were used to construct each growth curve.

3.2.4 Establishment of transformed hairy roots

Three different approaches were used with which to infect *E. natalensis* explants, and the bacteria were also prepared differently prior to inoculation. Nevertheless, where necessary, the explants used were sterilized 20 h before use, and once inoculated, regular passages were made into fresh medium every second week.

Single colonies of each strain were obtained on YEB or YMB agar plates (containing 10 µg ml^{-1} of rifampicin), then used to inoculate 5 ml of a similar medium and incubated overnight at 25 °C while shaken at 150 rpm. Thereafter, 250 µl of each bacterial suspension was used to inoculate 50 ml of the same medium (containing 5 µg ml^{-1} of rifampicin). These cultures were incubated at 25 °C, shaken at 150 rpm, and supplemented with different concentrations of acetosyringone (AS) (Sigma-Aldrich) at specific time points depending on the infection protocol used:

Protocol 1: Inoculation of leaf discs

To each growing 50 ml culture of *A. rhizogenes*, 100 µM AS was added, 10 h after starting the culture. The bacteria were incubated for another 14 h and then collected at room temperature (RT) over 10 min using 3000 rpm. Each bacterial pellet was gently re-suspended and washed in MS (half-strength macronutrients) medium containing 100 µM AS, then sedimented and re-suspended to a density of 0.3 - 0.5 (A_{600nm}) (DU-64 Spectrophotometer, Beckman). Leaf discs (with an 8 mm diammeter) cut from sapling leaves, as well as cotyledons, leaves, hypocotyls, stems and roots from *in vitro* seedlings were pricked with a needle and used as explants. These were incubated with each bacterial suspension for one hour, then blotted dry on sterile filter paper to remove excess bacteria, and placed either with the basal or apical side down on MS (half-strength macronutrients)

agar plates, pH 6.4, supplemented with 100 µM of AS (Herrera-Estrella and Simpson, 1988). The bacteria and explants were then co-cultivated in the dark at 25 °C for 48 h. Thereafter, the explants were rinsed in sterile water, blotted dry and orientated apical side up or down on MS (half-strength macronutrients) agar plates containing 700 µg ml^{-1} carbenicillin (Carb) (Sigma) and 300 µg ml^{-1} cefotaxime (Cef) (Sigma), pH 6.4. Control explants were treated as above, but incubated with medium lacking bacteria.

Protocol 2: Sonication-assisted *Agrobacterium*-mediated transformation (SAAT)

The second approach used was that described by Trick and Finer (1997). SAAT was used to infect leaf discs derived from *E. natalensis* saplings. Each bacterial strain was prepared as discussed above, except that the bacterial suspension was adjusted to a density of 0.1 - 0.3 (A_{600nm}). Leaf discs were placed in glass vials, inoculated with 10 ml of each bacterial suspension and then sonicated for 5, 10, 15, 35, 45, 60, 80 and 120 sec (Bandelin Sonorex, TK 52 H set at 100%) at RT, and gently shaken for 30 min. The explants were then blotted dry on sterile filter paper, and co-cultivated with bacteria in the dark, at 20 °C, for 48 h on MS (half-strength macronutrients) agar plates, pH 5.8, supplemented with 0.75% (w/v) polyvinylpolypyrrolidone (PVPP) (Sigma), 2 mg l^{-1} 6-benzylaminopurine (6-BA) (Sigma) and 200 µM AS. Explants were then moved to MS agar plates, pH 5.8, containing antibiotics in addition to 0.75% (w/v) PVPP. Control explants were treated as above, but in the absence of bacteria.

The effects of sonication on the viability of each strain was ascertained by sonicating a bacterial suspension (OD_{600} 0.1) as outlined above for 30 s and 120 s, then spread-plating (Prescott *et al.*, 1996c) 100 µl on YEB and YMB agar plates containing 10 µg ml^{-1} of rifampicin.

Protocol 3: Inoculation of sapling leaves

This approach was performed as recommended by Hamill and Lidgett (1997). Single colonies of each strain were used to inoculate 5 ml of YEB and YMB containing 5 μg ml^{-1} rifampicin, and agitated (150 rpm) overnight at 25 °C. Thereafter, 250 μl from each of these cultures were used to inoculate 25 ml of a similar medium. These were incubated in the dark at 25 °C for sixteen hours, after which they were supplemented with 200 μM AS and grown for another eight hours. Whole sapling leaves were then injected with each bacterial suspension, and inserted into tissue culture jars containing MS (half-strength macronutrients) medium and incubated under continuous light, 70 μmol m^{-2} s^{-1} (Li-Cor light meter, Li-Cor, USA) at 25 °C. Explants displaying bacterial growth were moved into a similar medium containing 700 μg ml^{-1} Carb and 300 μg ml^{-1} Cef. Control explants were injected with medium devoid of bacteria.

3.2.5 Initiation of hairy root cultures

In attempts to promote the growth of the transformed roots as hairy root cultures, roots over two centimetres in length were excised from each explant and regularly sub-cultured in 20 ml of MS (half-strength macronutrient) supplemented with 700 μg ml^{-1} Carb and 300 μg ml^{-1} Cef, and agitated at 150 rpm in the dark at 25 °C.

3.2.6 Light microscopy

Root material growing on an ATCC 8196-infected sapling leaf was cut into small sections (about 5 mm), and fixed over 24 h at 4 °C in 2.5% (w/v) glutaraldehyde in 0.1 M phosphate buffer containing 0.5% caffeine. Thereafter, the root sections were rinsed in 0.1 M phosphate buffer (pH 7.4) and dehydrated twice in an ethanol series (25 to 100% ethanol) and lastly acetone, with each dehydrating step taking 10 min. The samples were then

embedded in Spurr's resin (Spurr, 1969), cut into 5 µm sections using a Reichert Ultracut-S microtome (Leica) and mounted on glass slides. The histological arrangement was examined by staining with 1% toluidine blue (O'brien *et al.*, 1964) for 30 sec, and viewed using a light microscope (Leitz Diaplan). Three sets of 5 µm sections were obtained from different regions along the length of the root to verify that the root anatomy was consistently maintained.

3.2.7 Scanning electron microscopy (SEM)

Young leaves were removed from saplings and sterilised as described above. The leaves were then cut into small discs (with an 8 mm diammeter), placed in glass vials with 10 ml of MS (half-strength macronutrients) and sonicated for five to 120 sec as explained above. They were then placed in 2.5% glutaraldehyde in 0.1 M phosphate buffer containing 0.5% caffeine and fixed overnight. Thereafter the samples were critical-point dried, mounted and viewed using a scanning electron microscope (Leo SS440). Four explants (orientated in the same way on the stubs) were used to observe the effects of each length of sonication on the extent of microwouding.

3.2.8 Primer design

PCR primers were designed to amplify the *rol*C and *vir*C2 genes of *A. rhizogenes* ATCC 1724, and to amplify the ORF 13 of ATCC 8196. The gene sequences required to design the primers were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov). Using these sequences, the software programme DNAman was used to identify primers with a GC content between 50 - 60%, and selectively omit primer pairs that exhibited a calculated melting temperature (Tm) difference greater than 2 °C. The program was also used to perform a multiple sequence alignment of the nucleotide sequence of the *vir*C1 genes from

A. rhizogenes ATCC 1724 (pRi1724) and A4 (pRiA4). Degenerate primers were then designed to amplify a probable *vir*C1 gene region of strain ATCC 8196. All primer pairs were checked for self and primer-primer complementarities, and thereafter synthesised by the Oligonucleotide Synthesis Unit in the Dept. of Molecular and Cell Biology, UCT.

