MOLECULAR ANALYSES OF *SALVIA AFRICANA-LUTEA* L. TRANSGENIC ROOT CLONES FOR SECONDARY BIOACTIVES

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously submitted it in its entirety or in part, at any university for a degree.

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Watsie Princess Neo Ramogola

I declare that the above statement is correct.

_____

Doctor Nokwanda Makunga

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Date

DEDICATION

This thesis is dedicated to my Mother;

Matshediso Ramogola

"Mama ke a leboga"

SCIENTIFIC OUTPUTS

(i)   PUBLICATIONS IN PEER-REVIEWED JOURNALS

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(ii)   CONFERENCE OUTPUTS

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Makunga NP, Colling J, Horsthemke HR, **Ramogola WPN** and Van Staden J (2007) Changing the chemical mosaic of South African medicinal plants through biotechnological strategies.   South African Association for Botanists 33rd Annual Congress (University of Cape Town). *South African Journal of Botany* 73: 299

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ABBREVIATIONS

%	Percentage
$(C_2H_5)_2NCS_2Na.3H_2O$	Sodium diethyldithiocarbamate trihydrate
+ve	Positive
≤	Equal to or less than
≥	Equal to or greater than
®	Registered trademark
µCi	Micro Curie
µg	Micro grams
µl	Micro litre
µM	Micro Molar
µm	Micro metre
$\mu mol\ m^{-2}\ s^{-1}$	Micro moles per square metre area per second
½B5	Half strength Gamborg's B5 basal salts
½MS	Half-strength Murashige and Skoog basal salts
1 atm	1 atmosphere
^1H	Proton
2,4-D	2,4-dichlorophenoxyacetic acid
2iP	6-γ-γ dimethyl-allylaminopurine
6,7-V	Veliky and Martin basal salts

A	Adenosine
Ag$^+$	Silver ion
ags	Agropine synthase gene
ANOVA	Analysis of variance
AT-pair	A pair between adenosine and thymidine
B$_4$Na$_2$O$_7$	Sodium tetraborate
B5	Gamborg's B5 basal salts
BA	6-benzyladenine
BABA	ß-aminobutyric acid
BAP	6-Benzylaminopurine
bp	Base pairs
BSA	Bovine serum albumin
C	Cytidine
cDNA	Copy deoxyribonucleic acid
Ci/mmol	Curie per millimolarity
cm	centimetre
CRD	completely randomised
dATP	2'-deoxyadenosine 5'-triphosphate
DCM	Dichloromethane
dCTP	2'-deoxycytidine 5'-triphosphate
ddH$_2$O	Nuclease free water (double distilled)
dGTP	2'-deoxyguanosine 5'-triphosphate

dH$_2$O	Distilled water
DMAPP	dimethylallyl pyrophosphate
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DOXP	1-deoxy-D-xylulose
DPI	Diphenylene iodonum
dTTP	2'-deoxythymidine 5'-triphosphate
DXR	1-deoxy-D-xylulose reductoisomerase
DXS	1-deoxy-D-xylulose synthase
EDTA	Ethylenediaminetetraacetic acid
EO	Essential oil
EtBr	Ethidium bromide
EtOH	Ethanol
Fe-EDTA	Ferric ethylenediaminetetra acetic acid
G	Guanosine
g L^{-1}	Gram per litre
GA$_3$	Gibberellic Acid
GA-3P	D-glyceraldehyde 3-phosphate
GC-MS	Gas chromatography coupled with mass spectrometry
GC	A pair between guanosine and cytidine

HCl	Hydrochloric acid
H$_2$O$_2$	Hydrogen peroxide
HMG-CoA	3-hydroxy-3-methyl-glutaryl coenzyme A
HMGR	3-hydroxy-3-methyl-glutaryl coenzyme A reductase
HPLC	High performance liquid chromatography
IAA	indole-3-acetic acid
IBA	indole-butyric acid
INT	*p*-iodonitrotetrazolium violet
IPP	isopentenyl diphosphate
LAB	Lithospermic acid B
LC-MS	Liquid Chromatography-Mass Spectrometry
LE	Leaf extract
M	Moles per litre
m/z	Mass-to-change ratio
MeJA	Methyl jasmonate
MEP	2*C*-methyl-D-erythritol 4-phosphate
MetOH	Methanol
mg L^{-1}	Milligram per litre
mg ml^{-1}	Milligrams per millilitre
Mg^{2+}	Magnesium ion
MgCl$_2$	Magnesium chloride

MH	Mueller-Hinton
MHz	Mega hertz
MIC	Minimum inhibitory concentration
min	Minute(s)
ml	Millilitre
mM	Milli Molar
mm	Millimetre
MRC	Medical Research Council
MS	Murashige and Skoog basal salts
MS-NH$_4$	Murashige and Skoog basal salts without ammonium nitrate
MSoH	Murashige and Skoog (1962) basal salts with 1g L^{-1} casamino acids, 2% sucrose
MSTFA	N-Methyl-N-(trimethylsilyl) trifluoroacetamide
MVA	Mevalonate pathway
Na$_2$S$_4$O$_5$	Sodium metabisulfite
NAA	1-napthalene acetic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	nanogram
ng μl^{-1}	Nanogram per microlitre
NH$_2$SO$_4$	Ammonium sulphate

nM	Nano-molarity
nm	Nanometre
NMR	Nuclear magnetic resonance
NN	Nitsch and Nitsch basal salts
non-MVA	Non-mevalonate pathway
°C	Degree Celsius
ORFs	Open reading frames
PAL	Phenylalanine ammonia lyase
PCR	Polymerase chain reaction
PDB	potato dextrose broth
PEG	polyethylene glycol
PFG	Pulsed field gradient
PGRs	Plant growth regulators
pH	Measure of acidity or alkalinity
Phe	Phenylalanine
PPFD	Photosynthetic photon flux density
PPP	Pentose phosphate pathway
pRi	Root-inducing plasmid
PROMEC	Programme on Mycotoxins and Experimental Carcinogenesis
PVP	Polyvinylpyrrolidone (water-soluble)
PVPP	Polyvinylpolypyrrolidone (insoluble in water)

RA	Rosmarinic acid
Ri	Root-inducing
rol	Root locus gene
rpm	Revolution per minute
SANBI	South African National Biodiversity Institute
SDS	Sodium dodecyl sulphate
sec	Second(s)
SSC	Sodium salt citrate
T	Thymidine
T$_a$	Primer annealing temperature
Taq	*Thermus aquaticus*
TBE	Tris-borate-ethylenediaminetetraacetic acid
T-DNA	Transfer DNA
TDZ	Thidiazuron
TLC	Thin layer chromatography
T$_L$-DNA	Left-stretch of T-DNA
T$_m$	Primer melting temperature
T$_R$-DNA	Right-stretch of T-DNA
Tris	Tris(hydroxymethyl)aminomethane
Trp	Tryptophan
Tyr	Tyrosine
U	units

UV	Ultra violet
v/v	Volume ratio
-ve	Negative
vir	virulence
w/v	Mass per volume
WPM	Woody plant basal media
YE	Yeast extract

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ABSTRAK

Biotegnologiese toepassings is nuttig vir waarde toevoeging tot inheemse medisinale plante en kan 'n alternatiewe bron van farmakologies aktiewe verbindings verskaf wat bydrae tot die bewaring van populasies in die natuur. Die aromatiese krui *Salvia. Africana-lutea* is reeds vir 'n lang tydperk in volks medisyne deur tradisionele geneesheers in die Wes Kaap provinsie (Suid Afrika) vir 'n verskeidenheid kwale gebruik. 'n Kontinu *S. africana-lutea* lootkultuur in soliede Murashige en Skoog (1962) (MS) media wat BA (0.5 mg L^{-1}) en NAA (0.2 mg L^{-1}) bevat, is suksesvol as 'n *in vitro* konservasie strategie ontwikkel. Die regenerasie tempo van die *S. africana-lutea* plante was hoog en het ongeveer 720 plante in 20 kultuur bottels tydens 'n vier week siklus gelewer. Die mikrolote is op plant groei reguleerder vrye MS media gewortel voordat plante geaklimatiseer is. 'n Oorlewingstempo van 92% is vir die glashuis geaklimatiseerde lote waargeneem.

'n Transgeniese harige wortel kultuur vir *S. africana-lutea* is vir die eerste keer gevestig deur gebruik te maak van *Agrobacterium rhizogenes* lyne A4T en LBA9402. Vier harige wortel klone is in 'n vloeibare skud kultuur sisteem gevestig en die transgeniese toestand van die harige wortels is deur middel van die polimerase ketting reaksie bemiddelde amplifikasie van die genomiese DNA, bevestig. Die *rol* A (350 bp) en *rol* C (400 bp) gene van die linker grens van die T-DNA is in alle harige wortel klone waargeneem. Aan die ander kant, is die ags geen (1.6 kbp) van die regterkantste grens van die T-DNA in al drie klone behalwe die A4T (2) kloon ingevoeg. Die ingevoegde *rol* gene is stabiel in die *S. africana-lutea* genoom as enkel kopië geintegreer soos bevestig d.m.v. Southern hibridisasie. 'n Optimale kontinue *in vitro* kultuur sisteem wat wortel proliferasie en fenoliese verbinding produksie ondersteun is krities vir die verskaling na 'n industriele vlak. Om die optimale vloeibare basale medium te bepaal, is die effek van vyf verskillende vloeibare basale media; MS, MS-NH$_4$ (MS sonder ammonium nitraat), B5 (Gamborg 1968), Miller's (Miller 1965) en ½MS, wat algemeen vir *in vitro* kulture gebruik word, getoets, om die groei van die wortels te bestudeer. Slegs die $^1/_2$MS media het

wortel groei ondersteun terwyl al die wortel klone tipiese sigmoidale groei patrone getoon het. Al vier transgeniese wortel klone het verskillende groei patrone getoon met kloon A4T (3) wat die kortste sloer fase en kloon A4T (1) wat die hoogste eksponensiele groei oor die vier week periode getoon het. Die hoogste gemiddelde vars biomassa (1.61 g) het in die A4T (1) kloon geakkumuleer.

Aangesien die infusies van *S. africana-lutea* as tradisionele medisyne vir die behandeling van mikrobiese infeksies gebruik word, is die antimikrobiese aktiwiteit van die plante in weefselkultuur asook die transgeniese wortel weefsel met die van plante wat in die natuur groei (*ex vitro*) vergelyk. Slegs die *in vitro* propagules was aktief teen *Bacillus subtilis* met 'n MIC van 0.39 mg ml^{-1}. Omgekeerd, beide die harige wortel en *in vitro* plant ekstrakte was hoogs aktief teen die fitofungale patogene. Byvoorbeeld, die MIC waardes vir *Fusarium verticilliodes* het tussen 0.02 tot 0.64 mg ml^{-1} gewissel en vir *F. proliferatum* was dit tussen 0.08 to 0.64 mg ml^{-1}. Die metaboliet profiele vir beide loot en wortel kulture tydens TLC, NMR en GC-MS analise het interessante veranderinge in die chemiese vingerafdruk van *S. africana-lutea* aangedui. Die meeste van die verbindings wat in die *in vitro* blare geidentifiseer (bv kafeïensuur en tiosuur) is, was nie in die *ex vitro* blare teenwoordig nie. Kafeïensuur is 'n fenoliese verbinding wat algemeen in *Salvia* plante voorkom en is bekend vir antimikrobiese aktiwiteit, terwyl die soute van tiosuur tydens die produksie van swamdoders en antibiotika gebruik word. Dit wil voorkom asof die weefselkultuur mikro-omgewing die *de novo* biosintese van farmakologies aktiewe verbindings in die mikroplante induseer, aangesien die ekstrakte meer biologiese aktiweit gehad het in vergelyking met die plante wat nie gekultiveer is nie. Die chemiese profiele van beide die A4T (1) en A4T (3) wortel klone was relatief meer kompleks as die blaar ekstrakte (beide *in vitro* en *ex vitro*) terwyl die LBA9402 kloon minder verbindings gehad het. Al die harige wortel klone het 2-azathianthrene en verskeie suiker oximes geproduseer wat farmakologies belangrik is as antioksidante en tydens die behandeling van menslike chemiese vergiftigings.

Die hoë aktiwiteit van die *S. africana-lutea* ekstrakte teen beide bakteriële en fungus patogene is 'n betekenisvolle bevinding vir beide menslike gesondheid en die landbou sektor. Die *in vitro* sisteem wat tydens die studie gebruik is kan op 'n groot skaal gebruik

word tot voordeel van beide die farmakologiese en die landbou sektors. Dit sal die doel vir die konservasie van die medisinale gewilde krui verder dien.

ABSTRACT

Biotechnological applications are useful for adding value to the local medicinal plants and may provide an alternative source of pharmacologically-active compounds thus assisting with the conservation of wild populations. *Salvia africana-lutea* aromatic herb has long been used in folk medicine by traditional healers in the Western Cape Province (South Africa) for various ailments. As an *in vitro* conservation strategy, a continuous *S. africana-lutea* shoot culture was successfully established in solid MS medium containing BA (0.5 mg L^{-1}) and NAA (0.2 mg L^{-1}). The regeneration rate of the *S. africana-lutea* plants was high which produced approximately 720 plantlets in 20 culture bottles over a four week cycle. The microshoots were rooted in the MS medium without PGRs prior to acclimatisation. A survival rate of 92% was recorded for the greenhouse-acclimatised shoots.

For the first time, a transgenic hairy root culture of *S. africana-lutea* was established using *Agrobacterium rhizogenes* strains A4T and LBA9402. Four hairy root clones were established in liquid shake culture and transgenesis of the hairy roots was proven through amplification of genomic DNA via the polymerase chain reaction. The *rol A* (350 bp) and *rol C* genes (400 bp) from the left border of the T-DNA were detected in all hairy root clones. On the other hand, the *ags* gene (1.6 kb) from the right border of the T-DNA was inserted in all three hairy root clones except the A4T (2) clone. The inserted *rol* genes were stably integrated in the *S. africana-lutea* genome as confirmed by Southern hybridisation. An optimal continuous *in vitro* culture system supporting root proliferation and phenolic compound production is crucial for up-scaling to industrial level. To determine the optimal liquid basal medium, the effect of five different liquid basal media commonly used for the *in vitro* root cultures namely, MS, MS-NH$_4$ (MS without ammonium nitrate), B5 (Gamborg 1968), Miller's (Miller 1965) and ½MS on root growth were studied. Only the ½MS medium supported root growth with all root clones showing characteristic Sigmoidal growth pattern. All four transgenic root clones had varying growth rates with the A4T (3) having the shortest lag phase and the A4T (1) with the

highest exponential growth over a four week period. The highest mean fresh biomass accumulated was 1.61 g in the A4T (1) root clone.

As the infusions of *S. africana-lutea* are used for the treatment of microbial infections in traditional medicine, the antimicrobial activity of micropropagated plantlets along with the transgenic root tissue was compared with the wild-growing (*ex vitro*) plants. Only the *in vitro* propagules were more potent against *Bacillus subtilis* with the MIC of 0.39 mg ml^{-1}. Conversely, both the hairy root and *in vitro* plantlet extracts were highly potent against the phytofungal pathogens. For instance, the MICs ranged from 0.02 to 0.64 mg ml^{-1} for *Fusarium verticilliodes* and 0.08 to 0.64 mg ml^{-1} for *F. proliferatum*. The metabolite profiles of both shoot and root cultures using TLC, NMR and GC-MS showed interesting changes in the chemical footprint of *S. africana-lutea*. For instance, most of compounds identified in the *in vitro* leaves such as caffeic acid and thiocyanic acid (as examples) were not present in the *ex vitro* leaves. Caffeic acid is a phenolic compound commonly found in Salvia plants known for antimicrobial activities while the thiocyanic acid salts are used in the production of fungicides and antibiotics. The tissue culture microenvironment seems to induce *de novo* biosynthesis of pharmacologically-active compounds in microplants as these extracts are more biologically active compared to non-propagated plants. The chemical profiles of both the A4T (1) and the A4T (3) root clones were as complex as the foliage extracts (both *in vitro* and *ex vitro*) but the LBA9402 clones had fewer compounds. All hairy root clones produced 2-azathianthrene and various sugar oximes, which are important pharmacologically as antioxidant and treatment of human chemical poisonings respectively.

The high potency of the *S. africana-lutea* extracts against both the bacterial and fungal pathogens is a significant finding for both human health and agricultural sector. The *in vitro* system used in this research can be exploited on a larger scale to benefit both the pharmacology and the agricultural sectors. This will further serve as a conservation method for this medicinally popular herb.

CHAPTER 1

INTRODUCTION

South Africa is rich in plant biodiversity with more than 30 000 angiosperms which represent about 10% of all higher plants on earth (Goldblatt 1978). Among this diverse group of indigenous plants are the medicinal plants. In South Africa, the use of medicinal plants has been a fundamental part of South African culture for many generations (Cunningham 1993; Mander 1998). About 27 million South Africans are dependent on indigenous medicinal plants from about 1020 plant species (Meyer *et al*. 1996; Mander 1998) for their primary healthcare. This is approximately equal to 20 000 tonnes of plant material consumed every year in South Africa (Mander 1998). This is particularly the case in the rural communities where people have limited access to modern and well-resourced healthcare facilities. There is a high prevalence of poverty in these areas and people cannot afford to pay for basic healthcare and thus depend on traditional healer(s) or self-medication with traditional herbs (Cunningham 1993). Furthermore, people from these communities use medicinal plants as a source of income through trading ethnoherbal products in urban areas in informal *muthi* markets (Mander 1998). As a result, there has been an increase in the *muthi* markets in the urban areas. According to Mander (1998), the expansion of the *muthi* markets indicates the reliance of urban dwellers on traditional herbs. The high usage of traditional herbs in urban areas has been attributed to these herbs being affordable and easily accessible. The other reason is that usage of traditional herbs is still a culturally significant practice in urban areas (Mander 1998).

The South African population has been growing exponentially in the latter half of the 20th century and this has in turn led to exponential increase in the demand for medicinal plants (Cunningham 1993). An increasing number of plants are harvested from the wild to meet high demands. Harvesting for the majority of these plants is in an unsustainable manner with a large proportion being non-renewable (for example bark and underground storage organs) or in some instances, the whole plant has to be uprooted (Mander 1998). Unsustainable harvesting has put

an enormous pressure on the natural populations of these plants leading to many species being threatened and some being rare. To mention a few, according to Fennell *et al.* (2004[b]), plants such as *Warburgia salutaris* (Canellaceae), *Cassin transvaalensis* (Celastraceae), *Alepidea amatymbica* (Apiaceae) and *Erythrophleum lasianthum* (Leguminosae) have been recorded as extremely rare as early as 1938. Instilling and practicing sustainable harvesting of medicinal plants is not a practical solution anymore due to exponential demand of these plants. The only practical solution at the moment is the cultivation and small-scale farming (for details refer to van Staden 1999). However, only a few medicinal plants such as *Agathosma betulina* (Rutaceae) and *Harpophytum procumbens* (Pedaliaceae) are cultivated (Fennell *et al.* 2004[b]). There is still a big challenge that has to be overcome in cultivation of medicinal plants because some traditional healers in South Africa are still conservative about the use of cultivated traditional herbs. They still believe that plants grown under modern agricultural conditions (for example grown in straight lines on fertilized and irrigated fields) are less effective or powerful for healing, with fewer medicinal properties compared to wild-growing and harvested plants (Cunningham 1993).

There has been an increase in the integration of traditional medicine with primary healthcare worldwide (Fennell *et al.* 2004[a]) especially in China (see review by Zhou and Wu 2006) and southern Africa. Researchers are currently interested in not only validating efficacy of traditional herbs scientifically but also discovering novel compounds from these plants. These new compounds are beneficial particularly for the pharmaceutical industries. Scientists use traditional knowledge to guide them to identify target plants instead of using trial and error for drug discovery as it was in the past (Cox and Balick 1994). In 2001, Fabricant and Farnsworth reported that about 122 drugs have been discovered through ethnobotanical leads from 94 plant species. In order to validate and discover novel compounds from medicinal plants, researchers are using a variety of different scientific interdisciplinary approaches including biotechnological techniques (Makunga *et al.* 2007).