Table 3.1 Properties of PCR primers designed for *rol* and *vir* genes

A. rhizogenes	Gene target	Primer sequence, 5'-3'	Length, bp	GC %	Tm, °C	Product size, bp
ATCC 1724	*rol*C	FW: ATCCAGGCCTCAAAGG	19	57.9	60	468
		RV: ACTGCCATCACTCCATTCC	19	52.6	58	
	*vir*C2	FW: CCGGCCTCATCATATTAGC	19	52.6	58	455
		RV: CAAACGTCGTGACAACTTCG	20	50	60	
ATCC 8196	ORF13	FW: GAGCCCACTCGATTGAAGG	19	58	60	517
		RV: TTGCCGATTGCCAGTATGG	19	52.6	58	
	probable *vir*C1 region'	FW: AAGTTCTCGCTCAGCAGC	18	56	56	372
		RV: AAACCACGGC[AG]CTCATGG	18	55.6	56	

3.2.9 Bacterial and plant DNA isolation

Single colonies of ATCC 1724 and 8196 on YEB agar plates containing 10 μg ml^{-1} of rifampicin were used to inoculate 50 μl of TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA). Bacterial DNA was extracted as upon boiling each sample at 95 °C for 10 min, followed by centrifuging at 5000 rpm for five minutes. Each supernatant was then collected and transferred into a sterile micro-centrifuge tube for storing at -20 °C.

Plant genomic DNA was isolated from non-transformed roots derived from axenic *in vitro* grown seedlings, and due to the greater availability of transformed root material genomic DNA was also isolated from roots growing on whole-leaves infected with ATCC 1724 and 8196. Prior to extraction, the putative hairy roots were severed from the explants and incubated in 20 ml of MS (half-strength macronutrient) supplemented with 2000 μg ml^{-1} Carb and 1500 μg ml^{-1} Cef. The absence of bacteria was verified by streaking the roots over YEB containing 10 μg ml^{-1} rifampicin and incubating the plates at 27 °C for three days. DNA was extracted using the DNeasy Plant Mini kit (50) (Quiagen, USA) following the manufacturer's instructions, although in this case the roots were ground in a micro-centrifuge containing small amounts of carborundum and buffer AP1. The carborundum was subsequently removed during the first centrifugation step. The isolated DNA was re-suspended in TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA) and the purity and concentration were evaluated after absorbance at 260 nm and 280 nm.

3.2.10 PCR confirmation of transformation

PCR was performed on DNA isolated from putative hairy roots emerging on whole-leaves inoculated with ATCC 1724 and ATCC 8196. All the reactions were performed in 20 μl volumes containing 1 x PCR buffer, 0.5 μM of each primer, 0.2 mM of each dNTP, 1.25 U *Taq* DNA polymerase (SuperTherm, Southern Cross Biotechnology), 2.5 mM $MgCl_2$ and 30 ng of putative hairy root DNA. As controls, 1.4 μl of bacterial DNA (positive) and water (negative) were used.

The *rol* and *vir* genes were amplified using primers described in Table 3.1 in a thermal cycler (Thermo Cycler, Eppendorf, Germany) with an initial denaturation cycle at 94 °C for 2 min, and 30 cycles of 94 °C for 0.5 min, annealing at 53 °C for ATCC 1724 *rolC* and

*vir*C2 genes, 52.2 °C and 51.1 °C for the ATCC 8196 ORF 13 and 'probable' *vir*C1 gene region, respectively for 0.5 min, elongating at 72 °C for 0.5 min and a final elongation at 72 °C for 10 min. Using TBE buffer, the amplified products were separated on a 2% agarose gel alongside a λ-*Pst*I DNA marker.

3.2.11 Cloning of PCR products

Two bands (a and b) within the vicinity of the expected ATCC 8196 'probable' *vir*C1 region were viewed under long wavelength UV and cut out of the gel using sterile scalpels. The bands were then purified using a GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, U.K) as recommended by the manufacturer. PCR products a and b (24 ng of each) were cloned into the pDrive cloning vector (Quiagen), and 5 µl of the resulting ligation mix used to transform competent *Escherichia coli* JM109 cells , as described by the manufacturer. Transformed cells were plated on LB agar plates supplemented with 10 µg ml^{-1} IPTG, 5 µg ml^{-1} X-Gal, 100 µg ml^{-1} ampicillin and incubated overnight at 37 °C.

Successful insertions of fragments 'a' and 'b' were isolated by blue-white selection and confirmed by PCR (as described above) using primers designed to amplify the 'probable' *vir*C1 region (Table 3.1). Cells cloned with fragment 'a' were grown overnight at 37 °C in 5 ml LB containing 100 µg ml^{-1} ampicillin and thereafter plasmid DNA was isolated using a Miniprep kit (QIAprep 96 Turbo, Qiagen).

Sequencing of the cloned plasmid vector and PCR products were performed on a MegaBACE 500 sequencer (Molecular Dynamics). The PCR products were purified (as above) and sequenced using the appropriate primers, whereas the isolated plasmid was

sequenced using M13 forward and reverse primers. The BLASTn algorithm (Altschul *et al.*, 1997) was used to search the database and confirm for identity of the PCR products.

3.2.12 Extraction and analysis of diospyrin production

Four months after inoculation, hairy roots induced on whole leaves using four of the strains were severed from explants, frozen in liquid nitrogen and freeze-dried. Thereafter these samples were ground into a fine powder, macerated to exhaustion in $CHCl_3$ and then filtered (Advantec filter paper). Polar compounds present in the chloroform extracts were removed by partitioning repeatedly into water (3 x 80 ml volumes). The $CHCl_3$ portions were then collected and concentrated *in vacuo*. The medium in which hairy roots induced by ATCC 1724 were grown was also collected. Subsequently, the hairy root and medium chloroform extracts were analysed for diospyrin as previously described (Chapter two).

3.3 Results and discussion

3.3.1 *Agrobacterium rhizogenes* strains and growth curve patterns

Different *A. rhizogenes* strains display varying transformation abilities when it comes to different plant species (van de Velde *et al.*, 2003), and therefore five different strains were used in attempts to transform *E. natalensis*. Agropine and mannopine-type strains (Table 3.2) were included in order to accommodate the possible effects of endogenous levels of auxin in *E. natalensis* explants, and T-DNA arrangement on root initiation. These two factors interact in such a way that in some plant species the integration of the T_L T-DNA region of all five types of plasmids is sufficient to induce root growth. However, plants or tissues with low auxin levels may require the inclusion of the T_R T-DNA region, which

encodes auxin biosynthesis genes (Cardarelli *et al.*, 1985). As only agropine-type plasmids have the T_R T-DNA region, mannopine, cucumopine and mikimopine-type strains are less efficient at transforming tissues or plants with low levels of endogenous auxin (Cardarelli *et al.*, 1985; Filetici *et al.*, 1987).

Table 3.2 *Agrobacterium rhizogenes* strains used and their plasmid characteristics (from van de Velde *et al.*, 2003).

***A. rhizogenes* strain**	**Plasmid**	**T-DNA arrangement**	**Plasmid type**
ATCC 1724	pRi 1724	T_L	mikimopine
ATCC 2659	pRi 2659	T_L	cucumopine
ATCC 8196	pRi 8196	T_L	mannopine
ATCC 15834	pRi 15834	T_L and T_R	agropine
LBA 9402	pRi 1850	T_L and T_R	agropine

Growth curves were established for all the strains except LBA 9402 (Figure 3.1) in order to establish the time at which the bacteria reached the exponential phase of growth, and were therefore physiologically and biochemically fit for inoculation events (Prescott *et al.*, 1996e). *A. rhizogenes* strain LBA 9402 was assumed to enter the log phase after 24 h of growth. As plasmid loss has been observed to occur when the bacteria are grown between 28 and 30 °C, all the experiments were performed using bacteria grown at 25 °C (Sanita di Toppi *et al.*, 1997).