Plant biotechnology is an area of research that focuses on the manipulation of biological systems of plants (Kirakosyan 2006). It has revolutionised plant science in three major areas for

the past decades. These areas being, growth and development control (vegetative, generative and propagative); protection of plants against the environmental threats (biotic and abiotic stresses) and development of ways in which biochemicals and pharmaceuticals are produced (Kirakosyan 2006). Plant biotechnology has been applied extensively on agronomic crops because many companies invested in this technology for improvement of food crops for food security. For example, germplasm conservation, propagation and improvement of various traits through breeding have been applied on crop plants (Nessler 1994). Although biotechnology was initially viewed as a high-technology science which was only relevant to crop plants (Nessler 1994), researchers have found this technology to be equally relevant on wild-harvested medicinal plants as medicinal plant populations in their natural habitats still remain the main source of pharmaceuticals (Nigro *et al*. 2004). Even though biotechnological techniques have been applied successfully in American and Asian traditional medicinal taxa, application in the African species is still limited (Nigro *et al*. 2004). However, South African researchers are currently exploring the benefits of this 'high-tech' science on the traditional medicines' sector (Makunga *et al*. 2007).

To meet the ever-increasing commercial demands, large quantities of medicinal plants are desirable for the global dermaceutical, aromatherapeutical and pharmaceutical industries (Lange 1998). In addition, since regulating and instilling sustainable harvesting by law enforcement has proven to be impractical (van Staden 1999), mass micropropagation is useful for overcoming the problems associated with high demands for medicinal plants. *In vitro* propagation is a speedy process that provides multi-fold benefits; it serves as a conservation tool (Kintzios 2000; Nigro *et al*. 2004) by alleviating pressure off the highly harvested plants by providing alternative plant material (Nigro *et al*. 2004). The micropropagated plants can then be used as source of seedlings for field cultivation and small-scale farming (van Staden 1999). Furthermore, pathogen-free *in vitro* plant material can be used for further *in vitro* phytochemical and pharmacological studies since there is substantial evidence that plant secondary metabolism can be enhanced by induction of *in vitro* morphogenesis (Kintzios 2000).

Biotechnological techniques also facilitate in conservation of medicinal plants that are in high demand. Medicinal plants can be conserved through both *in vitro* propagation (refer to Nigro *et al*. 2004 for examples) and transgenic technologies (see Bajaj and Ishimaru 1999). In the case

of *in vitro* propagation, medicinal plants are mass-propagated under closely monitored and controlled conditions. Micropropagation is the largest component of plant biotechnology exploited on a commercial scale worldwide (Nigro *et al.* 2004). Continuous, consistent and uniform propagules of different plant species are mass produced through plant tissue culture (see Section 2.2.1) and these plantlets can be used for scientific validation of efficacy of these plants and more importantly also for discovery of novel compounds. Furthermore, these plantlets can be transferred from the *in vitro* environment into the field for continuous cultivation as an alternative source of plant material (van Staden 1999). In this way, the high demand pressure on the wild populations is alleviated and the wild populations are conserved (Grace *et al.* 2002).

Moreover, micropropagation plays an important role in modern conservation of rare and threatened medicinal plant taxa such as long term germplasm storage, establishing pathogen-free plant material (Fay 1996) and seedbanks (Smith *et al.* 2002). Germplasm conservation consists of propagation and breeding to improve various traits and cryopreservation (Nessler 1994). Although germplasm conservation has previously been focussed on crop plants, several African countries including South Africa have established germplasm collections of important medicinal plants (see Nigro *et al.* 2004). Seedbanks provide seed of living rare and threatened taxa, for habitat restoration and species re-introduction (Nigro *et al.* 2004). Biotechnology contributes to seedbanks via seed research such as dormancy studies, genetic fingerprinting and propagation studies (Smith *et al.* 2002). The need for *in vitro* propagation of medicinally important plants is not only from obvious implications resultant from unsustainable harvesting but also for *in vitro* optimisation of secondary metabolite production (Nigro *et al.* 2004).

Plant secondary compounds are metabolites derived from the plant's secondary metabolism. These compounds include alkaloids, terpenoids, phenolics, steroids and flavonoids and they are generally present in plant organs at low concentrations (Verpoorte *et al.* 1999). However, the level of secondary compounds in some plant organs such as roots, bark and heartwood may be constitutively high (Collin 2001). Initially secondary compounds were thought to be the products of metabolism with no specific function in plants. Secondary compounds were later shown to

have an active role in plants (Collin 2001). For example, secondary compounds are part of biochemical defence mechanisms of plants against pathogens and predators (Bennet and Wallsgrove 1994). Secondary compounds are not only important to plants but also of a significant value to humans. Isolated and purified secondary metabolites are a source of high value compounds used as flavour additives, cosmetics, perfumes, therapeuticals and pharmaceuticals (Collin 2001).

Pharmaceutical production is usually limited by several factors. Some source plants may not be readily accessible because the source plant might be severely endangered (Alfermann and Petersen 1995). Another limitation to pharmaceutical production is that the important secondary compounds may be present at very low concentrations in the source plant. These compounds can only be isolated at low yields resulting in the pharmaceutical products produced to be very expensive (Collin 2001). Plant and tissue culture has long been regarded as a potential solution to limitations on the production of secondary compounds. Large-scale plant tissue cultures are seen as a more convenient and reliable source of secondary compounds (Bajaj and Ishimaru 1999; Collin 2001). As plant biotechnology is popularly described as an important tool for manipulation of plants to suit Man's needs (Nigro *et al.* 2004), manipulation and optimisation of secondary metabolite production from medicinal taxa is one of many ways that Man manipulates plants. *In vitro* cultures are used for secondary metabolite optimisation studies (Bajaj and Ishimaru 1999; Collin 2001). In addition to alleviating pressure off the wild populations, *in vitro* cultures may also reduce inconsistencies in the quality and composition of the produced metabolite (Bajaj and Ishimaru 1999) which might be due to genotypic and phenotypic variation that occur in the wild. In this way, the yields can be raised or reduced and standardised by management practices (Bajaj and Ishimaru 1999; Grace *et al.* 2002). Different *in vitro* culture systems have been used for the production of secondary metabolites from rare and threatened plants each of them with its own advantages and disadvantages.

In vitro plant cell and callus cultures have been used for the production of a range of metabolites that have been patented (Dodds and Roberts 1985). Cell suspension cultures have been used for large volume productions due to their faster growth cycles (Fu 1998). Despite the considerable efforts, only a few commercial production systems (for example ginseng, shikonin, berberine, rosmarinic acid) have been achieved using plant cell suspension cultures (Bajaj and

Ishimaru 1999). The main challenge with the undifferentiated cell cultures is that they are not genetically stable and therefore may produce low yields of secondary compounds (Hamill *et al*. 1987). For instance, callus cultures often eventually lose their ability to produce specific secondary compounds after several sub-cultures (Flores and Medina-Bolivar 1995). In fact, poor secondary compound synthesis and genetic instability of callus or cell cultures have prevented commercialisation of many plant culture systems (Deus-Neumann and Zenk 1984; Sharp and Doran 1990). To solve this, differentiated cell cultures systems have since been used. The organ cultures are used for production of secondary metabolites synthesised only in differentiated specialised organs. The metabolite profiles of these specialised cultures resemble that of intact whole plants. These cultures are more genetically stable and produce metabolites consistently as compared to cell and callus cultures (Fu 1998). The controlled tissue culture environment offers benefits such as continuous and consistent natural product production free of any fluctuations that might be due to climate, agriculture, political and legal regulations of the respective country (Collin 2001).

Secondary metabolite production by organ cultures can be improved further by the use of transgenic technology. The ability to introduce foreign genes into plants (transformation) has revolutionized secondary compound production and synthesis. Since the 1990s, plants have been targets for genetic modification as they may be utilised as 'green factories' for the production of the ever increasing number of commercially useful compounds (Lindsey 1992). Transformation increases the capacity of differentiated tissue such as shoots and roots to synthesize secondary compounds (Collin 2001). Since these differentiated organs produce metabolites in a stable manner, this stable synthesis of the secondary compounds is transmitted to plantlets regenerated from the shoot or root culture (Collin, 2001). Hairy root culture (transgenic roots with the root inducing (Ri) genes of *Agrobacterium rhizogenes*, (refer to Section 2.2.4) is one of the genetically-modified differentiated organ cultures used for production and optimisation of the commercially important secondary compounds. It offers possible solutions to many problems associated with secondary compound production in untransformed plant cell cultures (Aird *et al*. 1988). In order to optimise secondary metabolite production in medicinal plants through transformation, about 70 medicinal plants were reported to be manipulated in transformation studies (for an overview see Bajaj and Ishimaru 1999). Both

direct and indirect gene transfer methods have been used on medicinal plants with *Agrobacterium*-mediated transformation being the most popular (Nigro *et al*. 2004).

All these above-discussed biotechnological tools have been coupled with the use of high technology methods for identifying the bioactive compounds from medicinal plants in order to contribute to pharmacopoeias worldwide (Wilson 2000). These techniques include high throughput chromatographic and spectrometry tools such as Gas chromatography-Mass spectrometry (GC-MS), Nuclear Magnetic Resonance (NMR), High Performance Liquid Chromatography (HPLC) (Wilson 2000) and Liquid Chromatography-Mass Spectrometry (LC-MS) (Wolfender *et al*. 2003) just to mention a few.

Biotechnological applications on South African plants are few particularly those using Ri transgenesis. *Salvia africana-lutea* was chosen as a target for transformation as it is an ethnobotanically important species of the Western Cape. Although most of medicinal plants endemic to the KwaZulu-Natal region have been micropropagated, biotechnological manipulation of the Western Cape medicinal plants is still limited. *S. africana-lutea* has long been used in folk medicine by traditional healers and early European settlers in the Western Cape Province for various ailments (Watt and Breyer-Brandwijk 1962). Despite all this, the scientific literature on this plant is very little with only few reports validating the pharmacological actions (Section 2.1.2) and phytochemistry (Section 2.1.3) of this plant. At this stage, there is only one publication on *in vitro* propagation of this plant (Makunga and van Staden 2008). This study was therefore undertaken to add to the literature on this species. Biotechnological approaches (incorporating both micropropagation and transgenic technology) were used to highlight the phytochemistry and pharmacology of this important South African medicinal plant.

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CHAPTER 2

LITERATURE REVIEW

2.1 BACKGROUND INFORMATION ON GENUS *SALVIA*

2.1.1 Botany and geographical distribution of *S. africana-lutea*

There are about 900 *Salvia* species (Lamiaceae) worldwide, 26 of which are found in South Africa especially in the western and/or eastern regions (Codd 1985). *Salvia africana-lutea* is endemic to the Western Cape Province in South Africa (Eliovson 1955). Its geographical distribution is mainly along the coast, extending from Namaqualand to the Cape Peninsula and eastwards to Port Alfred in the Eastern Cape Province (Codd 1985) (Figure 2-1). *S. africana-lutea* is commonly known as beach or brown dune sage because it naturally grows on coastal sand dunes. This hardy shrub also grows in arid fynbos on lower rocky slopes up to 800 metres (Codd 1985). The fynbos is species-rich with shrubland vegetation characterised by Mediterranean climate with winter rainfall (Holmes and Cowling 1997). The *S. africana-lutea* plants produce flowers clustered into an inflorescence with a long floral display (June-December). During this long floral display, the colour of the flowers changes from bright yellow, then they fade to rusty-orange (Figure 2-2B) and finally to reddish brown after which they wilt and fall-off (Phillips 1951; Eliovson 1955; Riley 1963). According to Makunga and van Staden (2008), the long floral display is making *S. africana-lutea* a highly valued ornamental plant especially in the coastal gardens and other areas across the country where wind and drought resistance is important. This is important because recently, planting of indigenous South African species which are accustomed to the South African climate has become highly fashionable (Makunga and van Staden 2008). Planting *S. africana-lutea* in garden landscapes attracts a wide range of wildlife including blue and bronze butterflies that use these sage plants as the host for their larvae (Viljoen 2002). The nectar in the base of the flowers also attracts bees, other insects and sunbirds all year round (Viljoen 2002).

S. africana-lutea is a highly branched shrub that grows up to two metres (Bohnen 1986). The leaves are densely covered with glandular trichomes forming an even white carpet-like layer, which gives them a grey-green leathery colour (Codd 1985). Soft younger branches are also densely covered with white trichomes thus appearing white in comparison to old woody branches (Figure 2-2A). The abundant trichomes covering leaves and branches give members of genus *Salvia* a strong distinctive aroma (Codd 1985). Therefore, the aromatic leaves of *S. africana-lutea* are used in potpourri as they retain their shape, colour and most of their aroma, and mix well with other ingredients (Viljoen 2002).
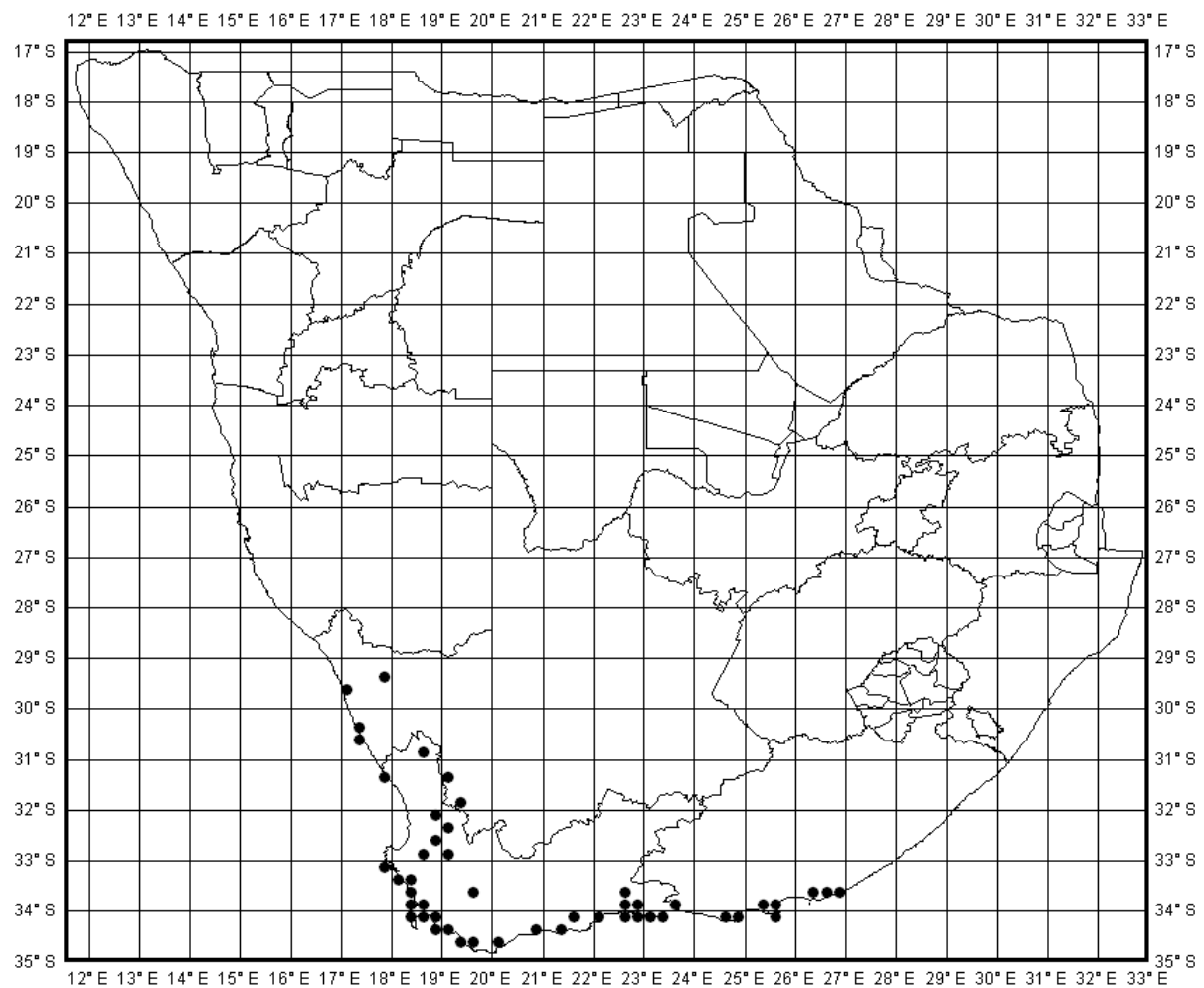
Figure 2-1 Geographical distribution of *Salvia africana-lutea* in South Africa (reproduced with kind permission of SANBI, PRECIS)

Figure 2-2 A) A bush of *S. africana-lutea* flowering during Spring (30th August 2006) at Stellenbosch University, SOUTH AFRICA B) Inflorescence after the petals have dropped (Note the seeds developing in the remaining bracts)

2.1.2 Biological activity of *Salvia*

Salvia species are aromatic plants because they are rich in essential oils. Essential oils are odorous and volatile products of aromatic plants' secondary metabolism that give them a distinctive characteristic aroma (Araújo *et al*. 2003). The volatile essential oils are produced in the glands of the trichomes. Essential oils produced in the *Salvia* foliage have long been used in the folk medicine (Watt and Breyer-Brandwijk 1962). In other parts of the world, *Salvia* species are traditionally used as a medication against perspiration, fever, rheumatism, female diseases and sexual incapacity, treating mental and nervous conditions and as an insecticidal agent (Baricevic and Bartol 2000). Some species such as *S. officinalis* (the common sage) are useful as flavouring agents in culinary dishes or as a source of essential oils used in perfumery and cosmetics (Werker *et al*. 1985; Tzakou *et al*. 2001). Different *Salvia* species studied in many parts of the world were found to possess anti-bacterial (Ulubelen *et al*. 2001), anti-oxidant, anti-

inflammatory, anti-cholinesterase (Perry *et al.* 2003), anti-cancer (Li *et al.* 2002) and anti-diabetic (Hitokato *et al.* 1980) activities amongst others.

Similarly, South African species have also been found to have anti-microbial (Kamatou *et al.* 2005; 2006[a]; 2006[b]; 2007[a]; 2007[b]), anti-inflammatory (Kamatou *et al.* 2005), anti-malarial (Kamatou *et al.* 2007; 2008) and anti-oxidant (Kamatou *et al.* 2005) activities. Recently, some South African *Salvia* species have been found to have anti-cancer (Kamatou *et al.* 2008) and anti-mycobacterial (Kamatou *et al.* 2007[b]) activities (Table 2-1). The latter is a very important and promising breakthrough for South Africa as a whole especially the Western Cape Province where there are many incidences of tuberculosis (TB) with current reports on the occurrence of the new extreme drug-resistant TB strain (XDR-TB) highest in the Western Cape (Health24.com, 2007). In addition, as reported by Kamatou *et al.* (2008), *Salvia* species are active against certain cancers such as colo-rectum, brain and breast cancer. In terms of breast cancer, this is an encouraging finding because according to Health24.com (2001), breast cancer is the most prevalent type of cancer in South African women. Among different pharmacological studies conducted, the majority of the South African species seem to have anti-microbial, anti-inflammatory and anti-malarial properties (Table 2-1). Interestingly, in the study conducted on three South African *Salvia* species namely, *S. stenophylla, S. runcinata* and *S. repens*, essential oils from all three species failed to inhibit Gram-negative bacteria, whereas the methanolic leaf extracts were active against all Gram-positive bacteria tested (Kamatou *et al.* 2005). However, essential oils of *S. albicaulis* and *S. dolomitica* are active against both Gram-negative and Gram-positive bacteria with the lowest activity against *E. coli*. Furthermore, these oils also show anti-inflammatory and anti-malarial (Kamatou *et al.* 2007[a]) activity. Variable bioactivity of essential oils of different *Salvia* species illustrates the variety in the essential oil constituents of the different species. In another study, *S. chamelaegnea* leaf extracts have synergistic anti-bacterial activity against Gram-positive bacteria while there is antagonistic synergism on Gram-negative bacteria for various ratios tested with *Leonotis leonurus* extracts (Kamatou *et al.* 2006[a]). Combination of extracts from different plants is not a new concept; for many years, traditional healers often prescribed more than one herb for treatment of the same condition (Iwu 1994). Similarly, pharmacists often use a synergistic combination commercially by prescribing more than one anti-microbial for the same infection (Kamatou *et al.* 2006[a]).

S. africana-lutea was used by early Europeans settlers in the Western Cape as an infusion for colds. Before the discovery of antibiotics, it was frequently prepared as a component of herbal tea mixtures, to treat tuberculosis and chronic. In southern Africa, *S. africana-lutea* is collected fresh when needed or sold in dried or semi-dried bundles comprising mainly of leaves or occasionally flowers and fruits. The traditional healers in the Western Cape Province prescribe the *S. africana-lutea* decoction to treat respiratory ailments, influenza, female diseases (Watt and Breyer-Brandwijk 1962), fever, headache and digestive disorders (Amabeoku *et al*. 2001).

Although *S. africana-lutea* has long been used in folk medicine for various ailments, very little scientific information has been published to validate claims about the effectiveness of this plant. The available pharmacological data indicates pharmacological activity including anti-pyretic activity in the *in vivo* tests performed on rats and analgesic activity in mice (Amabeoku *et al*. 2001). In the most recent studies, *S. africana-lutea* extracts have been found to have anti-malarial properties against the chloroquine-resistant *Plasmodium falciparum* FCR-3 strain. However, the extracts were less potent than the two anti-malarial reference drugs chloroquine diphosphate and quinine sulphate (Kamatou *et al*. 2008). Furthermore, the extracts were found to be significantly active against human colon cancer cells, with less cytotoxity against glioblastoma cells (Kamatou *et al*. 2008). In addition, the extracts are also active against the breast cancer cells (Kamatou *et al*. 2008). The methanol: chloroform (1:1 v/v) extracts of *S. africana-lutea* have anti-bacterial bioactivity against both Gram-positive (*Bacillus cereus* and *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*) (Kamatou *et al*. 2007[a]; 2007[b]). However, the best bioactivity was recorded against the Gram-negative bacteria (Kamatou *et al*. 2007[a]; 2007[b]). Furthermore, the extracts also have potent anti-mycobacterial activity (Kamatou *et al*. 2007[b]).

Table 2-1 Summary of pharmacological activities of some South African *Salvia* species

Species	Geographical distribution[a]	*In vitro* pharmacological assay	Extract type	Reference
S. *africana-caerulea* L.	Western and Eastern Cape	Anti-bacterial; anti-malarial; anti-cancer; anti-plasmodial; anti-oxidant	LE and EO	Kamatou *et al.* (2007[b]); (2008)
S. *africana-lutea* L.	Northern, Western and Eastern Cape (along the coast)	Anti-pyretic activity; anti-analgesic; anti-plasmodial; anti-malarial, anti-cancer; anti-bacterial; anti-mycobacterial; anti-oxidant	LE and EO	Amabeouku *et al.* (2001); Kamatou *et al.* (2007[b]); (2008)
S. lanceolata **Lam.**	Northern and Western Cape	Anti-cancer; anti-microbial; anti-mycobacterial; anti-malarial; anti-plasmodial; anti-oxidant	LE and EO	Kamatou *et al.* (2007[b]); (2008)
S. *albicaulis* Benth.	Western Cape	Anti-inflammatory; anti-bacterial; anti-malarial; anti-cancer; anti-plasmodial	LE and EO	Kamatou *et al.* (2007[a]); (2007[b]); (2008)
S. *aurita* L.f var. aurita	Gauteng, Limpopo, Western and Eastern Cape	Anti-malarial; anti-microbial; anti-cancer; anti-mycobacterial	LE	Kamatou *et al.* (2007[b]); (2008)
S. chamelaeagnea **Berg.**	Northern and Western Cape	Anti-microbial; anti-malarial; anti-cancer; anti-mycobacterial	LE and EO	Kamatou *et al.* (2006[a]); (2007[b]); (2008)

*S. diserma*s L.	Free State, Gauteng, Limpopo, Northern and Western Cape	Anti-microbial; anti-cancer; anti-malarial; anti-mycobacterial	LE	Kamatou *et al.* (2007[b]); (2008)
S. dolomitica Codd	Gauteng and Limpopo	Anti-microbial; anti-mycobacterial; anti-cancer; anti-inflammatory; anti-malarial; anti-plasmodial	LE and EO	Kamatou *et al.* (2007[a]); (2007[b]); (2008)
S. garipensis E. Mey. Ex Benth	Northern Cape	Anti-cancer; anti-microbial ; anti-malarial; anti-mycobacterial	LE	Kamatou *et al.* (2007[b]); (2008)
S. muirii L. Bol.	Western Cape	Anti-microbial; anti-cancer; anti-malarial	LE and EO	Kamatou *et al.* (2006[b]); (2007[b])
S. namaensis Schinz	Free State, Northern and Western Cape	Anti-cancer; anti-malarial	LE	Kamatou *et al.* (2008)
S. radula Benth.	Gauteng, Limpopo, North-West	Anti-cancer; anti-microbial; anti-mycobacterial; anti-malarial	LE	Kamatou *et al.* (2007[b]); (2008)
S. repens Burch. Ex Benth. var. *repens*	Eastern Cape, Free State, KwaZulu-Natal; Gauteng, Limpopo	Anti-inflammatory; anti-microbial; anti-malarial; anti-oxidant; anti-mycobacterial	LE and EO	Kamatou *et al.* (2005); (2008)

S. runcinata L.f.	Eastern Cape, Free State, Gauteng, KwaZulu-Natal , Limpopo, North-West, Mpumalanga	Anti-microbial*; anti-inflammatory; anti-malarial; anti-cancer; anti-mycobacterial	LE	Kamatou *et al.* (2007[b]); (2008)
S. schlechteri Briq.	Eastern Cape	Anti-cancer; anti-malarial; anti-microbial; anti-mycobacterial	LE	Kamatou *et al.* (2007[b]); (2008)
S. stenophylla Burch. Ex Benth.	Eastern Cape, Free State, Gauteng, KwaZulu-Natal, Limpopo	Anti-microbial; anti-mycobacterial; anti-cancer; anti-malarial; anti-inflammatory; anti-oxidant	LE and EO	Kamatou *et al.* (2005); (2007[b]); (2008)
S. *verbenaca* L.	North-West, Free State, Gauteng, Limpopo, Northern, Western and Eastern Cape	Anti-cancer; anti-microbial; anti-mycobacterial; anti-malarial	LE	Kamatou *et al.* (2007[b]); (2008)

[a]Geographical distribution of species is according to the Monographs by Kamatou (2006) whereas the summary of pharmacological studies in *Salvia* species has been collated from different sources. LE indicates the pharmacological analysis of non-volatile extracts prepared from leaf and stem material with aid of solvents whereas EO indicates analysis of volatile essential oil components obtained via Clevenger-type distillation. **NOTE:** All tabulated pharmacological activities were extracts of leaf material collected from the wild. This clearly indicates the lack of biotechnological manipulations of South African *Salvia* species for pharmacological and phytochemical studies. This list is not necessarily exhaustive as it was prepared from literature that was present at the time when this manuscript was written.

2.1.3 Phytochemistry of *Salvia*

The diverse medicinal uses of *Salvia* species has led to extensive phytochemical research of these plants worldwide. *Salvia* species have a large number of useful phenolic compounds that have been isolated displaying a wide range of biological activities as discussed above. These compounds are hydrophilic or hydrophobic (Chen *et al.* 1999[a;b]), belonging to various chemical groups such as essential (volatile) oils, alkaloids, terpenoids and other phenolic derivatives (Arikat *et al.* 2004). Secondary compounds from *Salvia* are found in pharmacopoeias of different countries worldwide (Tepe *et al.* 2007). Although *Salvia* species have the same phenolic compounds belonging to the same chemical groups, the constituents of the compounds are different due to their geographical distribution, and climatic conditions that they grow in but most importantly due to the genetic diversity (Arikat *et al.* 2004). For example, Skoula *et al.* (1999; 2000) found the essential oil composition of *S. fruticosa* populations to be different due to geographical distribution and genetic diversity. Many papers have been published on phytochemistry of different *Salvia* species in other parts of the world; Table 2-2 has a list of some of the publications with different phenolic compounds being identified.

Despite extensive phytochemical research on most *Salvia* species, studies on southern African species are still limited. In the study done by Kamatou *et al.* (2005), extracts from aerial parts of *S. stenophylla, S. runcinata* and *S. repens* were found to contain both volatile and non-volatile compounds. Ketones and alcohols were the most abundant non-volatile components with β-caryophyllene being the major volatile component present in large amounts (7-12%) in all three species. Rosmarinic acid, a strong anti-oxidant generally produced by *Salvia* species was found in all of three species, while carnosic acid was found only in *S. stenophylla* and *S. repens* (Kamatou *et al.* 2005). In another study, the constituents of essential oils of *S. albicaulis* and *S. dolomitica* are mainly oxygen-containing terpenoids with sesquiterpenes (47%) and monoterpenes (72%) respectively (Kamatou *et al.* 2007[a]). In the bioassay-guided fractionation of *S. radula*, anti-malarial compounds betulafolientriol oxide and salvigenin were identified (Kamatou *et al.* 2008). Moreover, five anti-bacterial constituents carnosol, 7-*O*-methylepirosmanol, oleanolic acid and ursolic acid were identified via bioassay-guided fractionation of *S. chamelaegnea* leaf crude extracts (Kamatou *et al.* 2007[b]). In the few

phytochemical studies done on *S. africana-lutea*, various chemical group compounds such as tannins, quinones, alkaloids, triterpenes, steroids, reducing sugars and saponins were detected in the foliage extracts (Amabeouku *et al*. 2001). In a recent study by Hussein *et al*. (2007), three abietane diterpenoids carnosol, rosmadial and carnosic acid were identified from ethanolic extracts of *S. africana-lutea*. Furthermore, carnosic acid was found to be more active against *Mycobaterium tuberculosis* and breast human cell-line (MCF-7) than carnosol (Hussein *et al*. 2007).

The main bioactive phenolic compounds in *Salvia* species are terpenoids and phenolics. The essential oils and their main constituents mainly monoterpenoids and sesquiterpenoids are important as flavouring and fragrance agents in food, cosmetics and perfumery. Terpenoids are also important as naturally-occurring pharmaceuticals such as artemisinin (anti-malarial), taxol (anti-cancer), sterol glycosides (for congestive heart disease), steroidal saponins (starting material for progesterone-like compounds for birth control pills) and carotenoids (vitamins) just to mention a few (Luthra *et al*. 1999). Different *Salvia* species have various terpenoid compounds and derivatives. For example, *S. albicaulis* and *S. dolomitica* have sesquiterpenes and monoterpenes respectively *(*Kamatou *et al*. 2007). Furthermore, *S. miltiorrhiza* hairy roots have mainly diterpenoid pigments predominantly the phenanthrofurane quinine derivates commonly known as tanshinones (Ge and Wu 2005[a]).

2.1.4 Biosynthesis of terpenes in *Salvia*

In vascular plants (higher plants), terpenes are synthesized via two different pathways mevalonate (MVA) and non-MVA occurring in the cytoplasm and plastids respectively (Laule *et al*. 2003) (Figure 2-3). The MVA pathway synthesizes mainly sterols, sesquiterpenes (C_{15}), ubiquinones whereas with the non-MVA pathway it is mainly hemi- (C_5) and mono- (C_{10}), and diterpenes (C_{20}) along with carotenoids (Dubey *et al*. 2003). Both these pathways are complex with several intermediate products being catalysed by different enzymes. For simplicity purposes, the key enzymes and intermediate products in production of terpenes are highlighted (for detailed reviews refer to Luthra *et al*. (1999), Dubey *et al*. (2003), Laule *et al*. (2003) and

Eisenreich *et al.* (2004)). Similarity between the two pathways is that the common precursor compounds for terpene biosynthesis are isopentenyl diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Dubey *et al.* 2003; Laule *et al.* 2003; Eisenreich *et al.* 2004).

MVA in brief involves condensation of three units of acetyl CoA to form 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) ①(Figure 2-3), and its subsequent reduction yields MVA. The reduction of HMG-CoA to MVA is catalysed by HMG-CoA reductase (HMGR) ② (Figure 2-3), which is a key regulatory enzyme of MVA pathway. MVA is then transformed to IPP via three sequential phosphorylation and decarboxylation steps ③ (Luthra *et al.* 1999; Dubey *et al.* 2003; Laule *et al.* 2003) (Figure 2-3).

In the other terpene biosynthesis pathway (non-MVA), pyruvate and D-glyceraldehyde 3-phosphate (GA-3P) are condensed to 1-deoxy-D-xylulose (DOXP) with catalysis by DOXP synthase (DXS) [**I**] (Figure 2-3). The DOXP reductoisomerase (DXR) rearranges and reduces DOXP to 2*C*-methyl-D-erythritol 4-phosphate (MEP) [**II**] (Figure 2-3) with MEP being converted into its cyclic diphosphate via sequential action of three enzyme activities. The cyclic diphosphate is transformed into IPP [**III**] (Figure 2-3) (Dubey *et al.* 2003; Eisenreich *et al.* 2004). In the second stage of both MVA and non-MVA pathways, IPP is isomerised to DMAPP (Luthra *et al.* 1999) ④ (Figure 2-3). These two isomers combine to yield terpenes via further condensations with additional IPP units followed by cyclisation, coupling and rearrangements to produce a parent carbon skeleton of the specific terpene (Luthra *et al.* 1999) ⑤ (Figure 2-3). The inhibitors of MVA and non-MVA are mevinolin and fosmidomycin respectively (Ge and Wu 2005[a]).

In recent studies, it has been established that compartmental separation of the pathways is not always absolute. It has been proven that the two independent pathways crosstalk in some plants (Dubey *et al.* 2003; Laule *et al.* 2003; Eisenreich *et al.* 2004) with small amounts of one or more unidentified metabolites being common to both pathways. Metabolites can be

exchanged in both directions via plastid membranes ⑥ (Eisenreich *et al.* 2004) (Figure 2-3). The terpenes derived from the MVA pathway become part of mono- and diterpenes in the plastid (Eisenreich *et al.* 2004) (Figure 2-3). In the study by Ge and Wu (2005[a]), the pathway for production of terpenoids (tanshinones) from *S. miltiorrhiza* was elucidated. Both biotic (yeast extract, YE) and abiotic elicitors (silver ions, Ag^+) were used to study the pathway. It was found that the activity of HMGR was only stimulated by Ag^+, and DXS was stimulated by both the YE and Ag^+, but more strongly by the YE. Furthermore, fosmidomycin (non-MVA inhibitor) inhibited the tanshinone accumulation induced by both elicitors, whereas mevinolin (MVA-pathway inhibitor) only suppressed the Ag^+-induced tanshinone accumulation. Although the results of this study suggest that the tanshinone accumulation induced by the two elicitors was synthesised mainly via the non-MVA pathway (Ge and Wu 2005[a]), tanshinone accumulation in *S. miltiorrhiza* hairy roots could also depend on crosstalk between the MVA and non-MVA pathways, however this remains to be elucidated.

On the other hand, phenolic compounds (aromatics) include a large group of chemical compounds possessing an aromatic ring bearing one or more hydroxyl groups together with a number of other side groups (Croteau *et al.* 2002; Taiz and Zeiger 2006). Although phenolic compounds have an aromatic ring, the important thing to note is that not all phenolics are aromatic. Phenolic compounds range from simple phenolics such as hydroxybenzoic acids to biphenyls (such as rosmarinic acid) to large condensed tannins with high molecular weights (Shetty *et al.* 2006). Most phenolics are readily oxidised to coloured quinine-containing products causing browning of plant tissue (Shetty *et al.* 2006). Phenolic compounds have many roles in plant growth and development. They function in defence mechanisms such as UV barriers, anti-fungal, anti-bacterial, anti-herbivory and anti-mitotic (in morphogenesis) (Lewis 1993). For instance, phytoalexins (anti-microbial phenolics) are synthesised upon pathogen attack on the infection site (Dixon *et al.* 1994; Dixon and Paiva 1995). Other phenylpropanoid compounds such as flavonoids, isoflavonoids, anthocyanins and polyphenols are also produced in response to wounding, nutritional stress, high visible light and cold stress (Lewis 1993). Among many functional roles of phytochemical phenolics is their anti-oxidant activity induced by abiotic and biotic stress. Stress initiates free radical generating processes, which change the cellular equilibrium towards lipid peroxidation (Shetty *et al.* 2006). Essential oils from various aromatic

plants have free radical-scavenging properties. In addition, flavonoids that give plants characteristic flavour and aroma have been found to have anti-cancer (anti-oxidant) properties by inhibiting lipid peroxidation of lipid cell membranes (Driver and Bhattacharya 1998). Therefore, as a defence mechanism, plant cells can be induced to produce anti-oxidant phenolics to scavenge the produced free radicals. Phenolic phytochemicals are clearly always present with important roles in higher plants and as a result they are targeted as a source of therapeutic and disease preventing in human health and wellness (Shetty *et al.* 2006).

Plant phenolics are synthesised through two basic pathways namely shikimic acid (Shetty *et al.* 2006; Taiz and Zeiger 2006; Wildman and Kelley 2007) and acetate-malonate (malonic acid) pathways (Shetty *et al.* 2006; Wildman and Kelley 2007) via several routes. The shikimic pathway is the main one in higher plants even though the malonic pathway (dominant in lower plants, fungi and bacterial) is present (Shetty *et al.* 2006; Taiz and Zeiger 2006; Wildman and Kelley 2007). Therefore, focus will be put only on the shikimic pathway. According to Taiz and Zeiger (2006) and Wildman and Kelley (2007), the precursors of the shikimic pathway are simple carbohydrate intermediates from the glycolytic and pentose phosphate pathways (PPP). These simple carbohydrates are converted to shikimic acid which is the biogenic precursor of aromatic amino acids phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) (Shetty *et al.* 2006; Taiz and Zeiger 2006; Wildman and Kelley 2007) (Figure 2-4). These aromatic amino acids are considered the primary products that are of great importance to the biosynthesis of secondary metabolites in plants (Shetty *et al.* 2006; Wildman and Kelley 2007). Most secondary phenolics are produced from the deamination of Phe to form cinnamic acid catalysed by phenylalanine ammonia lyase (PAL) (Figure 2-4). Subsequent reactions lead to the addition of more hydroxyl groups and other constituents to form simple phenolic compounds called phenylpropanoids (contain benzene ring and three carbon side chain) (Shetty *et al.* 2006; Wildman and Kelley 2007). Phenylpropanoids are building blocks of more complex phenolic compounds. Depending on the species requirements and different specific environmental growth conditions, the type of phenolics synthesised can include tannins, caffeic acids, and rosmarinic acids amongst others, which are highly anti-oxidative (Shetty *et al.* 2006). For instance, according to Wildman and Kelley (2007), during the fungal infection the expression of PAL is increased. It is therefore presumed that caffeic acid is one of the highly synthesised

phenolics in most *Salvia* species as Lu and Foo (2002) and Jiang *et al.* (2005) reported that caffeic acid is the building block of most sage phenolic compounds.

Since the phytochemical studies of *S. africana-lutea* are still at their infancy, specific biosynthetic pathway(s) of its secondary metabolites are not yet elucidated. For the purpose of this study, it is assumed that the terpenoids are biosynthesised via either one or both MVA and non-MVA pathway(s) whereas the aromatic phenolic compounds are synthesised via the shikimic acid and/or acetate-malonate pathway(s).

The phytochemical and pharmacological research on most of *Salvia* species has then led to the *in vitro* biotechnological manipulation for secondary metabolite production. There has been a considerable effort in the use of plant cell cultures as an alternative for production of pharmaceutically-active compounds unique to plants (Dornenburg and Knorr 1995).