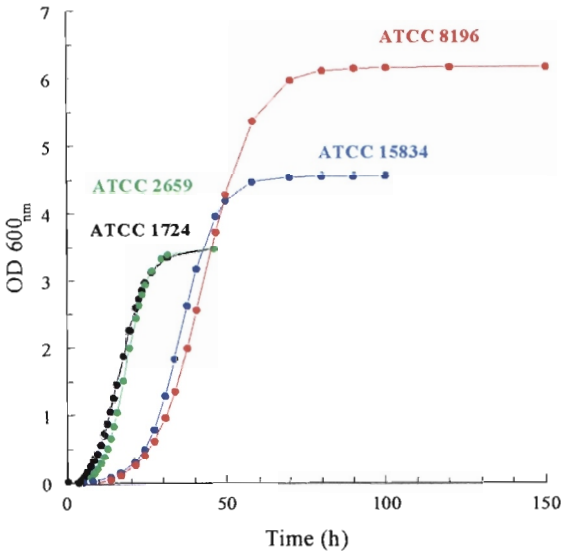

Figure 3.1 The growth curves of four of the *A. rhizogenes* strains used in this study. All strains were grown in yeast-extract broth supplemented with 5 μg ml^{-1} rifampicin. Values represent the average of three replicates.

3.3.2 Induction of hairy roots

The transformation conditions necessary to induce hairy root growth in any one plant species may differ from those of another. For this reason, an empirical approach, and three different transformation protocols, was used in order to induce root growth in *E. natalensis* explants. There were however, a number of factors that were common to all three protocols: Prior to inoculation, all *A. rhizogenes* strains were grown for 24 to 26 h, at which point all were within the exponential phase of growth (Figure 3.1). Since the inherent ability of *E. natalensis* to produce *vir*-inducing components is unknown, the bacterial growth media and the co-cultivation media were supplemented with AS, which is reputed to enhance the frequency of transformation in plants (Lipp João and Brown, 1994). Different parts of young explants were selected for inoculation with *A. rhizogenes* in order to increase the probability of isolating highly responsive tissue, and because young plants are known to

have a higher efficiency of hairy root formation (Senior *et al.*, 1995; Sanita di Toppi *et al.*, 1997).

Protocol 1: Inoculation of leaf discs

In this method, inoculated explants were placed either basal- or apical-end up during co-cultivation in an antibiotic-containing medium, in order to determine whether the orientation of the explants dictated the outcome of infection with these particular strains. Using carrot root discs, Ryder *et al.* (1985) concluded that *A. rhizogenes* strains can be grouped into those that are virulent on both sides of the explant, and those that are virulent only on the apical side. This discrepancy is perhaps as a result of different concentrations of auxin on either side of the explant, caused by the unidirectional movement of auxin from the basal to the apical surfaces (Goldsmith, 1977). Alternatively, as was the case in carrot roots, it is perhaps because auxin is synthesized on the apical surfaces and catabolised on the basal surfaces (Pilet, 1967). By orientating both sides of *E. natalensis* explants away from the medium it was presumed that the required levels of auxin, as dictated by the endogenous levels and the effects of T-DNA expression, would lead to initiation of adventitious root growth.

Four to six weeks after inoculation, all five strains were able to induce the growth of tumorous calli in all the explants used, regardless of orientation, but only on the side of the explant not in contact with the medium. The growth of tumorous calli, as opposed to hairy roots, is a function of the specific plant species being transformed (Taylor *et al.*, 1985), combined with the particular bacterial strain used (van de Velde, 2003). Since visible changes were not observed on *E. natalensis* control explants it is possible that the expression of T-DNA genes may have caused sufficient changes to the endogenous

phytohormones levels to induce callus growth, but not enough for these to differentiate into hairy roots at that point.

Although the appearance of tumorous calli was the result of infection with all five strains used, infection with ATCC 2659 did in a single instance lead to root formation (Figure 3.2). The greatest limitation with this protocol however, was the loss of over half the explants as a result of plant tissue necrosis. Necrogenesis was initiated on the cut edges of the leaf disks, as well as on the sites pricked with a needle, and by the fourth day after co-cultivation the necrosis was already severe. A similar reduction in transformation efficiency and explant death after inoculation with *A. rhizogenes* has been observed on sorghum and grape plants. In both cases, the necrotic effect started 2 days after co-cultivation with *Agrobacterium*, and had not occurred in response to the antibiotics used but as a result of the interaction of the explant with the *Agrobacterium* (Perl *et al.*, 1996; Carvalho *et al.*, 2004). *E. natalensis* control explants did not suffer necrotic damage as quickly, or to the same extent, as infected explants, which would suggest that *E. natalensis* develops a hypersensitivity response to *A. rhizogenes* similar to that in sorghum and grape plants, which probably jeopardizes the ability of transformed cells to differentiate into hairy roots.

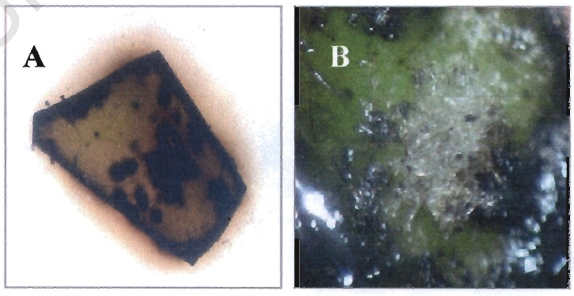

Figure 3.2 Necrogenesis and root growth on explants infected with *A. rhizogenes* ATCC 2659. (A) Localised tissue browning 4 days after co-cultivation, and (B), after 5 weeks appearance of branched roots showing negative geotropism, characteristics which are typical of hairy roots.

In grape explants, oxidation of polyphenols by peroxidase enzymes, and probably polyphenoloxidases, is thought to be involved in development of necrosis (Perl *et al.*, 1996). The tissue 'browning' resulting from oxidation of polyphenols has been controlled using antioxidants known to reduce the hypersensitivity response, and one of these, PVPP, was used in the development of the second transformation protocol

Protocol 2: Sonication–assisted *Agrobacterium*-mediated transformation

This protocol was developed in order to enhance the low transformation frequency of plants that are difficult to transform. Essentially, it involves ultrasound waves delivered by a sonicator which strike an explant, and the cavitation (Frizzel, 1988) results in the production of numerous microwounds on the surface and subsurface regions of the explant, thereby granting the bacteria access to many tissues including meristems (Trick and Finer, 1998).

In addition to using SAAT to transform *E. natalensis*, changes were also made to other factors known to affect the frequency of transformation. The concentration of the bacterial inoculum was reduced to 0.1 to 0.3 (A_{600nm}) so as to ensure that subsequent bacterial overgrowth and induction of necrosis (especially so with ATCC 2659, 1724 and LBA 9402) were reduced. Since pilus formation in *Agrobacterium* has been observed to occur at 19 °C, but not at higher temperatures (Fullner *et al.*, 1996), and since temperatures greater than 22 °C have been associated with decreased transformation frequencies (Boisson-Dernier *et al.*, 2001), the co-cultivation temperature was reduced to 20 °C. As media with high pH (e.g. pH 7) have been reported to inhibit the induction of *vir* genes (Stachel *et al.*, 1996), the pH of the co-cultivation and antibiotic-containing media was also reduced to pH 5.8. PVPP was also included in both media due to its ability to reduce necrogenesis (Perl *et*

al., 1996), and 6-BA was added to the co-cultivation media to induce host cell division, which is necessary for T-DNA integration into the host genome (Villemont *et al.*, 1997). The ability of all the strains after sonicating for 30 and 120 sec to continue growing indicated that the viability of the *Agrobacterium* was not affected by sonication. All five strains induced the growth of tumorous calli regardless of the length of sonication time. Two explants infected with LBA 9402, sonicated for either 30 or 70 sec, produced three to five thick adventitious roots from callus tissue five weeks after inoculation (Figure 3.3). An explant infected with ATCC 1724 and sonicated for 5 sec also produced similar types of root growth, seven weeks after inoculation.

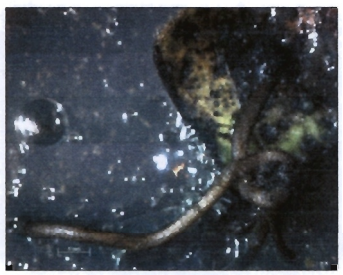

Figure 3.3 Adventitious root growth in sonicated, LBA 9402-infected leaf explants, and progressive necrogenesis five weeks after inoculation.

Although PVPP was included in the media, within 20 days most explants again developed necrotic responses that eventually lead to the death of the explant. These results, together with those of Carvalho *et al.* (2004), contrast those made by Perl *et al.* (1996), where necrosis was totally inhibited using PVPP together with dithiothreitol (DTT). The necrotic response in *E. natalensis* explants did not correlate with the degree of sonication, although explants sonicated for over 70 sec displayed a greater release of exudates (visual analysis) and necrosis in these samples was severe. Necrosis, extent of sonication and root induction ability also did not correlate with the degree of microwounding observed, making it difficult to select a level of sonication necessary to induce sub-lethal microwounding (Figure 3.4). Evidence of a direct relationship between sonication time, and extent of

microwounding, in soybean immature cotyledons (Santarém *et al.*, 1998) suggests that the developmental stage of the *E. natalensis* explants used may have played a role in their susceptibility to sonication.

Root induction using this protocol, was however, slightly more successful indicating that the changes implemented can perhaps be further explored, in an effort to minimize the effects of the two limiting factors, namely necrogenesis (by including a combination of antioxidants) and the inconsistency between sonication and microwounding (possibly by using even younger explants).

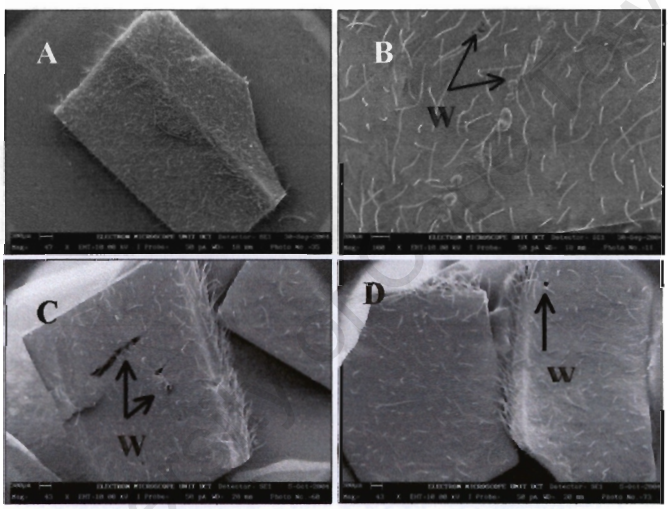

Figure 3.4 Scanning electron micrographs of *E. natalensis* leaf explants after sonication. (A) Abaxial surface of non-sonicated explant, and surface wounds on explants sonicated for 10, 60 and 120 sec (B, C and D, respectively). w, wounds.

Protocol 3: Inoculation of sapling leaves

Using this protocol, all five strains successfully induced root growth on *E. natalensis* leaf explants. Four weeks after inoculating each explant, tumorous calli developed from the site of infection, followed by an outgrowth of hairy roots four weeks later, often from the calli

or directly out of the inoculation site (Figure 3.5). A similar pattern of root formation has been reported by Sudha *et al.* (2003). Control explants, on the other hand, produced neither calli nor hairy roots.

All five strains induced the growth of at least five to eight thin adventitious roots (less, or equal to 2 mm in diameter), some of which displayed typical characteristics of hairy roots such as negative geotropism (Figure 3.5) (Chilton *et al.*, 1982). The morphological similarity of the hairy roots, regardless of the strain used, is in contrast to a report that dissimilar Ri plasmids, hosted by different bacterial strains, can induce hairy roots with different morphologies on the same plant species (Ionkova *et al.*, 1997). Conversely, hairy roots with different morphologies have been induced by ATCC 15834 on *Gmelina arborea* Roxb (Dhakulkar *et al.*, 2005) possibly as a result of variations in T- DNA copy number, integration site in the host genome, or expression of T-DNA genes (Cho *et al.*, 1998). This would suggest that the morphology of *E. natalensis* hairy roots may have been dictated not by the bacterial strain or infection protocol used, but possibly by the actual plant species used.

The susceptibility of the explants to root initiation varied according to the strain used, with LBA 9402 proving to be most virulent (80% chance of inducing root growth) under the conditions used, and ATCC 8196 the least virulent (Table 3.3). These differences in virulence, which have also been reported by Kumar *et al.* (1991) and Giri *et al.* (2001), are attributed to the nature of the plasmid within each strain (Nguyen *et al.*, 1992), the particular plant species infected (De Cleene and De Ley, 1981), and evidently also, the infection protocol used.

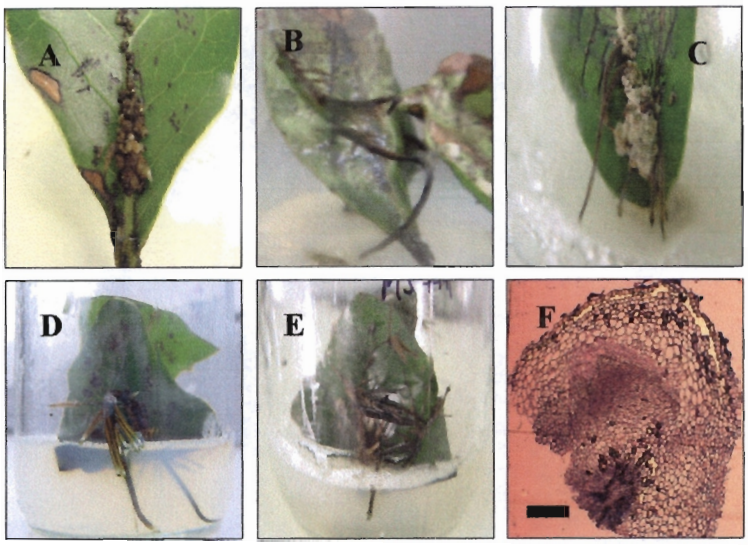

Figure 3.5 Morphological characteristics of *E. natalensis* hairy roots. (A) Emergence of root initials from calli eight to nine weeks after inoculation with ATCC 8196, and further development of adventitious root on explants transformed with (B) ATCC 15834, and (C) LBA 9402 13 and 14 weeks after inoculation, respectively. (C) Heavy branching and negative geotropism resulting from expression of Ri-TDNA derived from ATCC 1724 and (D) 2659. Cross-section of hairy root 10 weeks after infection with ATCC 1724, showing prominent vascular bundles. The bar represents 200 µm.

Table 3.3 Hairy root initiation in leaf explants after inoculation with various *A. rhizogenes* strains. The number of explants inoculated (n) is indicated in parenthesis.