Mevalonate pathway

Acetyl-CoA

①

HMG-CoA

Mevinolin —| HGMR

②

Mevalonate

③

④ DMAPP ← IPP

⑤

Sesquiterpenes

Triterpenes

Cytoplasm

Non-mevalonate pathway

Pyruvate+GA-3P

DXS | I

1-deoxy-D-xylulose 5-phosphate

II DXR |— *Fosmidomycin*

2-C-methyl-D-erythritol 4-phosphate

III

④ IPP ← → DMAPP

⑤

Diterpenes

Monoterpenes

Plastid

⑥
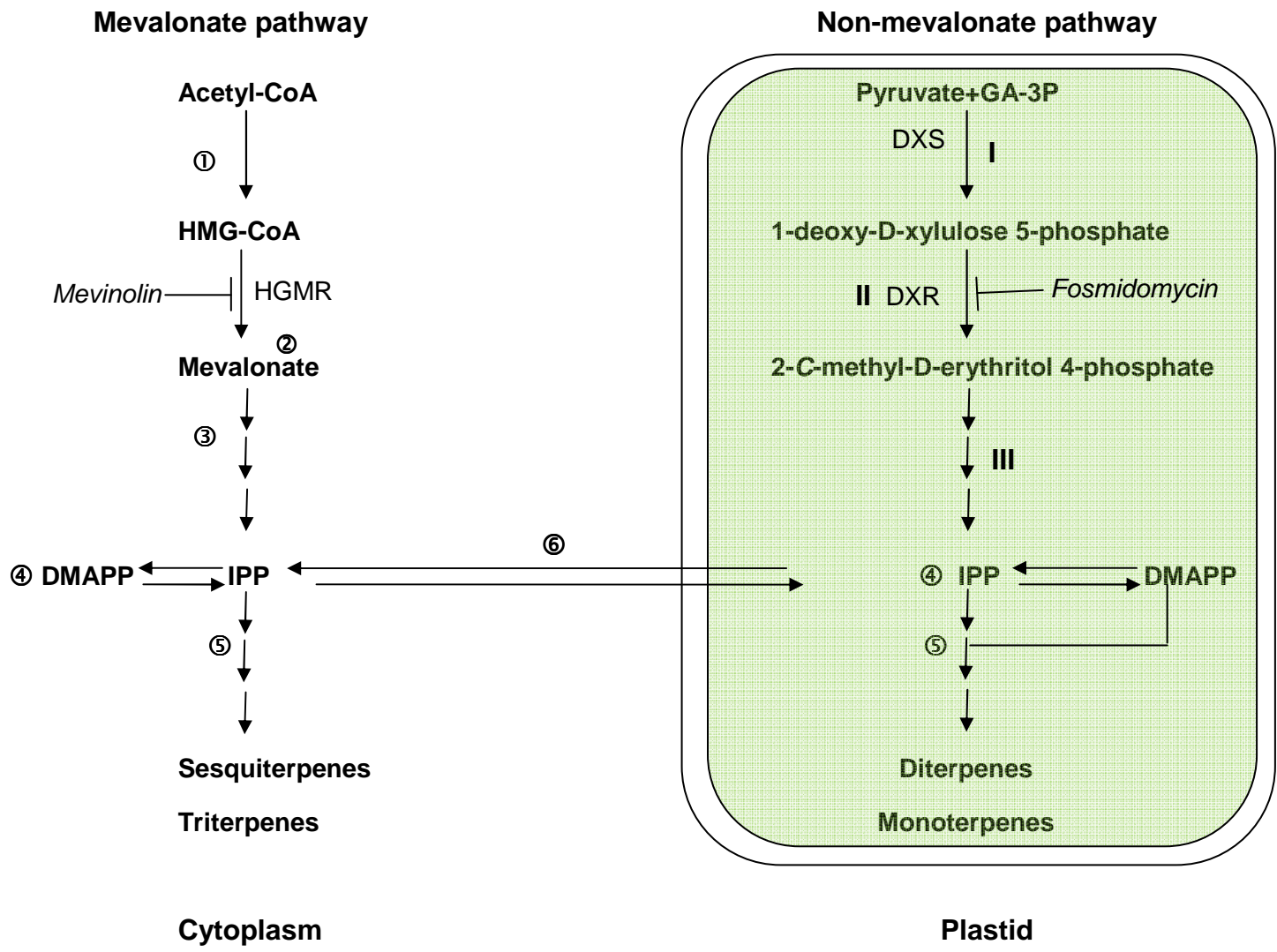
Figure 2-3 Summarised illustration of the cytoplasmic mevalonate (MVA) pathway and plastidial non-mevalonate (non-MVA) pathway for the biosynthesis of terpenoids in plant cells (Ge and Wu 2005[a])
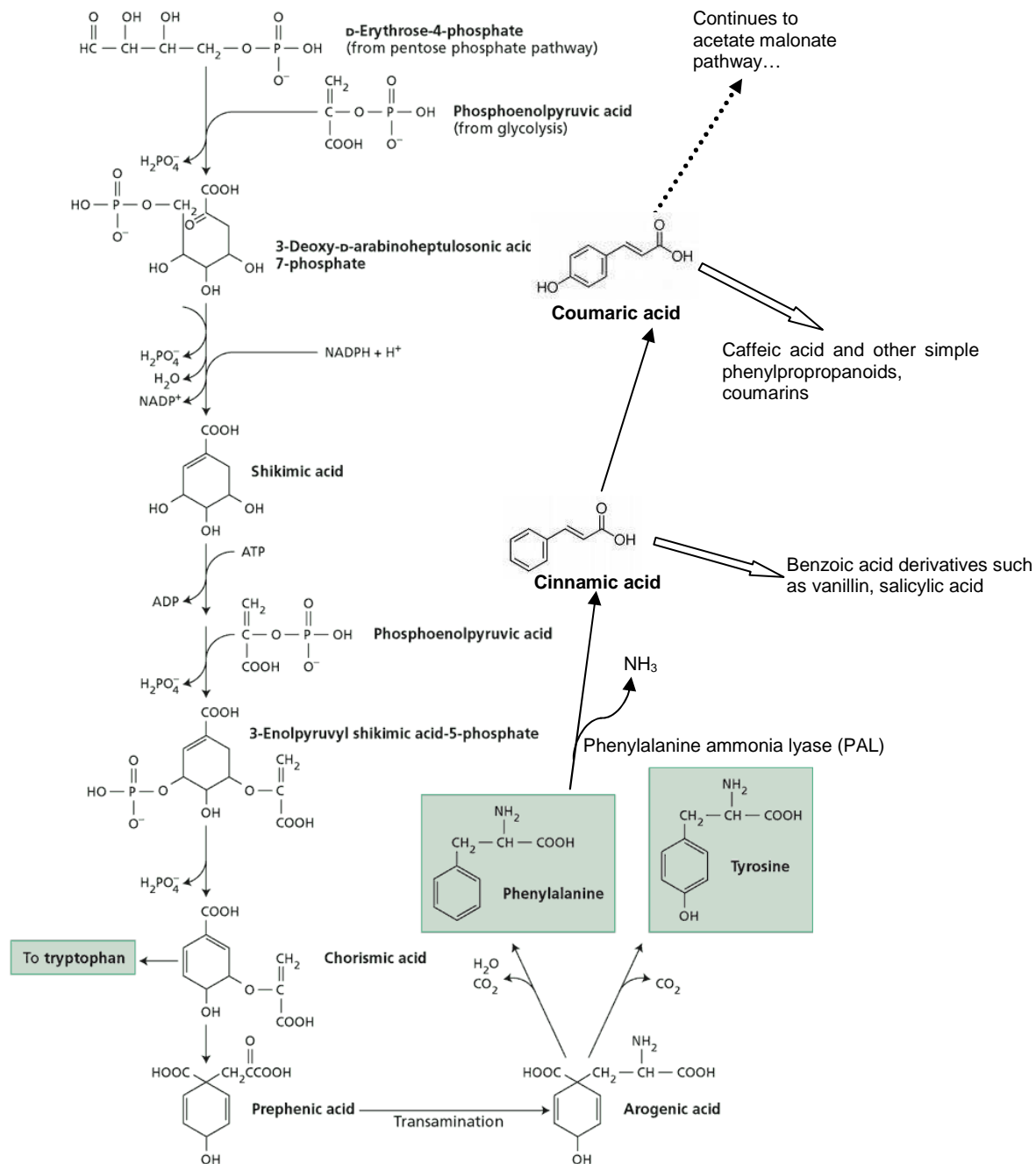
Figure 2-4 Shikimic acid pathway adapted from Taiz and Zeiger (2006) and Wildman and Kelley (2007)

2.2 BIOTECHNOLOGICAL APPLICATIONS ON *SALVIA* SPECIES

Most recently, there has been an enormous increase in the number of scientific reports outlining biotechnological applications on medicinal plants. The recent shift to application of biotechnological techniques on medicinal plants by scientists worldwide (among many reasons) relates to a growing reliance on pharmaceutical and aromatherapeutic products produced by plants. However, the main drawback of the natural products is that they are produced in relatively minute quantities from a large amount of plant material. Furthermore, some source plant species grow only under specific environmental conditions. Even so, they give a poor unreliable yield of the product (Panagiotopoulos *et al.* 2000) which is dependent on climatic conditions of the growing season. Scientists worldwide are therefore exploring biotechnological manipulations of medicinal plants. Biotechnology has wide applications in the medicinal plant industry with many advantages. Reliable and stable high quality bioactives are produced under controlled conditions without any climatic and geographical limitations (Collin 2001). Furthermore, the yield of the bioactives can be manipulated through genetic alterations of the medicinal plant(s) of interest (Bajaj and Ishimaru 1999; Nigro *et al.* 2004). Genetic modification allows further studies such as secondary metabolism by identifying the key regulatory enzymes catalysing production of the key metabolites. In this way, the pathway(s) can be manipulated for the optimisation and regulation for production of different metabolites *in vitro* (Nigro *et al.* 2004). The other advantage of application of biotechnological techniques on medicinal plants is that the biotechnologically-manipulated plants seem to produce novel compounds. Also, these *de novo* pathway(s) can be studied *in vitro* (Bajaj and Ishimaru 1999) with the possibility of subsequent breeding and production of high-producing lines of the novel compounds (Nigro *et al.* 2004). The novel compound-producing cultures along with other up-regulated compounds have the potential to be commercialised thus meeting the commercial natural product demands. Biotechnological tools such as micropropagation and transgenic technologies have been applied on various *Salvia* species.

2.2.1 Plant tissue culture

Plant tissue culture is a vital technique of plant science research and plant biotechnology. Plant tissue culture refers to the aseptic cultivation of any plant section (explant) such as single cell, a tissue or an organ in an enclosed vessel under controlled conditions (Hartmann *et al*. 2002). Other plant cultures include seedlings (whole plants), embryos (both embryonic and somatic) and protoplasts (Hartmann *et al*. 2002). Plant cells are biosynthetically totipotent with each cell retaining all genetic information *in vitro*, thus capable of producing a whole plant irrespective of the explant's origin (George 1993; Hartmann *et al*. 2002). Explant regeneration depends on the medium that it is placed onto. *In vitro* medium can be either solid (solidified by agar or gellan gum such as Gelrite®) or liquid, containing growth nutrients. The growth nutrients used in plant cell cultures include inorganic macro- and micro-elements, organic nutrients (vitamins and amino acids), plant growth regulators (PGRs) and a carbon source (George 1993). Media composition has a very important effect on cell growth and secondary metabolite accumulation. The media has to be optimized for specific cell line production (Giri and Narasu 2000). For instance, the media requirements for cell proliferation (primary metabolism) and secondary metabolite biosynthesis often require different nutrients (Giri and Narasu 2000). In the latter, it is because secondary metabolites are not associated with cell growth.

Plant tissue culture is used for mass propagation (micropropagation) of plant species. Micropropagation of plants for mass cultivation and production of plantlets in culture has been a useful vegetative propagation process used in agriculture, horticulture, forestry and recently for plant biotechnology. Micropropagation can be through somatic embryogenesis and cultivation of shoot tips or axillary buds and adventitious shoots (Hartmann *et al*. 2002).The development and application of micropropagation techniques for propagation of medicinal plants are important for rapid large-scale production of medicinal plant populations thus conserving the wild populations (Nigro *et al*. 2004). In addition, micropropagation can be combined with other biotechnological techniques to improve medicinal plants through somaclonal variation and genetic transformation (Nigro *et al*. 2004; Zhou and Wu 2006). Most studies for secondary metabolite production use a liquid culture system. Liquid cultures provide homogeneous culture conditions which support more rapid growth and potential for up-scaling the cultures (Collin 2001). Due to

the inherent totipotency in each explant, plant cells are theoretically capable of producing a range of secondary metabolites found in the parent plant (Fu 1998). In addition, plant tissue culture can also be used for production of compounds that are not normally found in the parent plant (novel compounds) (Bajaj and Ishimaru 1999). Furthermore, plant tissue culture can be used for the biotransformation of low-cost precursors into valuable compounds (Kirakosyan 2006). For experimental studies, small volume (10-100 ml) liquid cultures are usually maintained in Erlenmeyer flasks on shaking incubators (shake cultures). The common medicinal plant shake cultures are hairy roots (See Section 2.2.4) incubated in the dark for secondary metabolite synthesis.

2.2.2 Plant tissue cultures of *Salvia* species

The tissue culture of *Salvia* species is limited to a few species with mainly callus induction from various explants for secondary metabolite production (Table 2-2). Ruffoni *et al.* (2005) reported the *in vitro* culture and callus production of several *Salvia* species. So far, *S. miltiorrhiza* is the only species that is highly manipulated biotechnologically (Kintzios 2000). The first *Salvia* species to be micropropagated was *S. officinalis* using shoot buds as explants (Olszowska and Furmanowa 1990). The subsequent *in vitro* micropropagation protocols were on *S. leucantha* (Hosoki and Tahara 1993) and *S. canariensis* (Molina *et al.* 1997) using apical and axillary shoot explants respectively. In addition, 19 *Salvia* species were micropropagated using axillary and apical bud explants (Mascarello *et al.* 2006). Several explants have been used for establishment of tissue cultures (both plantlet and callus) systems from *Salvia* species. Some examples of explants include the seeds of *S. miltiorrhiza* (Kintzios 2000), shoot tips of *S. miltiorrhiza* (Morimoto *et al.* 1994), shoots with axillary buds of *S. canariensis* (Luis *et al.* 1992), young leaves of *S. officinalis* and *S. fruticosa* (Kintzios *et al.* 1999); zygotic embryos of *S. sclarea* (Kintzios 2000) and seedlings from *in vitro* germinated seeds of *S. miltiorrhiza* (Miyasaka *et al.* 1989; Gao *et al.* 1996). The main challenge associated with *in vitro* culturing of *Salvia* species is the browning of the explant, which leads to explant death in most cases (Kintzios 2000). The browning effect is both genotype- and culture medium-dependent. For instance, additions of 1-napthalene acetic acid (NAA) and 6-bezyladenine (BAP) to the *S. officinalis* culture medium promoted callusing and explant necrosis (Kintzios 2000). Explant browning was greatly reduced by the addition of

ascorbic acid (10 mg L^{-1}) to the culture medium (Kintzios 2000). Tissue culture protocols of *Salvia* species have and are still continually being optimised for different species. Manipulations include the use of different PGR types and combinations as well as environmental conditions such as light and temperature (Kintzios 2000).

The most frequently used basal medium is the Murashige and Skoog (MS) (1962). Occasionally, other basal media such as modified Nitsch and Nitsch (1969) (NN) (Olszowska and Furmanowa 1990) and medium containing MS major salts in combination with the Ringe and Nitsch (1968) minor elements (Hosoki and Tahara 1993) have been occasionally used. In addition to growth nutrients (macro and micro), PGRs such as cytokinins and auxins have been reported to be important for *in vitro* dedifferentiation and regeneration of *Salvia* species. Different cytokinins such as kinetin (Olszowska and Furmanowa 1990; Morimoto *et al.* 1994; Cuenca and Amo-Marco 2000), 6-γ-γ dimethyl-allylaminopurine (2iP) (Cuenca and Amo-Marco 2000), and 6-benzylaminopurine (BA) (Frett 1986; Tawfik *et al.* 1992; Hosoki and Tahara 1993) are effective for shoot proliferation in various *Salvia* species at specific concentrations along with auxins. The requirement of auxin for *in vitro* rooting of microshoots also varies with *Salvia* species (Olszowska and Furmanowa 1990; Hosoki and Tahara 1993; Cuenca and Amo-Marco 2000). In addition, an auxin 2,4-dichlorophenoxyacetic acid (2,4-D) at species-specific concentrations has been highly used in most *Salvia* species for *in vitro* induction of callus cultures (Table 2-2). Generally, the optimal culture conditions for various *Salvia* species are 25 ±2 $^{\circ}$C over a 16-hour photoperiod under photosynthetic photon flux density (PPFD) ranging between 25-250 µmol m^{-2} s^{-1} depending on the species (Kintzios 2000). Refer to Table 2-2 for more specific culture conditions of various *Salvia* species.

Biotechnological techniques do not only facilitate *in vitro* plant propagation but also the production of some important bioactive metabolites. Generally, it is preferable to establish a successful working tissue culture protocol to allow further molecular manipulation using biotechnological tools. However, tissue cultured plant material is not a prerequisite for all transformation techniques (Birch 1997).

Table 2-2 Summary of biotechnological applications on some *Salvia* species worldwide

Plant species	Explant	*In vitro* culture	Product	Medium	Reference
S. miltiorrhiza	*In vitro* shoots	Hairy root	**Phenolics:** lithospermic acid B, rosmarinic acid	**MS, MS (without NH₄NO₃)** (Murashige and Skoog 1962), **B5** (Gamborg *et al.* 1968), **WPM** Woody Plant media (Lloyd and McCown 1980) and **6,7-V** (Veliky and Martin 1970)	Chen *et al.* (1999[a])
S. miltiorrhiza	*In vitro* plantlets	Cell suspension culture	**Diterpenoids (tanshinones):** tanshinone I, tanshinone IIA, cryptotanshinone	**PGR-free MS, ½MS** (Murashige and Skoog 1962), **6,7-V** (Veliky and Martin 1970) and **B5** (Gamborg *et al.* 1968)	Chen *et al.* (1997)
S. miltiorrhiza	*In vitro* shoots	Cell suspension culture	**Phenolics:** lithospermic acid B, rosmarinic acid	**PGR-free B5** (Gamborg *et al.* 1968)	Chen *et al.* (1999[b])
S. miltiorrhiza	*In vitro* shoots	Cell suspension culture	**Diterpenoids (tanshinones):** cryptotanshinone	**PGR-free B5** (Gamborg *et al.* 1968) elicited with methyl jasmonate and/or salicylic acid	Chen and Chen (1999[a])

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S. miltiorrhiza	*In vitro* shoots	Cell suspension culture	**Diterpenoids (tanshinones):** tanshinone I, tanshinone IIA, cryptotanshinone **Phenolics:** lithospermic acid B, rosmarinic acid	**PGR-free 6,7-V** (Veliky and Martin 1970)	Chen and Chen (1999[b])
S. sclarea L.	*In vitro* shoots	Calli and cell suspension culture	**Deterpenoid:** Sclareol	**MS** (Murashige and Skoog 1962) supplemented with 2,4-D+kinetin **MS** (Murashige and Skoog 1962) supplemented with NAA+ BAP	Banthorpe *et al.* (1990)
S. miltiorrhiza	*In vitro* shoots	Cell suspension culture	**Deterpenoids:** cryptotanshinone and ferruginol	**MS** (Murashige and Skoog, 1962) without Fe-EDTA, supplemented with kinetin	Miyasaka *et al.* (1987)
S. miltiorrhiza	*In vitro* shoots	Cell suspension culture (two-stage)	**Deterpenoids:** cryptotanshinone and ferruginol	**1st stage:** **MS** (Murashige and Skoog, 1962) supplemented with 2,4-D **2nd stage:** **MS** (Murashige and Skoog, 1962) without Fe-EDTA, supplemented with 2,4-D	Miyasaka *et al.* (1986[a])

S. miltiorrhiza	*In vitro* shoots	Immobilized cells	**Deterpenoids:** cryptotanshinone and ferruginol	<u>1st stage:</u> **MS** (Murashige and Skoog, 1962) supplemented with 2,4-D <u>2nd stage:</u> **MS** (Murashige and Skoog, 1962) without Fe-EDTA, supplemented with 2,4-D	Miyasaka *et al.* (1986[b])
S. miltiorrhiza	*In vitro* shoots	Cell suspension culture	**Deterpenoids:** ferruginol	**MS** (Murashige and Skoog, 1962) supplemented with 2,4-D and kinetin	Miyasaka *et al.* (1985)
S. fruticosa Mill.	Shoot tips	Shoots	**Essential oils:** α-pinene, 1,8-cineole, camphor and borneol	Shoot induction: **MS** (Murashige and Skoog 1962), **NN** (Nitch and Nitch 1969) and **B5** (Gamborg *et al.* 1968) supplemented with BA and NAA Shoot multiplication: **MS** (Murashige and Skoog 1962) supplemented with BA and thidiazuron (TDZ)	Arikat *et al.* (2004)
S. sclarea L.	*In vitro* shoots	Hairy roots	**Abietane diterpenoids:** salvipisone, aethiopinone, 1-oxoaethiopinone and ferruginol	**PGR-free ½B5** (Gamborg *et al.* 1968)	Kuźma *et al.* (2007)

Species	Explant source	In vitro culture	Metabolites	Basal medium	References
S. sclarea L.	*In vitro* shoot internodes	Hairy roots	**Abietane diterpenoids:** salvipisone, aethiopinone, 1-oxoaethiopinone and ferruginol **Ursene triterpenoids:** 2a,3a-dihydroxy-urs-12-en-28-oic acid and 2a,3a,24-trihydroxy-urs-12-en-28-oic acid	**PGR-free ½B5** (Gamborg *et al.* 1968)	Kuźma *et al.* (2006)
S. miltiorrhiza	*In vitro* shoots	Calli and cell suspension	**Diterpenoid (tanshinone):** cryptotanshinone **Phenolics:** Rosmarinic acid	**PGR-free 6,7-V** (Veliky and Martin 1970) elicited with yeast extract	Chen and Chen (2000[a])
S. officinalis L.	<u>*In vitro* shoots:</u> *In vitro* seed germinated seedlings <u>Cell and calli:</u> *In vitro* shoots	Cell, calli and shoots	**Phenolics:** Rosmarinic acid **Diterpenoids:** carnosic acid and carnosol	<u>Calli and cells:</u> **MS** (Murashige and Skoog 1962) supplemented with NAA, 2,4-D and benzylaminopurine (BAP) <u>Shoots:</u> **MS** (Murashige and Skoog 1962) supplemented with indole-3-acetic acid (IAA) and BAP <u>Hairy roots:</u> **PGR-free WPM** (Lloyd and McCown 1980)	Grzegorczyk *et al.* (2007)

Species	Explant	Culture type	Metabolites	Medium/Elicitor	Reference
S. miltiorrhiza	*In vitro* shoots	Hairy roots	**Phenolic:** Rosmarinic acid	**PGR-free MS** (Murashige and Skoog 1962) elicited with yeast extract and Ag^{+}	Yan *et al*. (2006)
S. miltiorrhiza	*In vitro* shoots	Hairy roots	**Diterpenoids (tanshinones):** tanshinone I, tanshinone IIA, cryptotanshinone	**PGR-free MS (without NH$_4$NO$_3$)** (Murashige and Skoog 1962) elicited with yeast extract	Yan *et al*. (2005)
S. miltiorrhiza	*In vitro* shoots	Hairy roots	**Diterpenoids (tanshinones):** tanshinone I, tanshinone IIA, tanshinone IIB, cryptotanshinone, dihydrotanshinone I and methylenetanshiquinone **Phenolics:** lithospermic acid B, rosmarinic acid	**PGR-free 6,7-V** (Veliky and Martin 1970) elicited with yeast extract	Chen *et al*. 2001
S. miltiorrhiza	*In vitro* shoots	Hairy roots	**Diterpenoid (tanshinone):** cryptotanshinone	**PGR-free 6,7-V** (Veliky and Martin 1970) elicited with methyl viologen, hydogen peroxide (H$_2$O$_2$), diphenylene iodonum (DPI) and yeast extract	Chen and Chen (2000[b])
S. miltiorrhiza	*In vitro* shoots	Calli suspension	**Diterpenoid (tanshinone):** cryptotanshinone	**PGR-free B5** (Gamborg *et al*. 1968) elicited with methyl viologen, H$_2$O$_2$, DPI and yeast extract	Chen and Chen (2000[a])

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S. miltiorrhiza	*In vitro* shoots	Hairy roots	**Diterpenoids (tanshinones):** tanshinone I, tanshinone IIA, tanshinone IIB, tanshinone V, dihydrotanshinone I, cryptotanshinone, tanshinone VI **Other diterpenoid:** ferruginol.	**PGR-free MSoH[#]** (Murashige and Skoog 1962) without ammonium nitrate	Zhi and Alfermann (1993)
S. officinalis L.	*In vitro* Internodal segments	Calli and cell suspensions	**Phenolics:** gallic acid, 3-*O*-caffeoylquinic acid, 5-O-caffeoylquinic acid, caffeic aci, rosmarinic acid, hesperetin, epirosmanol, hispidulin, genkwanin, carnosol, carnosic acid and methyl carnosate.	**MS** (Murashige and Skoog, 1962) supplemented with 2,4-D in presence of BA or zeatin or kinetin	Santos-Gomes *et al.* (2003)
S. fruticosa	Calli: *in vitro* leaves Roots: *in vitro* non-transgenic roots	Calli, cell and non-transgenic root suspensions	**Phenolic:** Rosmarinic acid	Cell and calli: **MS** supplemented with TDZ and IAA Roots: **B5** (Gamborg *et al.* 1968) supplemented with NAA	Karam *et al.* (2003)

S. fruticosa **and** *S. officinalis*	<u>Calli</u>: *In vitro* and *ex vitro* leaves <u>Embryo</u>: *in vitro* callus	Calli and somatic embryo suspensions	**Phenolic:** Rosmarinic acid	<u>Calli</u>: **MS** (Murashige and Skoog, 1962) supplemented with 2,4-D and kinetin <u>Somatic embryos</u>: **PGR-free MS** (Murashige and Skoog, 1962)	Kintzios *et al.* (1999)
S. officinalis	*In vitro* shoots	Calli and cell suspension	**Phenolic:** Ursolic acid	**MS** (Murashige and Skoog, 1962) supplemented with NAA and BA	Bolta *et al.* (2000)
S. valentina **Vahl and** *S. blancoana* **Webb & Heldr subsp.** *marieolensis*	Apical shoot tips and nodal segments	Shoots	**Plant tissue culure only**	**MS** (Murashige and Skoog, 1962) supplemented with 2iP, BA and kinetin	Cuenca and Amo-Marco (2000)
S. chamelaeagnea	Leaf	Calli suspension	**Phenolics:** Rosmarinic acid	<u>Calli</u>: **MS** (Murashige and Skoog,1962) supplemented with 2,4-D	Huang and van Staden (2002)

Species	Explant	Product	Metabolites	Medium	Reference
S. miltiorrhiza	*In vitro* shoots	Hairy root	**Diterpenoids (tanshinones):** Tanshinone I, tanshinone II$_A$, tanshinone II$_B$, tanshinone V, dihydrotanshinone I, cryptotanshinone, tanshinone VI, and **Diterpene:** ferruginol	**PGR-free MS** (Murashige and Skoog, 1962)	Hu and Alfermann (1993)
S. canariensis	Axillary buds	Plantlet	**Diterpenoids:** 16-acetoxycarnosol, carnosic acid, 11-acetoxycarnosic acid and carnosol	**MS** (Murashige and Skoog, 1962) supplemented with BAP	Luis *et al.* (1992)
S. leucantha Cav.	Apical shoots	Shoot	**Plant tissue culture only**	**MS** major salts+ Fe-EDTA (Murashige and Skoog, 1962) combined with **Ringe and Nitch** minor salts (Ringe and Nitch 1968) supplemented with BA	Hosoki and Tahara (1993)
Several *Salvia* species (total number of 19)	*Ex vitro* axillary and apical buds	Plantlet	**Plant tissue culture only**	**MS** (Murashige and Skoog, 1962) supplemented with BA and kinetin	Mascarello *et al.* (2006)
S. nemorosa	*In vitro* shoot tips / *In vitro* leaves	Plantlet	**Plant tissue culture only**	**MS** (Murashige and Skoog, 1962) supplemented with BA and IAA	Skala and Wysokińska (2004)

S. officinalis L.	Apical and axillary buds	Plantlet	**Essential oils:** monoterpenes, oxygenated monoterpenes, sesquiterpenes, oxygenated sesquiterpenes and diterpenes	**BM** containing macronutrients according to Murashige and Skoog (1962), micronutrients of Nitsch and Nitsch (1969) supplemented with BAP	Avato *et al.* (2005)
S. miltiorrhiza	*In vitro* shoots	Hairy root	**Diterpenoids (tanshinones):** tanshinone I, tanshinone IIA, cryptotanshinone	**PGR-free MS** (Murashige and Skoog 1962) elicited with Ag+ and/or yeast extract	Ge and Wu (2005[a])
S. miltiorrhiza	*In vitro* shoots	Hairy root	**Diterpenoids (tanshinones):** tanshinone I, tanshinone IIA, cryptotanshinone	**PGR-free MS** (Murashige and Skoog 1962) elicited with YE and/or ß-aminobutyric acid (BABA), and methyl jasmonate (MeJA)	Ge and Wu (2005[b])
S. sclarea L.	Immature zygotic embryo cotyledons and calli	Plantlet	**Plant tissue culture only**	<u>Calli</u>**: MS** (Murashige and Skoog, 1962) supplemented with 2,4-D and NAA <u>Shoot</u>: **MS** (Murashige and Skoog, 1962) supplemented with BA, IAA, NAA and GA_3	Liu *et al.* (2000)
S. officinalis L.	*In vitro* shoot tips	Plantlet	**Plant tissue culture only**	**MS** (Murashige and Skoog, 1962) supplemented with TDZ	Tawfik and Mohamed (2006)

S. officinalis L.	Nodular meristematic callus	Plantlet	**Plant tissue culture only**	**MS** (Murashige and Skoog, 1962) supplemented with BA or ascorbic acid	Tawfik and Mohamed (2007)
S. officinalis L.	Shoot tips	Calli (solid medium)	**Plant tissue culture only**	**MS** (Murashige and Skoog, 1962) supplemented with TDZ	Tawfik and Mohamed (2007)
S. brachyodon **Vandas**	Shoot nodal segments	Plantlet	**Plant tissue culture only**	**MS** (Murashige and Skoog, 1962) supplemented with BAP and TDZ	Mišić *et al.* (2006)
S. chamelaeagnea	Calli and nodal shoots	Plantlet	**Phenolics:** Rosmarinic acid	**MS** (Murashige and Skoog, 1962) supplemented with BA	Huang and van Staden (2002)

#MSoH- PGR-free MS (Murashige and Skoog, 1962) with 1g L^{-1} casamino acids, 2% sucrose

2.2.3 Recombinant DNA technology

The aim of genetic modification is to add or enhance beneficial characteristics in plants in order to produce high metabolite-producing varieties that would otherwise be slow, costly, or impossible to achieve through conventional plant breeding (Sonnewald 2003). The plants' genome can be modified by the inclusion of foreign gene(s), which gives plant biotechnologists unique opportunities to up- or down-regulate metabolite biosynthesis thus revealing the nature, functional and physiological importance of secondary compounds in plants. Moreover, the emergence of recombinant DNA technology opened possibilities of directly modifying the expression of genes related to natural product biosynthesis (Kirakosyan 2006). With reference to medicinal plants, recombinant DNA technology focuses on metabolic and genetic engineering of biosynthetic pathways in order to improve production of highly valuable secondary compounds in plant cells (Kirakosyan 2006).

Furthermore, plant biotechnology has since developed from focussing only on *in vitro* cell and tissue culture and production of important products to more advanced approaches, which include high-throughput methodologies for functional analyses at transcript, protein and metabolite levels. Current plant biotechnology methodologies also allow genome modification by both homologous and site-specific recombination in a more precise and controlled manner (Kirakosyan 2006).

Various genetic engineering tools such as the recombinant DNA technology and transgenic cultures such as callus, cell suspension, immobilized and hairy root cultures have been applied on different *Salvia* species to facilitate production of secondary compounds. Recombinant DNA technology was used to study the production of the essential oil monoterpene component (+)-3-carene in the peltate glandular trichomes of *S. stenophylla* (Hoelscher *et al.* 2003). Furthermore, the key enzyme (3-carene synthase) catalysing production of the (+)-3-carene was elucidated from the cDNA library prepared from mRNA of the trichomes (Hoelscher *et al.* 2003).

2.2.4 *Agrobacterium*-mediated genetic modification

In addition to the use of recombinant DNA technology, direct and indirect gene transfer methods have been used in medicinal plants (Nigro *et al.* 2004). Amongst these transformation methods, *Agrobacterium*-mediated gene transfer is the mostly used (Nigro *et al.* 2004). The use of *Agrobacterium rhizogenes,* which induces hairy root syndrome in the host plant, is preferred in medicinal plants (Chilton *et al.* 1982). The hairy root syndrome occurs on some dicotyledonous plants after being infected by the soil bacterium, *A. rhizogenes.* This disease is associated with abundant proliferation of adventitious roots (Moore *et al.* 1979) at the infection site. The molecular basis of the hairy root syndrome is the transfer and stable integration of a defined fragment from the root-inducing plasmid (Ri plasmid) called the transfer DNA (T-DNA) into the plant nuclear genome leading to plant cell transformation (Bevan and Chilton, 1982). During the transfer of the T-DNA, certain phenolic compounds such as acetosyringone and alpha-hydroxy-acetosyringone present in the acidic plant wound sap attract *A. rhizogenes* (White and Nester 1980; Tepfer 1984; Tepfer and Casse-Delbart 1987). These phenolic compounds serve as inducers or co-inducers of the bacterial virulence (*vir*) genes located on the Ri plasmid (Gelvin 2000). The *vir* genes are genetic factors that are not transferred to the plant cell but instead they code for factors (proteins) that lead to the recognition of the wounded plant host, excision, transfer and integration of the T-DNA into the host plant genome (White and Nester 1980; Tepfer 1984). The integration and expression of the genes encoded by the T-DNA into genomic DNA of the host plant cells induces massive proliferation of adventitious transgenic roots characterized by abundant root hairs thus the name 'hairy roots' (White and Nester 1980; Tepfer 1984; Tepfer and Casse-Delbart 1987). Once produced, hairy roots can then be cultured aseptically in a liquid medium without any exogenous supply of plant growth regulators.

The induction of hairy roots is due to the expression of the oncogenes found on the Ri plasmid (Tepfer 1984; White *et al.* 1985; Spéna *et al.* 1987). The *A. rhizogenes* strains are divided into four classes: agropine, cucumopine, mannopine (Petit *et al.* 1983) or mikimopine (Casanova *et al.* 2005) based on the type of opine they catabolise (Petit *et al.* 1983). Opines are bacterial metabolites (Petit *et al.* 1983) secreted by host cells into the soil for catabolism by agrobacteria (Nilsson and Olsson 1997). In this sense, the transformation process benefits agrobacteria by transforming the host plant into a

producer of its custom-made food (Nilsson and Olsson 1997). Each of the *A. rhizogenes* strains have genes encoding for specific opine synthases that are also integrated and expressed in the host cells. The most studied Ri plasmid is the agropine A4 plasmid (Nilsson and Olsson, 1997). It can transfer two T-DNAs denoted T_L (left-stretch of T-DNA) and T_R (right-stretch of T-DNA) to the plant cell (Tepfer 1984; White *et al.* 1985). The T_R-DNA contains genes homologous to auxin biosynthesis (*tms$_1$* and *tms$_2$*) and genes encoding synthases for agropine (*ags*) (Nilsson and Olsson 1997). The sequence of the T_L-DNA of RiA4 plasmid has 18 open reading frames (ORFs) (Slightom *et al.* 1986). Functional analysis of the T_L-DNA of the agropine A4 plasmid revealed four loci out of the potential 18 loci on the T_L-DNA that play a crucial role in the formation of hairy roots (White *et al.* 1985). These loci were denoted root locus (*rol*) genes *A* (ORF 10), *B* (ORF 11), *C* (ORF 12) and *D* (ORF 15) (White *et al.* 1985; Spéna *et al.* 1987) (Figure 2-5). It must be noted that the T_R-DNA is only found in the agropine-type Ri plasmids. Other *A. rhizogenes* strains producing mannopine, cucumopine or mikimopine transfer a single fragment of T-DNA homologous to the agropine T_L-DNA but without the *rol D* gene (Meyer *et al.* 2000).

In the agropine strains, the T_L-DNA (carrying the *rol* genes) is adequate in itself to induce the hairy root disease (Jouanin *et al.* 1987). However, in some cases, the T_L-DNA is not enough and the presence of T_R-DNA is necessary as it improves the host range for infection to a great extent (White *et al.* 1985). In fact, the T_R-DNA enhances the endogenous biosynthesis of auxin of the plant, which in turn enhances the action of *rol* genes on T_L-DNA (Nilsson and Olsson 1997), controlling the intensity of the hairy root syndrome.
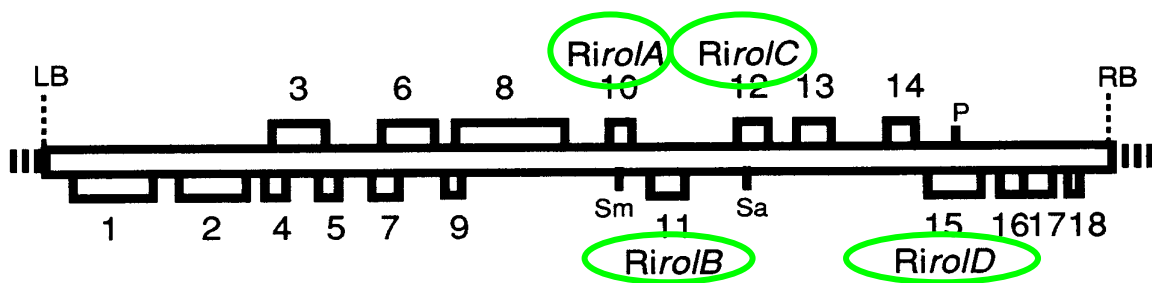
Figure 2-5 Schematic map of ORFs in the T$_L$-DNA of pRiA4b of *A. rhizogenes* (Aoki and Syōno 2000). LB-left border; RB-right border of T$_L$-DNA. Circles indicate the *rol* genes.

White *et al.* (1985) studied the role of individual *rol* genes on hairy root development using insertional mutagensis on *Kalanchoe* leaves. The roots produced by the *rol A* mutant T-DNA grew straight instead of curly compared to the wild-type. Root initiation was completely inhibited in the *rol* B mutants whereas the *rol* C mutants retarded root growth. Similarly, the *rol* D mutants caused severe root retardation and increased callusing (White *et al.* 1985). Total inhibition of root induction by *rol* B mutants illustrated the crucial role of *rol* B in promoting root differentiation. Both *rol* C and *rol* D mutants retarded root growth, suggesting their key role in root elongation (White *et al.* 1985). Spéna *et al.* (1987) repeated infections done previously by White *et al.* (1985) on *Kalanchoe* leaves using a gain-of-function approach instead of a mutagenesis strategy. Furthermore, different *A. rhizogenes* strains bearing different combinations of *rol* genes were co-infected with auxin-synthesizing genes. The absolute requirement of *rol B* on induction of hairy roots was again verified when no root formation was initiated from any construct without *rol B* gene. In addition, significant hairy root production was induced when *rol B* was expressed alone. The strongest response however, was produced by combined action of the three *rol* genes namely *rol* A, *rol* B and *rol C* (Spéna *et al.* 1987). Similarly, Spéna *et al.* (1987) provided the same evidence when tobacco leaf disc

explants were infected with *A. rhizogenes*. However, co-inoculations with auxin-synthesizing genes were not performed. The *rol* B gene did not only induce hairy root formation by itself, but also required the expression of the *rol* A *gene*. On the other hand, the *rol* C gene could induce root proliferation independently in tobacco under the control of the strong constitutive cauliflower mosaic virus 35S promoter (Spéna *et al.* 1987). Transformation of tobacco with *rol* gene pairs indicated that these genes act synergistically to produce stronger hairy root production than single genes (Spéna *et al.* 1987) acting alone.