***A. rhizogenes* strain**	**Root initiation (%)**
LBA 9402	80 (5)
ATCC 1724	67 (9)
ATCC 15834	50 (10)
ATCC 2659	50 (8)
ATCC 8196	26 (19)

Despite growth conditions that included constant light, none of the hairy roots turned green, as is often the case (Bhadra *et al.*, 1998; Giri *et al.*, 2001). Light-adapted green roots have been documented to produce metabolites characteristic of the aerial parts of the plant (Giri *et al.*, 2001) and exhibit a secondary metabolite profile different from roots grown in the dark (Bhadra *et al.*, 1998). This is of particular relevance if hairy roots are to be used as a source of diospyrin, since the compound accumulates predominantly in *E. natalensis* roots and bark, but in insignificant amounts only in the aerial parts.

The greater degree of transformation observed in these samples may be attributed to the changes adopted in this protocol. For example, bacterial suspensions were grown in the absence of antibiotics as these can potentially reduce the frequency of transformation (van de Velde, 2003), and AS was included eight hours prior to using the strains because longer periods (more than 14 h) in AS-containing growth medium tended to induce a degree of bacterial flocculation. With this protocol necrogenesis was also significantly reduced, probably because the area engaged in an *Agrobacterium*-explant interaction was reduced to the inoculated site only, which in turn may have resulted in a decreased hypersensitivity response.

3.3.3 Initiation of hairy root cultures

The use of hairy root cultures as a source of secondary metabolites has been especially attractive as a result of their ability to secrete certain of their metabolites into the liquid medium surrounding the hairy roots. Documented examples of this property include the secretion of rhizonone by *L. erythrorhizon* hairy root cultures (Fukui *et al.*, 1999) and verbascoside by *Gmelina arborea* hairy root cultures (Dhakulkar *et al.*, 2005). Since the

desired compound can be extracted from the liquid medium, this ability enables the hairy root cultures to be used continuously without destroying them.

Once hairy roots had been established from all five strains, growth was continuous while still attached to the explant, but ceased and the roots died once they were severed from the explants and moved into liquid media. The exception was those roots induced by strain ATCC 1724, which showed an initial period of growth which eventually also ceased. This is not unusual, many cases of successful hairy root initiation, but inadequate continuous development as hairy root cultures *in vitro* have been documented for several plant species. For example, Handa (1991) induced root growth in *Prunus incise*, *Brassica campestris* and a few other plants inoculated either with *A. rhizogenes* ATCC 1724, A13, or A4, but was unable to establish them as hairy roots cultures. Mugnier (1988) also encountered similar difficulties when using *A. rhizogenes* A4 to transform various species of the genus *Euphorbia*. Numerous hairy root cultures have been established though, making it theoretically possible to establish *E. natalensis* hairy root cultures. A number of factors can be manipulated, such as the tissue culture media used (Hamill and Lidget, 1997), oxygen supply (Yu *et al.*, 1997) or the use of phytohormones (Robins *et al.*, 1991), all of which have been reported to help establish healthy and fast growing hairy root cultures.

3.3.4 Primer design

To confirm the transformed nature of the roots, PCR primers were designed using the *rol*C and *vir*C2 sequences published for *A. rhizogenes* ATCC 1724, and ORF 13 sequence published for ATCC 8196. The virulence regions of ATCC 8196 has not been sequenced, nevertheless given the highly conserved nature of *vir* regions (Engler *et al.*, 1981; Risuleo *et al.*, 1982; Hooykaas *et al.*, 1984; Yanofsky *et al.*, 1985), degenerate primers were

designed to amplify a 'probable' *vir*C1 by aligning *vir*C1 regions of *A. rhizogenes* A4 and ATCC 1724 (Figure 3.6).

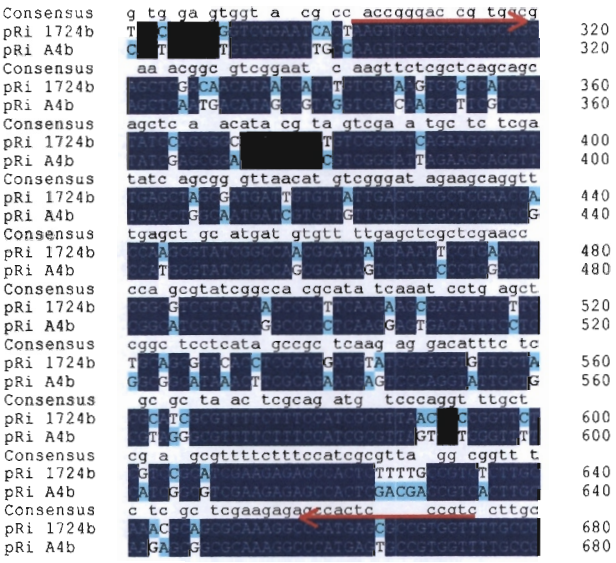

Figure 3.6 Sequence alignment of *vir*C1 regions of *A. rhizogenes* strains 1724 and A4.The red arrows delineate the regions between the forward and reverse primers.

3.3.5 PCR confirmation of transformation

The transgenic nature of hairy roots has usually been confirmed by assaying their ability to synthesize opines, and by observing the morphological phenotypes displayed by the roots. However, because some hairy roots have occasionally lacked or lost the ability to produce opines (Cho *et al.*, 1998; Tepfer, 1984), and displayed morphological phenotypes no different than wild-type roots (Berthomieu and Jouanin, 1992), PCR was used to detect the integration of T-DNA in the host genome.

Prior to amplification of *rol* and *vir* genes the absence of contaminating *Agrobacterium* on the selected hairy roots was confirmed by their inability to grow on YEB growth medium. Since *vir* genes do not integrate into the host genome, amplification of this region was also used to corroborate the absence of contaminating *Agrobacterium*.

Amplification of *rol* genes integrated in the host genome confirmed that the roots were indeed transformed. Primers specific to *rol*C amplified an expected product of 468 bp (Figure 3.7, A, lanes 2 - 3) from the genomic DNA of ATCC 1724-initiated roots whereas a 455 bp *vir*C2 fragment was not amplified from the same roots (Figure 3.7, A, lanes 8 - 9). Primers for the ORF 13 also amplified an expected product of 517 bp from the genomic DNA of ATCC 8196-initiated roots (Figure 3.7, B, lanes 1 - 2). However, the 'probable' *vir*C1 primers produced a slight smear within the range of the expected 'probable' *vir*C1 region 372 bp fragment (Figure 3.7, B, lane 7), yet the primers were specific to the region, as shown by the presence of a clear 372 bp fragment amplified from the DNA extracted from the bacterium (Figure 3.7, B, lane 9).