Hairy roots produced from genetic transformations of medicinal plants have the capacity to synthesize secondary compounds that the intact parent plant usually produce but at increased levels. Sometimes these hairy roots produce novel compounds (Murakami *et al.* 1998) not normally accumulating in the wild type. As a result, transformed root cultures have been exploited as a potential way for continuous production of important secondary compounds used as pharmaceuticals (Hamill *et al.* 1987; Yoshikawa and Furuya 1987), particularly for large-scale production of secondary phytochemicals (Shanks and Morgan 1999; Choi *et al.* 2000). For example, in the study by Murakami *et al.* (1998), the hairy root culture of *Hyssopus officinalis* for the production of rosmarinic acid (RA) and lithospermic acid B (LAB) contained 8.03% RA, which was over 8 times greater than that of the leaf material from a parent plant. The highest levels of RA and LAB (3.96%) were observed at the end of culture period (Murakami *et al.* 1998). Furthermore, hairy roots can also synthesize more than a single metabolite and therefore prove economical for commercial production purposes (Giri and Narasu 2000). Hence, hairy root cultures offer a promise for the production of valuable secondary metabolites in many medicinal plants. The transgenic root system also offers great potential for the introduction of additional genes along with the Ri T-DNA genes for alteration of metabolic pathways (metabolic engineering of pharmaceutical compounds) (Giri and Narasu 2000; Zhou and Wu 2006). Introduction of these additional genes allows manipulation of the limiting factors in secondary metabolic pathways at a molecular level, making metabolic engineering a powerful tool for improving the biosynthesis of therapeutically useful compounds in medicinal plants (Zhou and Wu 2006). Hairy root cultures may thus be suitable for harnessing interesting commercially important secondary metabolites and thus providing alternative plant material instead of

harvesting limited or threatened natural medicinal plants from the wild. In this way, hairy root cultures have the potential to facilitate the conservation of medicinal taxa from increasing demands from the pharmaceutical industry (Shi and Kintzios 2003). Hairy root cultures are preferred because of their higher growth rate in hormone-free media, rapid production of secondary compounds and genetic and biochemical stability in comparison to the cell suspension cultures (Flores *et al*. 1987; Rhodes *et al*. 1987).

The *Agrobacterium*-mediated transformation is advantageous over other transformation methods because *Agrobacterium* efficiently integrates lower copy number of transgenes lessening the chances for the transgene inactivation and silencing (Komari *et al*. 2004). The phenomenon of transgene silencing is speculated to result from multiple integrations of similar DNA sequences (Komari *et al*. 2004). As *Agrobacterium*-mediated transformation introduces few copies into the plant genome, *Agrobacterium*-derived transformants are genetically more stable. The inherent genetic stability of hairy roots results in stable production of secondary compounds over successive generations in contrast to cell suspension cultures. For this reason, transgenic root cultures are considered to be a better option for the continuous commercial production of secondary compounds than callus cultures (Aird *et al.* 1988). In addition to the production of secondary metabolites *in vitro*, hairy roots are able to regenerate genetically stable transgenic plants (Tepfer 1984).

2.2.5 Transgenic plants with *rol* genes

The regenerated plants transmit the Ri T-DNA to their progeny in a Mendelian fashion (Tepfer 1984). Transgenic plants produced from hairy roots are usually non-chimeric (Ohara *et al*. 2000) because hairy roots originate from a single cell and each hairy root consists of uniformly transformed cells (Nilsson and Olsson 1997; Ohara *et al*. 2000). Plants regenerated from hairy roots however, often have abnormalities such as short internodes, highly wrinkled leaves, abundant adventitious root mass (Tepfer 1984), reduced apical dominance with consequent bushy appearance, low fertility, and late flowering (Cho *et al*. 1998). These abnormalities are due to the expression of *rol* genes from the T-DNA (Cho *et al*. 1998). Casanova *et al*. (2005) reviewed studies investigating

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the individual effect of *rol* genes on plant growth and development, especially for phenotypic manipulation of important floricultural plants. To illustrate the individual effect of *rol* genes on the plant's phenotype refer to Table 2-3 for a summary of *rol* gene expression in tobacco plants. However, these phenotypic characters are not necessarily the same for all *rol*-transgenic plants.

Table 2-3 Phenotypic characteristics produced by single effect of *rol* genes on transgenic tobacco plants

Inserted gene	ORF and gene size	Phenotype	References
Rol A	ORF 10 (300 bp)	Small dark green severely wrinkled leaves with a low length-to-width ratio, stunted with extremely shortened internodes, late flowering with reduced number of flowers.	Schmülling *et al.* 1988; Sinkar *et al.* 1988
Rol B	ORF 11 (777 bp)	Increased adventitious root formation with branched anti-gravitropic roots, alteration in leaf morphology (wider leaf blades), bigger flowers with protruding pistils and early leaf necrosis.	Cardarelli *et al.* 1987; Schmülling *et al.* 1988
Rol C	ORF 12 (540 bp)	Reduced apical dominance with increased branching, increased leaf length to width (narrow shape), short internodes (dwarfness), early flowering, reduced flower size, reduced pollen viability and low seed production. Also increased number of lanceolate pale-green leaves and male sterility in some cases.	Oono *et al.* 1987; Schmülling *et al.* 1988
Rol D	ORF 15 (1032 bp)	Early flowering followed by production of larger number of lateral inflorescences, smaller and thinner leaves	Mauro *et al.* 1996

2.2.6 Secondary metabolism in *rol*-transgenic plants

The r*ol* genes may produce, in addition to phenotypic changes, alterations in plants' secondary metabolism. Transgenic medicinal plants synthesize the secondary compounds mainly in the roots (Sevón and Oksman-Caldentey 2002). Due to rapid growth rate of hairy roots, the induction frequency of transgenic plantlets regenerated from hairy roots is high with a consequent rapid growth rate as discussed previously in Section 2.2.4. Regeneration of transgenic plantlets from hairy roots is a method used for improving or increasing secondary compound synthesis in intact plants since *rol* genes are able to modify plant secondary metabolism indirectly (Casanova *et al.* 2005). Transgenic plants regenerated from hairy roots usually accumulate secondary compounds at higher levels than non-transgenic plants. For example, transgenic *Vinca minor* had twice the level of vincamine than wild plants. Furthermore, once these transgenic plants were placed in the glasshouse, their altered metabolism maintained the increased level of vincamine production (Tanaka *et al.* 1995). The *rol* genes have also been found to have an indirect effect on the increase in the essential oil content in certain ornamental aromatic plants such as lemon geranium (Pellegrineschi *et al.* 1994). This specific role of *rol* genes together with other biosynthetic genes coding for certain aromatic components, opens up a new angle on improving scent in aromatic and ornamental plants (Casanova *et al.* 2005).

The other applications of hairy roots are in tree biotechnology (Giri and Narasu 2000) which has been difficult in the past. Transformation of tree with *A. rhizogenes* improves the root system of may tree species. This enables manipulation of trees at cellular and molecular level, which serves as a good potential for clonal propagation and genetic improvement of tree species (Giri and Narasu 2000). Moreover, hairy roots can be used for breeding some crops such as sweet potato (Otani *et al.* 1993) and ornamental plants (Otani *et al.* 1996; Godo *et al.* 1997; Hoshino and Mii 1998; Casanova *et al.* 2005). Hairy roots are also considered useful for improving nitrogen fixation in legumes (Ohara *et al.* 2000).

2.2.7 Transformation of *Salvia* species

The commonly used transformation method in *Salvia* species is *Agrobacterium*-mediated transformation. *A. tumefaciens* and *A. rhizogenes* are the commonly used agrobacteria. *A. tumefaciens*-transformed cells are used for production of callus and cell suspension cultures, which can be subsequently immobilized to make an immobilized cell culture. Several cell and callus suspension cultures (not necessarily *A. tumefaciens*-transformed) of *Salvia* species have been reported, for example *S. officinalis* (Funk *et al*. 1992; Tawfik *et al*. 1992; Hippolyte *et al*. 1992; Santos-Gomes *et al*. 2003; Avato *et al*. 2005), *S. canariensis* (Luis *et al.* 1992) and *S. sclarea* (Skrzypek and Wysokinska 2002; Kuzma *et al.* 2007).

Most of the pharmacological bioactivity and phytochemical analyses of *Salvia* species worldwide have been done using extracts from callus suspension and dried aerial parts (both *in vitro* and *ex vitro*). A cell suspension culture of *S. miltiorrhiza* was established by transformation with *A. tumefaciens* C58 strain for production of a group of terpenes tanshinones (Chen and Chen 1999[a,b]; Chen *et al*. 1997; 1999[a,b]). The effect of initial sucrose concentrations on cell growth and different basal media were studied. Furthermore, tanshinone production was performed in two-stage culture; the first stage culture of fast growing cells in Gamborg's (1968) B5 medium (with low levels of tanshinones) were transferred to the fresh YE-containing medium (second stage culture) where the levels of tanshinones increased rapidly. Light had an inhibitory effect on tanshinone biosynthesis (Chen *et al*. 1997). In contrast, *S. miltiorrhiza* hairy roots in Veliky and Martin (1970) (6,7-V) medium produced both phenolic acids (lithospermic acid B and rosmarinic acid) and tanshinones simultaneously in a single culture system (Chen and Chen 1999[b]). In addition to using different basal media for continuous culture of Ti-transformed cultures of *Salvia* species, chemical elicitation of suspension cultures with methyl jasmonate and salicylic acid up-regulated secondary bioactives (for example Chen and Chen 1999[a]). Despite many successful cell and callus suspension cultures of *Salvia* species, there has been an increased interest in the use of transgenic root culture because in comparison to the cell and callus suspension cultures, production of secondary metabolites is relatively stable (Hamill *et al*. 1987).

Although hairy root cultures are commonly used for large-scale production of pharmaceutical compounds from medicinal plants, induction of hairy root culture in *Salvia* species has been limited to *S. miltiorrhiza* (for example, Hu and Alfermann 1993; Zhi and Alfermann 1993; Chen *et al.* 1999[a;b]; Chen *et al.* 2001; Arikat *et al.* 2004; Ge and Wu 2005[a]; Yan *et al.* 2005; 2006) for phytochemical and pharmacological studies. Nevertheless, most recently, hairy root cultures have been successfully established in *S. sclarea* (Kuźma *et al.* 2006; 2007). However, *S. miltiorrhiza* still seems to be the highly manipulated species in terms of genetic transformation (both callus and transgenic root systems). For induction of transgenic roots in *S. miltiorrhiza*, various *A. rhizogenes* strains such as ATCC 15834 (Hu and Alfermann 1993; Chen *et al.* 1999[a;b]; Chen *et al.* 2001; Ge and Wu 2005[a]; Yan *et al.* 2005; 2006; Grzegorczyk *et al.* 2007), LBA 9402, TR 105, R 1601 and A4 1027 (Hu and Alfermann 1993) were used. Similarly, *S. sclarea* hairy root cultures were established by infection with the LBA 9402 strain (Kuźma *et al.* 2006; 2007). *S. miltiorrhiza* transgenic root culture was used for production of various terpenoids and phenolic compounds (Table 2-2). The *S. sclarea* hairy roots culture was used to produce abietane diterpenes (Kuźma *et al.* 2007), diterpenes and triterpenes (Kuźma *et al.* 2006).

For optimal secondary metabolite production in hairy root cultures, the influence of different factors has been explored. For example, Weathers *et al.* (2004) found that filter-sterilized in contrast to autoclaved media produced both consistent growth and increase in yield of the secondary product from *Artemisia annua* hairy roots. Other studies investigated the effect of both biotic and abiotic elicitors on the up-regulation of secondary compounds *in vitro*. For instance, Ge and Wu (2005[a]) studied the effect of YE (biotic) and silver ions (abiotic) on the accumulation of secondary compounds in *S. miltiorrhiza* hairy roots. In similar studies, *S. miltiorrhiza* was also elicited with YE (Chen *et al.* 2001; Yan *et al.* 2005; 2006) and silver ions (Yan *et al.* 2005) in different basal media. In all these studies, it is generally reported that both biotic and abiotic elicitors improve or up-regulate production of secondary metabolites (phenolic acids and tanshinones) but more significantly by the biotic elicitor (Yan *et al.* 2005; 2006). Other studies focused on media optimization to up-regulate *in vitro* compound biosynthesis (Weathers *et al.* 2004). For example, out of five different basal media tested on root growth and phenolic compound production in *S. miltiorrhiza*, Murashige and Skoog

(1962) medium without ammonium nitrate (MS-NH$_4$) and 6,7-V (Veliky and Martin, 1970) were both optimal (Chen *et al.* 1999[a;b]). Generally, all *Salvia* hairy root cultures are maintained in PGR-free basal media supplemented with sucrose. Out of many different basal media used for maintenance of hairy root culture of *S. miltiorrhiza*, Murashige and Skoog (1962) MS is commonly used (Table 2-2). On the contrary, *S. sclarea* liquid hairy root cultures are maintained in half strength (½B5) Gamborg *et al.* (1968) (Kuźma *et al.* 2006). Table 2-2 shows other different liquid basal media used for maintenance of hairy root cultures of *Salvia* species.

To our current knowledge, there are no transgenic root applications on South African *Salvia* species for increased phytochemical and pharmacological studies. In the few papers published on phytochemistry and pharmacology of South African *Salvia* species, dried aerial parts harvested from the wild plants were used (Table 2-1). Even so, in the two papers published on biotechnological manipulation of the South African species S. *chamelaeagnea* (Huang and van Staden 2002) and *S. stenophylla* (Hoelscher *et al.* 2003), studies were done using leaf material. Although *in vitro* callus and leaf tissue were used for *S. chamelaeagnea*, in *S. stenophylla* wild (*ex vitro*) leaf tissue was used. Clearly, more biotechnological applications such as *in vitro* micropropagation and transgenic cultures are needed on South African *Salvia* species for conservation purposes and, phytochemical and pharmacological studies. This is an initial study on the induction of transgenic root culture coupled with high throughput technologies for phytochemical and pharmacological studies of South African *Salvia* species and *S. africana-lutea* is used as a model species.

2.3 CURRENT BIOTECHNOLOGICAL CHALLENGES AND FUTURE PROSPECTS

Although the hairy root system is an advantageous system for production of secondary metabolites from medicinal plants in comparison to the cell suspension cultures, there are few successes of commercialization of hairy root culture systems. Even though a variety of ideas such as elicitation, immobilization, stirring and air-lifting of cultures have been developed, it is still difficult to develop the practical method of up-scaling hairy root cultures to an industrial level. Despite the considerable efforts, only a few commercial production systems for example shikonin (Tabata and Fujita 1985) and berberine (Nakagawa *et al*. 1984) have been achieved using plant cell cultures. The main challenge with the cell cultures is that they are not genetically stable therefore produce inconsistent yields of secondary metabolites (Hamill *et al*. 1987). Callus cultures often eventually lose their ability to produce specific secondary compounds after several sub-cultures (Flores and Medina-Bolivar 1995). In fact, poor secondary compound synthesis and genetic stability of callus or cell cultures have prevented commercialisation of many plant cell culture systems (Deus-Neumann and Zenk 1984; Sharp and Doran 1990).

Furthermore, limitation in the successes of phytochemical production systems *in vitro* is due to inadequate knowledge of biosynthetic pathways and enzymes involved in secondary metabolism (Nigro *et al*. 2004). More progress in molecular biology of secondary metabolism will have a renewed understanding of the complexity of biosynthetic pathways in terms of their regulation and exploitation for the production of important phytochemicals (Facchini 2001).

2.4 AIMS AND OBJECTIVES

The main motivation of this study is that *S. africana-lutea* is an inherent part of traditional healing in the Western Cape Province, South Africa for various ailments but scientific studies on this plant are still limited. These plants face many threats related to indiscriminate harvesting for traditional medicines and diminishing natural habitat as many coastal areas in South Africa, particularly in the Western Cape are being converted to urban developments for holiday resorts. Therefore, biotechnological manipulations of this plant serves as a conservation solution as well as provide an alternative source of pharmacologically important secondary metabolites. For instance, the *in vitro* produced plant material can be used as an alternative *S. africana-lutea* plant tissue that can be used for both *in vitro* phytochemical and pharmacological studies. Furthermore, transgenic root cultures not only conserve the plant, but the culture conditions can be manipulated for the continuous production of the pharmacologically important metabolites. Continuous culture has a great potential for large-scale production of bioactives at a commercial level, to meet the ever increasing demand for bioactives from this herb. At this stage, there is no published literature on the induction of *S. africana-lutea* hairy roots for production of secondary compounds or for pharmacological and phytochemical studies. Therefore, the main aims of this study are to:

- contribute to the conservation of *S. africana-lutea* plants through use of biotechnological techniques such as micropropagation and transgenic root culture;
- determine transgenesis in the different hairy root clones using molecular biology techniques and its consequent influence on the secondary metabolite profile of the respective root clones;
- investigate the influence of the tissue culture microenvironment on the accumulation of secondary metabolites in *S. africana-lutea*;
- compare the chemical profile and pharmacological activities of transgenic roots to non-transgenic plants and;
- elucidate the chemical structure of the bioactive compound(s) using GC-MS and NMR.

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CHAPTER 3

MICROPLANT PROPAGATION AND HAIRY ROOT CULTURE OF *SALVIA AFRICANA-LUTEA*

3.1 INTRODUCTION

3.1.1 *In vitro* plantlet culture

Kintzios (2000) reported the limited application of biotechnological methods for propagation of *Salvia* species possibly because some of these species are easily propagated vegetatively through cuttings from three-year-old plants especially in spring and autumn. For example, *S. officinalis* has been propagated through cuttings (Nicola *et al.* 2003). Also, propagation by cuttings for most *Salvia* species is because of the reported low seed germination rate (Kintzios 2000). However, there has been increasing interest in the development of *in vitro* propagation of *Salvia* species in order to create a relatively fast system for production of uniform true-to-type clonal plants (Kintzios 2000). Several micropropagation studies of *Salvia* species using various basal media have been reported as discussed in Section 2.2.2 (Table 2-2). For example, *S. officinalis* (Santos-Gomes *et al.* 2003; Avato *et al.* 2005), *S. nemorosa* (Skała and Wysokińska 2004), *S. fruticosa* (Arikat *et al.* 2004; Mišić *et al.* 2006), *S. valentina* Vahl, *S. blancoana* Webb & Heldr subsp. *Marieolensis* (Cuenca and Amo-Marco 2000), *S. fruticosa* (Arikat *et al.* 2004; Mišić *et al.* 2006) and other several *Salvia* species (refer to Mascarello *et al.* 2006) have been micropropagated successfully. For South African *Salvia* species, only *S. chamelaeagnea* (Huang and van Staden 2002) and most recently *S. africana-lutea* (Makunga and van Staden 2008) have been micropropagated successfully.

There is a need for a micropropagation protocol that produces large numbers of *S. africana-lutea* plants in a short time to overcome the possible decline of these medicinally important herbal plants on the Western Cape coast. The biodiversity of *S. africana-lutea* plants is threatened as they are displaced from their natural habitats due to the increase in suburban developments along the Western Cape coast (Makunga and van Staden 2008). In addition, an increasing number of alien/invasive species on the ecologically fragile fynbos vegetation

disturbs the natural seedbank of *S. africana-lutea*. These factors are likely to reduce the natural succession of these plants, which might cause a remarkable decline of *S. africana-lutea* plants in the fynbos biome (Makunga and van Staden 2008). Therefore, this chapter focuses on improvement of the Makunga and van Staden (2008) micropropagation protocol of *S. africana-lutea* plantlets focussing on the reduction of time taken in culture through improving the rooting and acclimation steps.