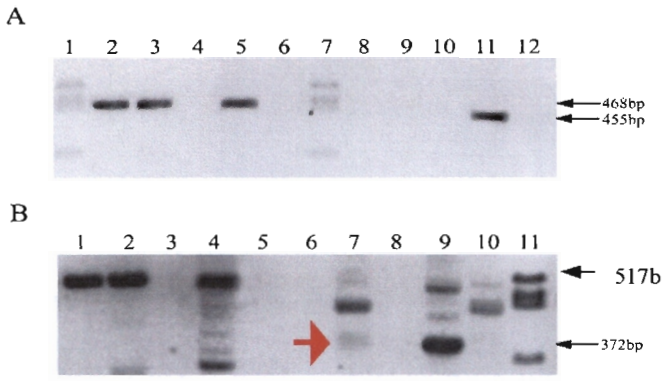

Figure 3.7 Amplification of T-DNA and *vir* genes of *A rhizogenes* ATCC 1724 and 8196. (A) ATCC 1724-induced hairy roots. Lanes 1 and 7; λ-*Pst*I DNA marker, Lane 2-3; amplification of *rol*C from hairy roots, Lane 4; *rol*C PCR reaction without DNA template, Lane 5; amplification of *rol*C from ATCC 1724, Lane 6; amplification of *rol*C from untransformed roots, Lane 8-9; amplification of *vir*C2 from hairy roots, Lane 10; *vir*C2 PCR reaction without DNA template, Lane 11; amplification of *vir*C2 from ATCC 1724, Lane 12; amplification of *vir*C2 from untransformed roots. (B) ATCC 8196-induced hairy roots. Lane 1-2; amplification of ORF13 from hairy roots, Lane 3; ORF 13 PCR reaction without DNA template, Lane 4; amplification of ORF13 from ATCC 8196, Lane 5; amplification of ORF13 from untransformed roots, Lane 7; amplification of 'probable' *vir*C1 region from hairy roots, Lane 8; 'probable' *vir*C1 PCR reaction without DNA template, Lane 9; amplification of probable *vir*C1 region from ATCC 8196, Lane 10; amplification of 'probable' *vir*C1 from untransformed roots, Lane 11; λ-*Pst*I DNA marker. The red arrow points to a smear of 372 bp, which is likely to indicate the presence contaminating bacteria.

3.3.6 Cloning of PCR products

To establish whether the smear was the result of an amplification of a *vir*C1 region, the smear was cut out of the gel, purified and amplified using a modified version of the original PCR conditions. The following changes were made; 1.5 mM $MgCl_2$, 38 ng of purified smear DNA, 4% DMSO, 15 μg ml^{-1}, initial denaturation at 94 °C for 1 min and an elongation period for 20 sec at 72 °C. These changes resulted in the appearance of two distinct bands within the vicinity of the 372 bp region (Figure 3.8, A, lanes 1 - 2). Each of

these bands, labelled 'a' and 'b', was cut and purified from the gel, and after cloning into pDrive cloning vector, *E. coli* JM109 clones with each of the bands were obtained (Figure 3.8, B, lanes 2 - 3) .

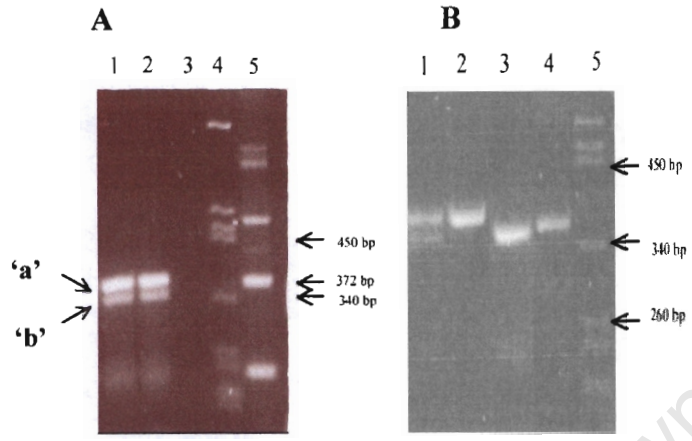

Figure 3.8 Amplification of fragments 'a' and 'b' using 'probable' *vir*C1 primers. (A) Separation of amplified smear on agarose. Lane 1 - 2; fragments 'a' and 'b', Lane 4; λ-*Pst*I DNA marker, Lane 5; amplification of probable *vir*C1 region of ATCC 8196. (B) Separation cloned fragments 'a' and 'b'. Lane 1; mixture of fragments 'a' and 'b', Lane 2; fragment 'a'-containing JM 109 clone, Lane 3; fragment 'b'-containing JM 109 clone, Lane 4; amplification of probable *vir*C1 region of ATCC 8196, Lane 5; λ-*Pst*I DNA marker.

3.3.7 Sequence analysis

Using the BLASTn algorithm (Altschul *et al.*, 1997) the 468 bp PCR product amplified from roots initiated by *A. rhizogenes* ATCC 1724 displayed high sequence similarity (100%) to the *rol*C gene of the same bacterium, and the 517 bp PCR product from roots initiated by *A. rhizogenes* ATCC 8196 also displayed high sequence similarity (99%) to *A. rhizogenes* ATCC 8196 (not shown). Multiple sequence alignment of PCR products 'a' and 'b' (Figure 3.8) revealed that although both bands were approximately the size of an expected 372 bp, PCR products 'a' and 'b' had 39% and 42%, respectively, sequence

homology to the *vir*C1 regions of *A. rhizogenes* strains 1724 and A4 from which the primers were designed (Figure 3.9).

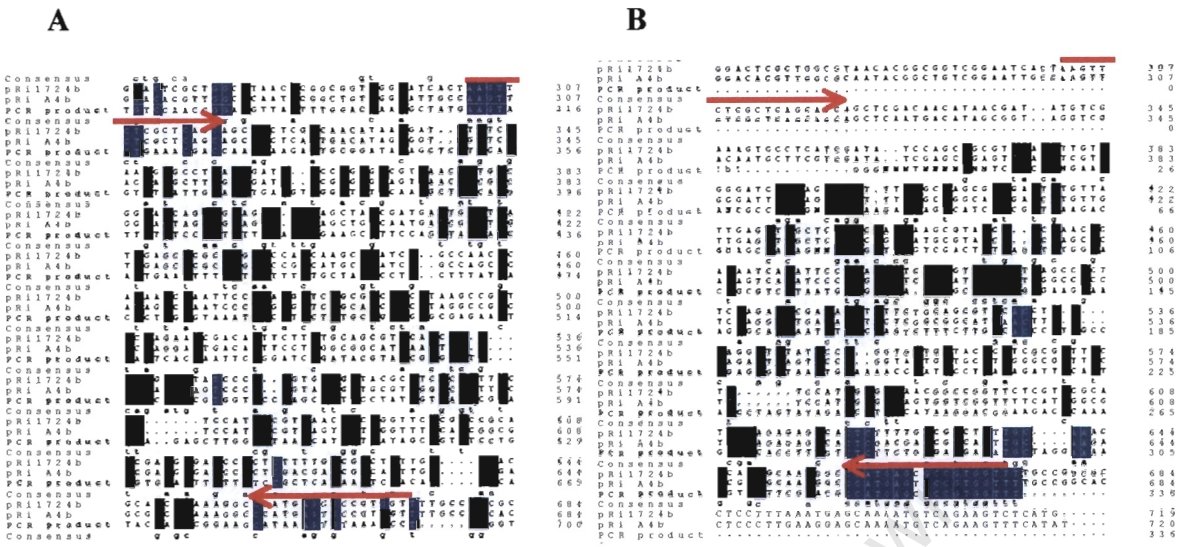

Figure 3.9 Sequence alignment of PCR products (A) 'a' and (B) 'b' with *vir*C1 regions of *A. rhizogenes* ATCC 1724 and A4. The red arrows delineate the regions between the forward and reverse primers.

Using the BLASTn algorithm (Altschul *et al.*, 1997), PCR products 'a' and 'b' were checked for sequence similarity to published sequences. A segment of product 'a' displayed 98% sequence identity to *Escherichia coli* pCA4 plasmid microsatellite, whereas a segment product 'b' displayed 100% sequence identity to *Mus musculus* BAC clone RP23-658. Overall, both products did not display sequence identity to published vir genes, and since this region is highly conserved in *Agrobacterium* (Engler *et al.*, 1981; Hooykaas *et al.*, 1984; Yanofsky *et al.*, 1985) this suggests that both products may have been amplified from sequences other that the *vir*C region of *A. rhizogenes* ATCC 8196.

3.3.8 Diospyrin production by hairy roots of *E. natalensis*

Hairy roots obtained after transformation of sapling leaves (protocol three) with four of the *A. rhizogenes* strains provided sufficient material for HPLC analysis to determine the presence and amount of diospyrin. Chloroform extracts confirmed the presence of diospyrin by sample co-migration with authentic diospyrin in Silica gel 60 TLC plates (not shown), and by determining the retention time of the extract in a HPLC column and comparing to that of a standard (not shown). Diospyrin accumulated in varying amounts in the sets of roots analysed using HPLC (Table 3.4 and Figure 3.10), but all fell within the range of diospyrin concentration in mature roots (0.01 to 1%, DW, van der Kooy, pers. comm., 2005) and seedling organs (roots, 0.17%; stem, 0.23%; leaf 0.006%).

Table 3.4 Diospyrin accumulation in hairy roots induced by various *A. rhizogenes* strains. Except for LBA 9402-infected hairy roots, the amount of diospyrin, and other naphthoquinones, represents the average of two replicates (where possible, standard errors are given in parenthesis). Percentages were calculated on a dry mass basis.

A. rhizogenes strain	Diospyrin	7-methyljuglone	Isodiospyrin	Neodiospyrin
	%			
LBA 9402	0.071	*	*	*
ATCC 2659	0.13 (± 0.09)	0.03	0.73	*
ATCC 15834	0.22 (± 0.04)	0.073	1.02	*
ATCC 1724	0.15 (± 0.03)	0.057	*	*

* Level below detection limit.

There were initial indications that the hairy roots were also able to exude diospyrin (amongst other naphthoquinones) into the surrounding media: traces of diospyrin (0.05%) were found in media in which ATCC 1724-induced roots were grown (in media where all the hairy roots failed to grow diospyrin was not detected). While these levels are very low,

it is certainly theoretically possible that with modifications to the medium, they can be increased. A thorough exploration was, however, not within the scope of this project. This property requires further analysis which may only be achieved once the viability of the hairy roots in liquid medium is improved.

The variations in metabolite production observed in *E. natalensis* hairy roots are not unusual, and have been reported for hairy root culture lines derived from the same transformation event (Bonhomme *et al.*, 2000). Different hairy root cultures have also been reported to produce greater or smaller amounts of certain metabolites in comparison to the intact, un-infected plant (Bonhomme *et al.*, 2000; Dhakulkar *et al.*, 2005). Variations in biosynthetic capacity have been attributed to varying copy numbers, as well as the sites into which the T-DNA may integrate in the host genome (Dhakulkar *et al.*, 2005). Although hairy root culture lines of *E. natalensis* that survive separation from the explant have yet to be established, the levels of diospyrin accumulated in the hairy roots are promising indications of the biosynthetic potential of potential hairy root cultures. Encouraging too, is the observation that all five strains of *A. rhizogenes* induced hairy roots, four sets of which were capable of producing diospyrin.

In certain hairy root cultures, the ability to synthesize specific secondary metabolites and the degree to which they do so has been attributed to the morphological phenotype displayed by the particular culture. Moyano *et al.* (1999) for example, observed a greater accumulation of alkaloids in *Datura metel* cultures with the classical hairy root phenotype when compared to the levels in hairy root cultures with a callus-like morphology, and this was attributable to the differentiated state of the former. In the case of diospyrin synthesis and accumulation, the association with the differentiated state seems to be reversed: greater

levels accumulate in dedifferentiated, un-transformed calli (1 to 5%) compared to the levels found in transformed roots. This being the case, *E. natalensis* hairy roots could potentially be induced to dedifferentiate in response to hormonal stimuli, so that transformed, yet callus-like, *E. natalensis* hairy roots can generate quantities of diospyrin comparable to those in the untransformed calli.

In addition to producing diospyrin in amounts comparable to those in untransformed seedling roots, secondary metabolite profiles of hairy roots induced by all four strains differed from that of seedling roots. In the hairy roots, where there was a greater accumulation of diospyrin, there was also a greater metabolic emphasis on the synthesis of the compound, since the levels of 7-methyljuglone, the precursor of diospyrin, were low (Figure 3.10, A, B, C, D). The inverse relationship between diospyrin and 7-methyljuglone was observed in seedling roots, with a greater accumulation of 7-methyljuglone instead of diospyrin (Table 3.4 and Figure 3.10, E). Presumably, in hairy roots there exists a greater degree of enzyme activity involved in the conversion of 7-methyljuglone to diospyrin.

In comparison to other naphthoquinones co-extracted along with diospyrin (e.g. 7-methyljuglone, isodiospyrin, neodiospyrin, shinanolone, and mamegakinone), the fact that high amounts of diospyrin accumulated in most of the hairy roots, regardless of bacterial strain used, suggests that this may have been in response to expression of *rol* genes, rather than either the integration sites, or the T-DNA copy numbers within each hairy root, events which occur randomly in any case (Tzfira *et al.*, 2004). Common to all the hairy roots though is the presence of *rolB* and *rolC* genes, both of which have been shown to activate the synthesis of secondary metabolites such as alkaloids in *Catharanthus roseus* (Palazón *et al.*, 1998) and anthraquinones in *Rubia cordifolia* (Bulgakov *et al.*, 2002). As suggested by

Bulgakov *et al.* (2004), expression of these genes induce a defence-like response possibly by an unknown mechanism in plants, or by acting downstream of signal pathways involved in defence responses (Ca^{2+}, octadenoid, salicylic-acid mediated and NADPH-oxidase signalling pathways), the result of which is synthesis of phytoalexins. Since naphthoquinones such as diospyrin play a protective role in plants, changes in metabolism could conceivably have occurred in response to *rol* gene expression.