3.1.2 *In vitro* hairy root culture

A transgenic liquid–shake root culture of *S. africana-lutea* was established in order to study the production of secondary metabolites and their medicinal properties. Secondary metabolite biosynthesis in plants is genetically controlled, nutritional and environmental factors also influence metabolite biosynthesis (Giri and Narasu 2000). Therefore, growth and normal metabolism of hairy root cells can be affected by the composition of the culture medium. As a result, it is essential to optimise the basal growth medium for continuous culture. Optimisation of the medium involves manipulation of nutritional constituents. An optimal growth medium has to support hairy root growth, optimal secondary compound production and accumulation. For instance, sucrose (carbon source) level, exogenous supply of plant growth regulators, the nature of the nitrogen source and the presence of other chemicals can affect growth, total biomass yield and secondary metabolite production in the hairy root cultures (Rhodes *et al.* 1987). Sucrose is taken up (assimilated) by hairy roots and hydrolyzed to glucose and fructose. New root cells are produced at the apical meristems (root tips) and lateral roots form behind the elongation zone due to sucrose assimilation. Such a defined growth pattern leads to steady accumulation of biomass in hairy root cultures (Srinivasan *et al.* 1995). Although hairy roots grow as they assimilate nutrients, the secondary metabolite production may not be growth related (Giri and Narasu 2000). Despite this, hairy roots are still able to synthesize stable quantities of secondary compounds, however the desired compound(s) may be poorly released into the medium and their accumulation in the roots may be limited by negative feedback inhibition (Giri and Narasu 2000). As a result, this may vary the levels of secondary compounds produced by *in vitro* transgenic root cultures, making it difficult to obtain consistent, statistically significant improvements in secondary compound production in cultured tissues (Weathers *et al.* 2004).

Therefore, the aims of this chapter were two-fold: firstly, to improve a previous tissue culture protocol by Makunga and van Staden (2008), especially the last two stages of *in vitro* rooting and acclimation. This would thus shorten the time taken to transfer *in vitro* propagated plantlets *ex vitro*. Secondly, to examine the effect of different medium components on the growth of *S. africana-lutea* liquid-shake root culture.

3.2 MATERIALS AND METHODS

3.2.1 Micropropagation of plantlets

To establish a continuous plantlet production system, *S. africana-lutea* plantlets (see Makunga and van Staden 2008) kindly donated by Dr N. Makunga in February 2006 were sub-cultured from glass culture tubes (20 mm X 100 mm) into glass culture bottles (59 mm X 98.5 mm, 175 ml capacity) to promote multiple shooting. Three internodes (± 1 cm each) were placed horizontally in contact with the medium (30 ml) and sealed with plastic lids (Magenta® B-cap, SIGMA). Full strength Murashige and Skoog (1962) medium (Appendix A1 (i) supplemented with 0.5 mg L^{-1} BA, 0.2 mg L^{-1} of NAA, 30 g L^{-1} sucrose, 0.1 g L^{-1} myo-inositol and solidified with 8 g L^{-1} agar (MERCK) was used for continuous adventitious shoot production. The pH of the medium was adjusted to 5.8 with 1 M NaOH and autoclaved at 121°C for 20 min. The resultant adventitious shoots were sub-cultured on a laminar flow bench cabinet every four weeks and kept in a growth chamber at 25 ± 2 ^{o}C illuminated by cool white fluorescent tubes (OSRAM L 58W/740, USA) for 24 hours light (70 µmol m^{-2} s^{-1} PPFD).

3.2.2. Rooting of plantlets

In order to induce rooting, four-week old multiple *in vitro* shoots (5 cm) (Section 3.3.1) were transferred to rooting media. Activated charcoal (5% w/v) was tested for its ability to increase the rooting efficiency of *Salvia* plants. Plantlets were also placed onto medium without charcoal (control). Both media were supplemented with 30 g L^{-1} sucrose, 0.1 g L^{-1} myo-inositol and 8 g L^{-1} agar (MERCK, South Africa) without any PGRs (pH 5.8) and autoclaved at 121 ^{o}C for 20 min. Two shoots were placed per bottle and returned to the same growth chamber conditions in Section 3.3.1. However, in this case, culture bottles were sealed with

vented caps. The vent was a 10 mm polypropylene membrane with a 0.22 µm pore size (Magenta® B-cap, vented SIGMA; USA). The root number and root length were recorded after four weeks, prior to transplantation from *in vitro* to *ex vitro* environment. For the effect of auxins (at various concentrations) on *in vitro* rooting *S. africana-lutea* plantlets refer to Makunga and van Staden (2008).

3.2.3. Acclimatisation of plantlets

Four-week old rooted and non-rooted plantlets (Section 3.3.2) were transferred from the culture bottles into the plant pots (95 mm X 105 mm). Rooted plantlets were pulled out gently from the agar medium ensuring that the fragile roots do not break, rinsed under running tap water to remove any residual agar. The un-rooted plantlets were dipped in 10 mg L^{-1} indole-butyric acid (IBA) for 30 min before potting. All plantlets were potted in autoclaved potting mix consisting of sand: vermiculite: soil (1:1:1 v/v). Each potted plantlet was covered with a transparent plastic bag to maintain plantlets at high relative humidity. These covered pots were kept in a thermostatically-controlled glasshouse. The glasshouse thermostat was set at 15-25 $^{\circ}$C (minimum and maximum temperature respectively). At the time of acclimatisation (30 August 2007) the natural midday PPFD range was 540-800 µmol m^{-2} s^{-1}. The relative humidity around the newly transplanted plants was reduced gradually (99-70%) by cutting an opening through the plastic cover every third day. During this gradual acclimatisation, plantlets were drenched with 2.5% (v/v) Kelpak (*Ecklonia maxima* seaweed concentrate with 2.2 mg L^{-1} auxin and 0.0062 mg L^{-1} cytokinin as active ingredients; STARKE AYRES, South Africa) every third day. Plastic covers were removed completely two weeks after *ex vitro* transfer of the plantlets. Plants were then watered with distilled water every third day and the foliage sprayed with 1% (v/v) Kelpak after removal of plastic covers.

3.2.4 Liquid hairy root culture

A continuous *S. africana-lutea* transgenic root culture was produced using hairy root clones kindly provided by Dr N. Makunga in February 2006 (Makunga *et al.* 2007). These hairy root clones were sub-cultured in two different half strength MS liquid media one supplemented with 0.1 mg L^{-1} IAA and the other without PGRs. Both media were supplemented with 30 g L^{-1} sucrose and 0.1 g L^{-1} myo-inositol (pH 5.8). The inoculum's size was about 0.1 g fresh

weight root tips in 250 ml Erlenmeyer flasks containing 50 ml of liquid media. The Erlenmeyer flasks were sealed with a cotton wool bung covered with tin foil. The total number of replicates per each treatment was 15 flasks. Flasks were placed on an orbital shaker at 140 rpm in the dark at 25 ± 2 ^{o}C. Roots were sub-cultured at 6 weekly intervals. Stock cultures of all four root clones were grown on Petri dishes in solid ½MS supplemented with 1 mg L^{-1} IAA, solidified with 8 g L^{-1} agar in the dark at 25 ± 2 ^{o}C.

3.2.5 Effect of different basal media on hairy root growth

Five liquid basal media full strength MS without ammonium nitrate (MS-NH$_4$), Gamborg's B5 (B5) (Gamborg *et al.* 1968), full strength MS (Murashige and Skoog 1962), Miller's (Miller 1965) and half strength MS (½MS) (see Appendix A1) were examined as growth media to test influence on continuous *S. africana-lutea* transgenic root culture. The ½MS was used as the control medium. No PGRs were added in all four different media used. For each basal medium, 30 Erlenmeyer flasks (100 ml) were filled with 30 ml of media containing 0.1 g (fresh weight) root tips and shaken on an orbital shaker at 140 rpm in the dark at 25 ± 2 ^{o}C. The growth rate of roots in different basal media was monitored over a 30-day period by harvesting three flasks for biomass measurement every three days. Hairy roots were harvested from the culture medium by filtration on Whatman® filter paper N° 1 (SCHLEICHER AND SCHUELL, USA) and blot dried with paper towels before weighing. Dry mass of harvested roots was determined by oven-drying them at 50 ^{o}C for two days. The growth index was determined from the fresh and dry masses.

3.2.6. Data collection and statistical analyses

Both the rooting and acclimatisation experiments were completely randomised and repeated twice. For the rooting of plantlets, randomly chosen multiple shoots from the maintenance media were distributed randomly in 15 bottles per treatment with two explants (replicates) in each bottle. Therefore, the total number of replicates was 30 per treatment. For the acclimatisation experiment a total of 30 replicates (15 bottles with 2 explants) per bottle were used. Survival was recorded as a percentage after 8 weeks in the glasshouse. For the growth pattern study of different *S. africana-lutea* transgenic root clones in different liquid basal media, 30 flasks (replicates) were used per treatment (total of five treatments) and the

mean fresh and dry masses were determined by weighing and oven-drying roots at 50°C from three flasks every third day respectively.

Unless stated otherwise all experiments were designed in a completely randomised manner (CRD). All quantitative data was analysed using the data analysis software system StatSoft, Inc. (2007) STATISTICA version 8.0. www.statsoft.com. All data sets were first tested for normal distribution using the Shapiro Wilk's test. The data set for rooting of plantlets was not normally distributed therefore it was log-transformed. Despite the log-transformation, this data set was still not normally-distributed. Therefore a non-parametric statistical analysis (Mann-Witney test) was used to compare the means. Both the change in fresh and dry mass data sets were normally distributed therefore one way analysis of variance (ANOVA) with Tukey's Studentized Range HSD and Bonferroni Dunn post-hoc tests were used to compare the means. All means were compared at a 5% significance level.

3.3 RESULTS AND DISCUSSION

3.3.1. Micropropagation of plantlets

Shoot initiation from the internodes was observed as early as seven days after sub-culture. Dark green callus, which eventually turned dark brown, formed at the base where the explant was in contact with the medium. Fully grown multiple shoots with fine hairs on the micro-branches were formed after four weeks (Figure 3-1A). As reported by Makunga and van Staden (2008), occasional *in vitro* flowering was observed on some of the fully-grown plantlets. Microshoots with hairs (Figure 3-1D) had a strong characteristic *Salvia* aromatic fragrance.

Figure 3-1 Micropropagation of *S. africana-lutea* plantlets A) Adventitous shoots after four weeks in BA:NAA (0.5:0.2 mg L^{-1}) **B)** Numerous root proliferation in PGR-free MS **C)** Up-rooted microplant with few elongated roots (from the PGR-free MS supplemented with activated charcoal) **D)** Microshoot immediately after potting (four weeks after *in vitro* rooting) with fine hairs on the microbranches **E)** Fully acclimated plantlet in the glasshouse (four weeks) **F)** Same plantlet eight weeks after transplantation in the glasshouse

3.3.2. Rooting of plantlets

According to Mencuccini (2003), *in vitro* rooting depends on endogenous and exogenous factors such as the genetic origin, ontogenetic phase of the plantlet, environment (for

example light) and the nutrient composition. The *S. africana-lutea* plantlets rooted successfully in PGR-free MS medium (see Makunga and van Staden 2008 for the effect of auxins on rooting of *S. africana-lutea*). Similarly, successful rooting of *Salvia* plants in a PGR-free MS medium has been reported before. For example, *S. blancoana, S. valentine* (Cuenca and Amo-Marco 2000) and *S. miltiorrhiza* (Morimoto *et al.* 1994) produced the highest percentage rooting in the PGR-free medium in comparison to the auxin-supplemented media. Similarly, in the study by Hosoki and Tahara (1993) it was concluded that IBA was not necessary for rooting *S. leucantha* as percentage rooting and the number of roots were high irrespective of the concentration of IBA.

Table 3-1 Rooting of *S. africana-lutea* plantlets in PGR-free MS media

	Mean root number per plantlet	Mean root length (cm)
Agar	2.79 ± 1.54[a]	0.79 ± 0.38[b]
Activated Charcoal	1.25 ± 0.56[a]	1.21 ± 0.54[b]

Same lettered values within a column are not significantly different from each other; $p>0.05$ (STATISTICA 8.0)

Addition of activated charcoal to the rooting basal medium can be used to study the effects of light on *in vitro* rooting (Mencuccini 2003). The translucent medium promoted the proliferation of numerous stunted roots (Figure 3-1B) whereas the darkened medium (activated charcoal) supported *in vitro* root elongation in *S. africana-lutea* microplants (Figure 3-1C). However, there was no significant difference between the two media in terms of root production and elongation at 5% significance level (Mann-Witney test) (Table 3-1). Activated charcoal has been reported to promote *in vitro* rooting (Pierik 1987). According to Pan and van Staden (1998), the commonly well-documented effects of activated charcoal are its root-promoting properties in different plants. These include the increase in the rooting rate, the root number and root length (Pan and van Staden 1998). Activated charcoal is also reported to improve adventitious root formation in woody plants such as *Pinus pinaster* (Dumas and Monteuuis 1995). *In vitro* rooting is improved by activated charcoal as it provides a dark growth environment that has been proven to stimulate rooting and root elongation (Pan and

van Staden 1998). According to Pan and van Staden (1998), the dark environment at the base of the plantlet is suitable for accumulation of auxin and co-factors which are light sensitive. Although activated charcoal is an important additive for rooting plants in culture (Pan and van Staden 1998), its effect on *Salvia* species has not been determined except most recently by Makunga and van Staden (2008). Similar to the findings by Makunga and van Staden (2008), roots in the activated charcoal were few and elongated (Figure 3-1C). Abnormal, prolific rooting observed in the transparent medium (Figure 3-1B) could be due to effect of light on rooting. Roots form naturally underground in a dark environment; therefore an illuminated environment has been reported to cause stunting of roots (Golaz and Pilet 1985) or sometimes it inhibit roots completely in some plant species (Mencuccini 2003).

3.3.3 Acclimatisation of plantlets

Table 3-2 Improvement in the acclimation process of the *S. africana-lutea* plantlets

	Previous protocol Makunga and van Staden (2008)	Current protocol
Rooting	**Two-stage rooting** • 2.5 or 5 µM IBA (2 weeks) then, • 5% activated charcoal (2 weeks)	**2-in-1 rooting/acclimation** (4 weeks) • PGR-free 5% activated charcoal and/or agar
Pre-acclimation	**Two-stage acclimation** (4 weeks) • vermiculite only • sand: soil: vermiculite: peat moss (1:1:1:1, v/v)	
Potting	sand: soil: peat moss: vermiculite (1:1:1:1, v/v)	sand: soil: vermiculite (1:1:1, v/v)
Acclimation period	8 weeks	4 weeks
Glasshouse survival	88%	92%

The acclimatisation protocol for *S. africana-lutea* has been greatly improved. For instance, the time taken to acclimatise *S. africana-lutea* plantlets was shortened (from eight to four weeks) by combining both the rooting and pre-acclimation into a single step. This was

achieved by rooting plantlets in the vented cultures bottles eliminating the need for elaborate two-stage pre-acclimation steps. The polypropylene membrane covering the vents on the caps improves the acclimation process by gradually reducing the relative humidity in the culture bottles from approximately 99 to 70%. Furthermore, the anatomic changes such as formation of hairs, waxy cuticle and reduction of the number and size of stomata on plantlets are initiated which also aid with successful acclimatisation to *ex vitro* conditions (Cuenca and Amo-Marco 2000). As a result, these already hardening off plants (both rooted and unrooted) were transferred straight from the *in vitro* environment into the potting mix (*ex vitro*). However, it was still necessary to cover the newly transplanted plants with plastic bags, for gradual acclimation to the glasshouse conditions and prevention of desiccation (Cuenca and Amo-Marco 2000; Mišić *et al.* 2006). The acclimation protocol was a success as a total of 92% glasshouse survival of plantlets was recorded after four weeks (Table 3-2) (Figure 3-1E). Successful transplantation (survival rates ≥75%) of *in vitro* rooted *Salvia* plants directly to *ex vitro* environment without a pre-acclimation step have been reported (for example, Cuenca and Amo-Marco 2000; Huang and van Staden 2002; Arikat *et al.* 2004; Avato *et al.* 2005; Mišić *et al.* 2006). Potted *S. africana-lutea* plantlets were watered with Kelpak as it is reported to increase plantlet vigour and reduce any stress that may occur after transplanting plants from *in vitro* to a harsher *ex vitro* environment (Kowalski *et al.* 1999). Acclimatised plants had no abnormalities eight weeks after transplantation to the *ex vitro* environment (Figure 3-1F).

This current micropropagation protocol is commercially viable as it is relatively time-efficient, less labour-intensive without many elaborate pre- and post-acclimation steps. Also, plantlets are in culture for relatively shorter time thus reducing any possibilities of *in vitro* somaclonal variation. Lastly, this protocol is cost effective since some rooting and pre-acclimatisation steps were omitted. As a result, costs were reduced since these eliminated steps use costly reagents such as IBA and Gelrite® which were used in the pre-rooting medium and the MS watering solution (Makunga and van Staden 2008).

3.3.4 Liquid hairy root culture

The growth pattern of all *S. africana-lutea* transgenic root clones was similar, characterized with lateral branching resulting in a large network of cream-coloured laterals. The

proliferation of the laterals ceased four weeks after sub-culture that was followed by darkening (from cream to dark brown) indicating increased secondary metabolic activity. Sub-culturing was done at this stage. The darkening of the roots spread from the centre of the root network (old inoculum) to the periphery (**Error! Reference source not found.**-2). The hairy root stock cultures showed an interesting growth pattern on solid medium. The A4T(1) roots were highly branched whereas the other three clones had elongated primary roots with very little secondary branching (Figure 3-3).

3.3.5 Effect of different basal media on hairy root growth

There was no difference in growth rate of hairy roots grown in half-strength liquid MS medium supplemented with IAA or half-strength liquid MS medium without IAA (data not shown). Hairy roots were cultured in hormone-free media. Amongst all five liquid basal media tested (Miller's, $MS-NH_4$, B5, MS and ½MS (control)), hairy root growth was observed only in the control medium (½MS) for all root clones. As a result, all subsequent studies on the root culture were performed in the ½MS medium.