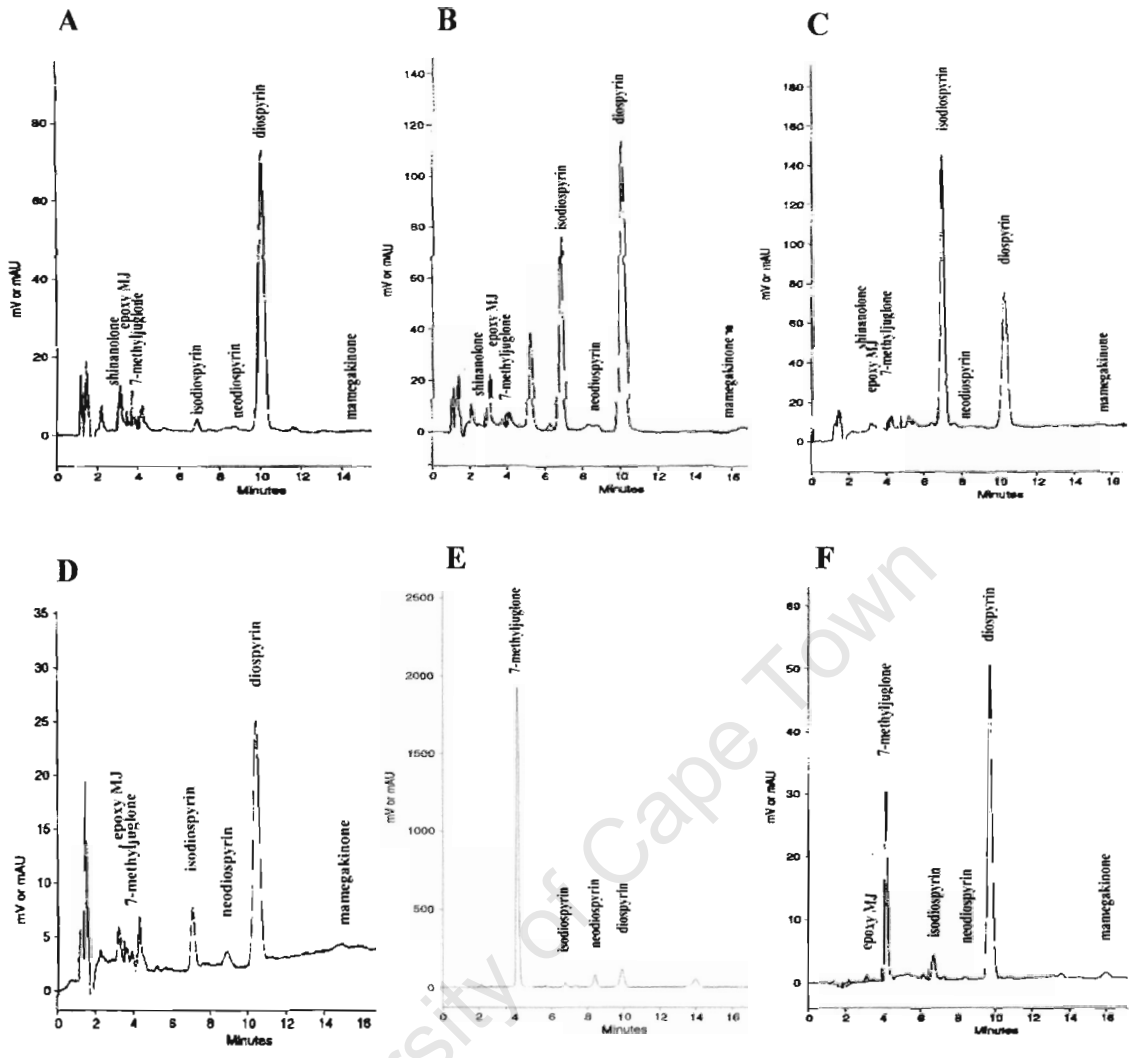

Figure 3.10 HPLC elution profiles of chloroform extracts from hairy roots (detected at 254 nm), 3 month-old seedling roots, and hairy root incubation medium (detected at 430 nm). (A) ATCC 1724-induced roots; (B) ATCC 2659-induced roots; (C) ATCC 15834-induced roots; (D) LBA 9402-induced roots; (E) seedling roots; (F) incubation medium of ATCC 1724-induced roots. The retention time of diospyrin is approximately 9.8 min, and co-extracted naphthoquinones are identified by their names.

3.4 Conclusions

All five strains of *Agrobacterium rhizogenes* used in this study successfully induced the formation of transformed hairy roots on *Euclea natalensis* leaf explants. The extent to which roots developed depended on the transformation protocol employed and the virulence of the particular strain. Between the three protocol used, the inoculation of sapling leaves was associated with greater rates of hairy root formation, and LBA 9402 proved to be the most virulent strain of *A. rhizogenes* under these conditions. Roots grew rapidly while still attached to the leaf explant, but either ceased to grow once transplanted into liquid culture media, or grew slowly only. The hairy roots were able to produce diospyrin at levels comparable to those reported for mature roots and *E. natalensis* seedlings, but less than the levels found in *E. natalensis* calli. Small amounts of diospyrin were also detected in one of the liquid culture media, clearly as exudates of the hairy roots. The establishment of hairy roots from *E. natalensis* explants, and confirmation of diospyrin production ability, serve as a platform from which an empirical approach may be used in order to improve the characteristics of the growth medium so as to generate viable hairy root cultures, thereby offering a potentially renewable source of diospyrin.

Concluding remarks

The search for novel compounds active against *Mycobacterium tuberculosis* has included medicinal plants, among them *Euclea natalensis*, traditionally used to cure the symptoms of tuberculosis (TB). Unfortunately, the harvesting method to obtain the antimycobacterial compound, diospyrin, is ultimately unsustainable since it involves destroying the mature roots. Attempts to generate more sustainable means of harvesting the compound involved exploring *E. natalensis* seedlings, calli and hairy roots for their diospyrin, and other antimicrobial compound-producing abilities.

Assays on leaf, stem and root extracts of *E. natalensis* seedlings confirmed the presence of diospyrin and other antimycobacterial compounds, and the precursor of diospyrin, 7-methyljuglone. Diospyrin specifically, accumulated mostly in the stem (0.23%) and root (0.17%) of the seedlings, mimicking the same in mature trees. The levels of diospyrin accumulated in the roots and stem are comparable to those found in the roots of mature trees (0.1 to 1%), which presently serve as the major source of diospyrin. Since *E. natalensis* seeds germinate easily, seedlings can quite easily serve as a sustainable source of diospyrin at the same concentrations traditionally extracted from mature roots. By virtue of the fact that they are cultivated in greenhouses, seedlings may also be more amenable to experimentation with various growth conditions (e.g. temperature, nutrients) designed to maintain, or enhance their capacity to accumulate diospyrin.

Calli of *E. natalensis* were also successfully established, using leaf explants, on modified Murashige and Skoog and Fujii and Nito tissue culture media. Calli grown on both media

types accumulated between 1.2 and 5.2% of diospyrin, concentrations that are clearly higher than those found in the seedlings. This suggests that in the dedifferentiated state *E. natalensis* plant cells have either an ability to synthesize, or to accumulate, more diospyrin when compared to differentiated cells. It also implies that the production of diospyrin is highly responsive to the nutrient or hormonal composition of the media. These qualities make calli a superior source of the compound, compared to the seedlings. However, it is comparatively easier to generate and maintain *E. natalensis* seedlings, and possibly cost effective, than it is to produce calli. Nevertheless, the attractiveness of calli lies not only in their inherent abilities to produce more diospyrin, but also in the fact that they can be used to produce *E. natalensis* cell suspensions. As such, cell suspensions may be investigated for their ability to secrete diospyrin into the surrounding media, they may be studied for their ability to use exogenous 7-methyljuglone to produced diospyrin, and they may be more efficiently used to study the effect of elicitors on the synthesis of diospyrin. Although the abilities of calli make them more attractive sources of diospyrin compared to the seedlings, cell suspensions of *E. natalensis* are likely to represent a preferable source of the compound.

In addition to seedlings and calli, hairy roots of *E. natalensis* were also successfully established using five different strains of *Agrobacterium rhizogenes* and they were able to produce diospyrin. The ability to induce root formation depended greatly on the bacterial strain and the infection protocol used, with the greatest limitation being necrogenesis. Nevertheless, all hairy roots produced diospyrin at levels comparable to those found in the stem and roots of *E. natalensis* seedlings, but much less than those found in the calli. Attempts to induce hairy root cultures were unsuccessful once separated from the explant, although the roots seemed to be able to secrete diospyrin into the surrounding growth

medium. Despite the failure to induce sustainable hairy root cultures, the attractiveness of these cultures lies in their enhanced genetic and biosynthetic abilities, thus making them possibly better sources of diospyrin than calli. Since hairy root induction is dependent on transformation conditions, there is a possibility that with the creation of correct conditions, cultures of *E. natalensis* may indeed be established.

This study demonstrates that seedlings, calli and hairy roots of *E. natalensis* all clearly offer a sustainable means of diospyrin production, together with a number of other important compounds. Further investigations with the aim of identifying optimal growth conditions for seedlings, calli and hairy roots would hopefully eliminate the need for harvesting of mature *E. natalensis* roots in order to obtain these important anti-TB compounds.

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