Figure 3-2 An example of transgenic liquid-shake root culture of *S. africana-lutea* in ½MS after four weeks of sub-culture
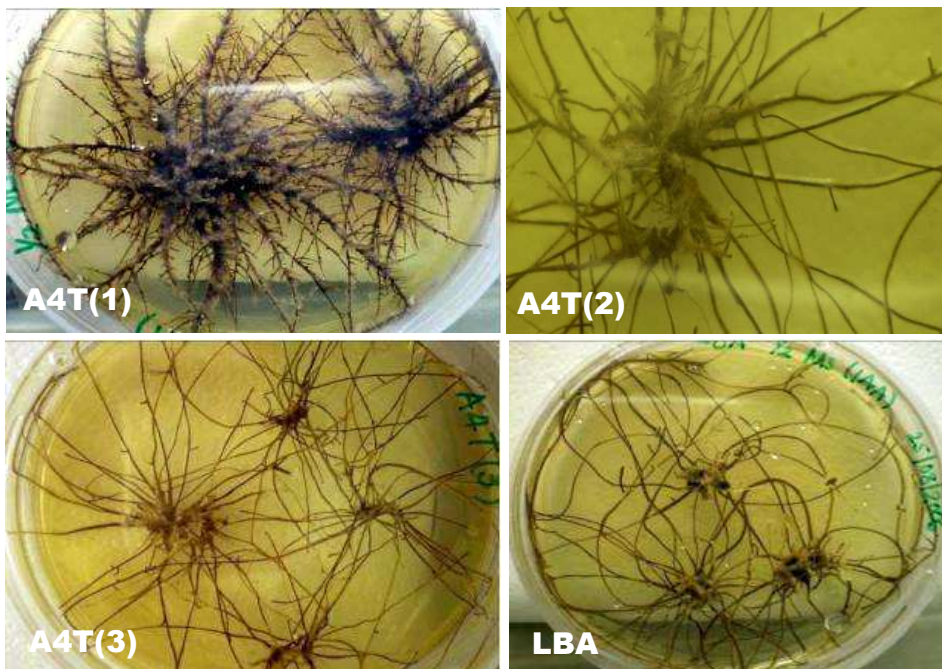
Figure 3-3 Growth pattern of *S. africana-lutea* hairy root clones on solid ½MS medium
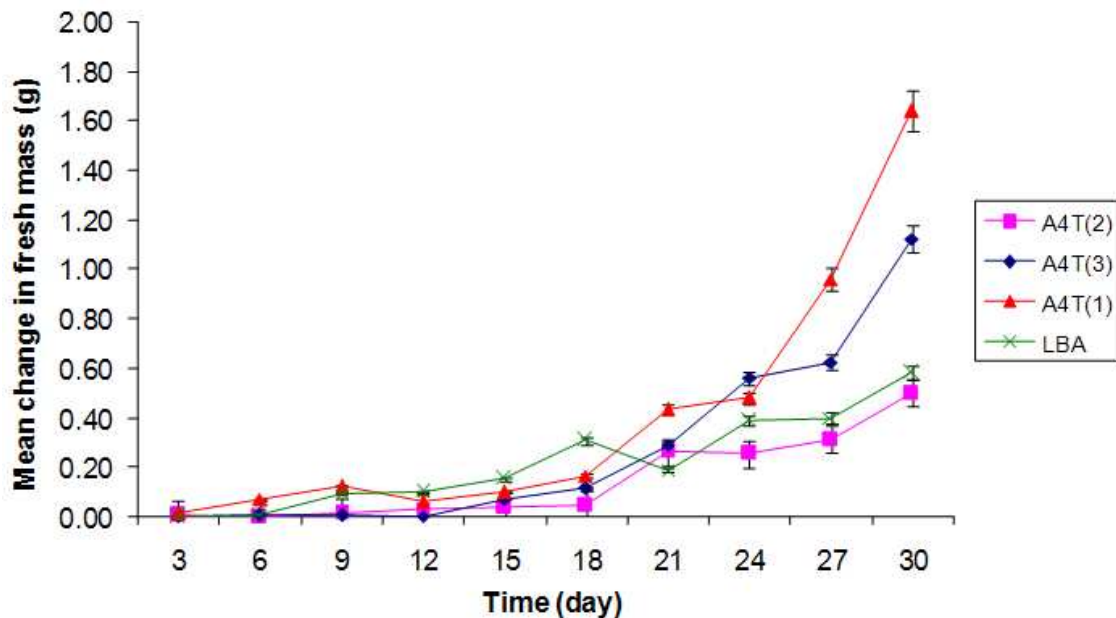
Figure 3-4 Growth curves of different *S. africana lutea* hairy root clones in liquid PGR-free ½MS medium over 30-day period. Each point represents the mean of three replicates. Vertical bars represent standard error. Same lettered values are not significantly different from each other; $p > 0.05$ (SAS®)

The growth curves of the transgenic *S. africana-lutea* roots was a typical sigmoid (Figure 3-4). The longest lag phase was observed in the A4T(1) and A4T(2) cultures (18 days). Although A4T(3) had the shortest lag phase (12 days), A4T(1) had the highest overall growth rate. Generally, proliferation of lateral roots (exponential phase) for all clones was three weeks after sub-culture. All four clones had not reached the stationery phase at the termination of the experiment. Although the A4T(1) root clone grew faster than the other three hairy root clones, it was only significantly different from A4T(2) ($p = 0.39$). Even though the growth curves of A4T(3) and LBA were different, they were not significantly different each from each other statistically nor to A4T(1) and/or A4T(2) (Figure 3-4).

Various basal media such as MS (Murashige and Skoog 1962), B5 (Gamborg *et al.* 1968), MS-NH$_4$ (Murashige and Skoog 1962), MSoH without NH$_4$NO$_3$ (Murashige and Skoog 1962), 6,7 V (Veliky and Martin 1970) and WPM (Lloyd and McCown 1980) have been used to study the hairy root growth and phenolic compound production of different *Salvia* hairy roots.

In most cases no additional PGRs were added in the basal media (Table 2-2) however, there have been reports of elicitation (for example Chen and Chen 2000; Chen *et al*. 2001; Ge and Wu 2005[a;b]; Yan *et al*. 2005; 2006). It seems that the optimal basal media for transgenic root growth depends on the plant species and the strength of the basal salts. For instance, MS supported optimal hairy root growth in *S. miltiorrhiza* (Hu and Alfermann 1993; Ge and Wu 2005[a;b]; Yan *et al*. 2006). Although MS and B5 were reported to be the worst for growth of *S. miltiorrhiza* hairy roots (Chen *et al*. 1999), on the contrary, B5 was favourable for growth of *S. sclarea* L. hairy roots (Kuźma *et al*. 2006; 2007) but the basal media components were halved. Although MS-NH4 (Murashige and Skoog 1962) proved futile in the growth of *S. africana-lutea* hairy roots, MS-NH4 was one of the best basal media that supported the growth of *S. miltiorrhiza* hairy roots (Chen *et al*. 1999; Yan *et al*. 2005).

These varying growth responses of different *Salvia* species' hairy roots in a variety of basal media could also be due to genetic variation. This is because the transformation process is random with foreign DNA being integrated at random positions on the host genome thus producing independent transgenics (Bhat and Srinivasan 2002). These independent clones (carrying the same 'cassette' of foreign gene sequences) may exhibit variable morphologies and growth patterns depending on their position on the host genome (Bhat and Srinivasan 2002) as illustrated by A4T(1), A4T(2) and A4T(3) clones. Molecular analysis indicated the *rol* C and *rol* A present in all clones. It is thus imperative to follow such studies with molecular analysis in order to gain a better understanding of transgene integration (Section 4.2).

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CHAPTER 4
MOLECULAR ANALYSIS OF *S. AFRICANA-LUTEA* HAIRY ROOTS

4.1 INTRODUCTION

Plant transformation is an important tool which offers means of manipulating plants to produce pharmaceuticals, nutraceutical and novel products (Bhat and Srinivasan 2002) amongst many others. There are four popular methods that are commonly used for the introduction of foreign DNA into plant genomes. These are polyethylene glycol (PEG)-mediated, electroporation, biolistic bombardment and *Agrobacterium*-mediated transformation. Plant transformation methods do not have any specific mechanism for introduction of foreign DNA into a specific intracellular location (Skinner *et al.* 2004) or a defined locus (Bhat and Srinivasan 2002). The localisation of foreign DNA into appropriate cellular compartment or organelle is therefore entirely by chance. Thereafter, the DNA is integrated into host DNA usually by a non-specific recombination process (Skinner *et al.* 2004). As a result, foreign DNA is inserted at random locations in the host genome (Bhat and Srinivasan 2002; Skinner *et al.* 2004). Furthermore, the copy number of transgene cannot be controlled during the transformation process (Bhat and Srinivasan 2002).

The commonly used transformation method for medicinal plants is the *Agrobacterium*-mediated system (Yang and Choi 2000). The *Agrobacterium*-mediated system is favoured over other gene transfer methods for several reasons. For instance, many of the gene transfer methods are labour-intensive because they involve preparation of the host tissue for DNA uptake and also they produce variable transformation efficiencies (Skinner *et al.* 2004). *Agrobacterium*-mediated transformation is a complex process which involves chemical signal communication between the bacterium and plant cells. The interaction between the two organisms is still not understood, and there have been a number of reviews on this topic (Winans 1992; Zambryski 1992; Zupan and Zambryski 1995; Gelvin 2000; 2003). Thus far, the bacterial factors involved in the infection process are well-studied and identified. Also, the complete sequence of the T-DNA of the *Agrobacterium* has been fully characterized. *Agrobacterium*-mediated transformation is superior in comparison to other gene transfer

methods because it delivers DNA specifically into the nuclear compartment with high efficiency.

The effectiveness of the *Agrobacterium*-mediated transformation is due to the interaction between plant-encoded gene products with the incoming T-DNA from the *Agrobacterium*. This thus makes the *Agrobacterium*-mediated transformation to be comparatively efficient due to a better control of the T-DNA transfer (Gelvin 2000) by plant-encoded gene products. As a consequence of all these factors, T-DNA integration is less random with fewer occurrences of multicopy, inverted repeats that may result in gene silencing (Skinner *et al*. 2004; Gelvin 2000; Komari *et al*. 2004). The commonly used agrobacteria for plant transformation are *A. tumefaciens* and *A. rhizogenes*. The latter is preferred for transformation of medicinal plants because it rapidly induces hairy root syndrome (see Section 2.2.4). These transgenic roots can be up-scaled for industrial production of pharmaceutical compounds from medicinal plants (Georgiev *et al*. 2007). Although a hairy root syndrome is a phenotypic indication of transgenesis of the roots, it is however critical to confirm the transgenic status of these roots before biological manipulations.

4.2 MOLECULAR ANALYSIS OF HAIRY ROOTS

To confirm transgenesis and transformation efficiency, different molecular techniques are used. For instance, polymerase chain reaction (PCR) amplification of the transgene is an indication of transgenesis (Brown 1995). In the case of *A. rhizogenes* transformation (hairy root syndrome), positive PCR amplifications with *rol* genes that are exclusive to the Ri plasmid is a verification of the successful incorporation of the *rol* genes in the host genome (White and Nester 1980; Tepfer 1984; Tepfer and Casse-Delbart 1987). In order to determine the number of independent insertions of the transgene (copy number), Southern hybridisations (Southern 1975) are useful in this case. The size of the transgene can be determined by both PCR and Southern hybridisations.

4.2.1 Polymerase chain reaction

The PCR confirms the presence of recombinant DNA inserted in the genomic DNA of the prospective transgenic plant tissue (Kawata *et al.* 2003) by amplification of a specific region of DNA from a complex genome (Saiki *et al.* 1988). The PCR is a very sensitive procedure and its outcome is influenced by numerous parameters. The influential factors include template quality, the temperature regime, concentration of $MgCl_2$, the amount of the polymerase and the presence of certain additives (Weising *et al.* 2005). There are two main undesired outcomes of a PCR that depend on above-mentioned factors. A PCR can yield no product or the reaction may be unspecific with many unwanted products.

The quality of the template has a significant influence in the success of a PCR. Isolation and purification of PCR-quality DNA from medicinal plant tissue can be a limiting step for downstream molecular analyses (Kawata *et al.* 2003). Difficulty in obtaining PCR-quality DNA from medicinal plants is due to the presence of large amounts of polyphenols and polysaccharides (Pateraki and Kanellis 2004; Weising *et al.* 2005) in their tissues. The presence of such compounds in the tissue interferes with the isolation process of the nucleic acids (Pateraki and Kanellis 2004). Furthermore, the presence of polyphenols and polysaccharides in the template DNA is highly inhibitory to the PCR amplification (Weising *et al.* 2005). Obviously, the best possible way to avoid such inhibitory effects is to isolate a very clean template. However, isolation of a clean template especially from tissue high in polyphenols and polysaccharides can be laborious. As a result, there have been several studies on the fastest and least laborious methods for isolation of DNA from different plant species' tissue high in these inhibitory compounds (for example, Lodhi *et al.* 1994; Kawata *et al.* 2003; Mitchiels *et al.* 2003; Pateraki and Kanellis 2004; Rouhibakhsh *et al.* 2008). Furthermore, different commercial companies have taken advantage of the problems surrounding isolation of clean DNA from difficult plant tissues. Therefore, several commercial DNA isolation kits have since been developed to solve this problem.

Isolation of pure inhibitory compound-free DNA is not always a guarantee for successful PCRs. More often, multiple unwanted PCR products are generated in addition or exclusion of the desired fragment (Weising *et al.* 2005). In such instances, the optimisation of the PCR is required for optimal yield and specificity. The standard approaches applied to increase the

specificity of the PCR include raising the annealing temperature (T_a) of the primer(s) and/or reducing the concentration of $MgCl_2$. The T_a is the most important point of attention in the temperature regime of a PCR. If the T_a is too high, no amplification takes place because the binding of primers to the template is not stable. Too low T_a increases the chances of primer-template mismatches thus resulting in unspecific amplification (Dale and von Schantz 2002). The T_a depends on the composition and the length of the primer (Metzenberg 2007). A general rule of thumb for calculation of T_a is 2 ^{o}C for each AT-pair and 4 ^{o}C for each GC-pair originally calculated in a 1 M salt concentration for primer hybridisation assays (Newton and Graham 1997; Weising *et al.* 2005).

4.2.2 Southern hybridisation

In transformed tissues, the Southern blot is used as a definitive test to be certain that a transgene has been successfully and stably incorporated into the host genome (Dale and von Schantz 2002). Furthermore, it can be determined whether different transgenic tissues carry the transgene on the same size of the DNA fragment (single transformation event) or on different sized fragment (independent transformation events) (Skinner *et al.* 2004).

Southern hybridisation localises specific DNA fragments carrying a gene of interest from a mixture of other DNA fragments after separation by electrophoresis (Dale and von Schantz 2002; Watson *et al.* 2004). The Southern analysis of hairy roots involves the digestion of the genomic hairy root DNA with specific endonuclease restriction enzymes such as *Hind*III and *Eco*RI. The labelled *rol* genes from the plasmid DNA amplified by PCR are usually used as the probes. The *rol* genes hybridise complementarily to the DNA bands on the membrane producing one or more bands (single- or multi-copy gene integration respectively).

The objective of this part of the study was to develop an optimal and efficient DNA isolation protocol for subsequent downstream applications. The PCR and Southern hybridisations of the *rol* genes were therefore used to facilitate molecular characterisation of *S. africana-lutea* hairy root clones. As a result, genetic variation amongst the hairy root clones was determined.

4.3 MATERIALS AND METHODS

4.3.1 Genomic DNA extraction from hairy roots

Salvia africana-lutea like other medicinal plants contain high amounts of metabolites such as terpenoids and polyphenols (Amabeouku *et al.* 2001; Hussein *et al.* 2007) which may interfere with nucleic acid isolation. In addition to the presence of polyphenolics complicating DNA extractions, it interferes with enzymatic reactions such as PCR and endonuclease restriction digestion (Lodhi *et al.* 1994; Michiels *et al.* 2003). Therefore, a suitable protocol that removes these metabolites effectively is a pre-requisite for getting pure nucleic acids for downstream molecular analyses.

4.3.1.1 Basic DNA isolation protocol

Fresh root tissue (0.1 g) was ground to a fine powder in liquid nitrogen using a pestle and mortar. The ground tissue was transferred into a 1.5 ml microfuge tube. Freshly prepared modified urea-based extraction buffer (500 µl) [5 M NaCl; 1 M trishydroxymethylaminomethane chloride (Tris-Cl pH 8.0); 0.5 M EDTA; 20% (v/v) sarkosyl; 20 mM sodium metabisulfite ($Na_2S_4O_5$); 20 mM sodium diethyldithiocarbamate trihydrate $(C_2H_5)_2NCS_2Na.3H_2O$; 20 mM sodium tetraborate ($B_4Na_2O_7$) and 420 g L^{-1} urea crystals] (Gegenheimer 1990) was added to the ground tissue immediately before it thawed and vortexed thoroughly. To precipitate the protein, polysaccharides and the detergent from the mixture, an equal volume of Tris-saturated phenol and chloroform: isoamyl alcohol (24:1 v/v) was added to the extract and shaken on a table top shaker at room temperature for an hour. Thereafter, the samples were centrifuged at 15 000 rpm for 15 min at room temperature. After three phenol-chloroform precipitations, the cleared supernatant was transferred into a new microfuge tube and the volume was noted. To precipitate nucleic acids, a tenth of the total volume of the supernatant of 4.4 M ammonium acetate and equal volume of ice-cold isopropanol were added to the cleared lysate. The tubes were inverted gently to precipitate the DNA. Further DNA precipitation was done by leaving the tubes at -20 $^{\circ}$C for 20 min. The precipitated DNA was collected by centrifuging at 15 000 rpm for 20 min at 4 $^{\circ}$C. The DNA pellet was rinsed with 1 ml of 70% (v/v) ethanol (EtOH) and centrifuged at 15 000 rpm for 30 sec at 4 $^{\circ}$C to remove ethanol. Further centrifugation for 30 sec at 15 000 rpm (4 $^{\circ}$C) was repeated to remove any residual ethanol. The DNA pellet was air-dried by leaving the

microfuge tubes open on a sterile laminar flow bench. The air-dried DNA was dissolved in 30 µl of ultra-pure water (nuclease-free).

Several changes were made on the above mentioned protocol in attempts to optimise total genomic DNA extraction from *S. africana-lutea* hairy roots. A method that produced high yield and quality of DNA was chosen after several modifications. Optimisation steps of the basic protocol were as follows:

4.3.1.2 Cell lysis at 65 ^{o}C

To improve the total yield of the isolated DNA, cell lysis of the previous protocol was changed by incubating the mixture of ground tissue (urea extraction buffer) in a 65 ^{o}C waterbath for 10 min (with occasional inversion of the tube 2-3 times) prior to Tris-saturated phenol-chlorofom protein precipitation.

4.3.1.3 Use of polyphenol-binding extraction buffers

The mass of the root tissue was increased to 0.5 g. A urea-based extraction buffer was replaced with two extraction buffers coined A [200 mM NaCl; 100 mM Tris-Cl (pH 8.0); 20 mM EDTA; 4% (v/v) sarkosyl; 0.02 M $Na_2S_4O_5$; 0.002 M $(C_2H_5)_2NCS_2Na.3H_2O$; 16 mM ß-mercaptoethanol; 1.5% (w/v) polyvinylpolypyrrolidone (PVPP) and 5% (w/v) bovine serum albumin (BSA)] and B [same as Buffer A including 10 mg ml^{-1} Proteinase K (Roche) added just before use with exception for BSA and PVPP]. These buffers both contain reagents that have high affinity for the polyphenols (Gegenheimer 1990) which is a common problem when extracting DNA from tissues such as roots with high phenolic content. Buffer A (2 ml) was added to ground tissue immediately before it thawed. In addition, 6.5 ml of Buffer B was added to the mixture and left on ice for a minute. Afterwards, the mixture was incubated in a 37 ^{o}C waterbath for 20 min. The mixture was mixed occasionally (2-3 times) during the incubation by inverting the tube. The lysate was centrifuged at 10 000 rpm for 10 min at 4 ^{o}C. The cleared lysate was added into a new tube (without disturbing the pellet) and one tenth of the volume of RNase (10 mg ml^{-1}) was added to the supernatant for RNA digestion in a 37 ^{o}C waterbath for 10 min. An equal volume of Tris-saturated phenol was added to the RNA-digested lysate and shaken briefly on a table top shaker and centrifuged at 10 000 rpm at room temperature. After three phenol extractions, chloroform: isoamyl alcohol (24:1 v/v)

extractions followed. The chloroform: isoamyl alcohol (24:1 v/v) extractions were repeated until the white interface (protein) was not visible. Nucleic acid precipitation and washing was done as described in the basic protocol (Section 4.3.1.1).

4.3.1.4 Polyphenol-binding extraction buffers and Qiagen Plant DNeasy Mini Kit

The first steps of this protocol are the same as outlined in Section 4.3.1.3. However, after the phenol-chloroform precipitation steps, the supernatant was passed through a DNA-binding column from the Qiagen Mini Kit (Mini Kit). The kit was used to thoroughly clean any residual organic solvents that could have detrimental effect on downstream molecular analyses. Therefore, the DNA extraction was continued from Step 14 on the Mini Kit protocol (refer to the Qiagen Plant DNeasy Mini Kit Booklet).

4.3.1.5 Elimination of phenol extractions

The three Tris-saturated phenol extractions before the chloroform: isoamyl alcohol (24:1 v/v) extractions were eliminated because it was suspected that the kit could not remove some residual phenol completely. However, chloroform: isoamyl alcohol (24:1 v/v) extractions were still applied with the rest of the protocol as outlined in Section 4.3.1.4 (using buffers A and B along with the Mini Kit).

4.3.1.6 Use of Qiagen Plant DNeasy Maxi Kit

To increase the yield of the isolated DNA, the Mini Kit was replaced with Qiagen Plant DNeasy Maxi Kit (Maxi Kit). The replacement of the Mini Kit with the Maxi Kit was an attempt to isolate relatively high amounts of the DNA necessary for Southern analysis. The self-made extraction buffers (Buffers A and B) were replaced with AP1 Buffer (supplied in the Maxi Kit). The Maxi Kit protocol was followed systematically. However, the DNA was eluted with ddH_2O (nuclease-free) instead of the supplied AE Buffer [Tris-Cl pH 8.0].

(i) In order to concentrate the isolated DNA even further, the DNA eluted with Maxi Kit was re-precipitated with salt/EtOH solution [1/10 volume of 1M NaCl/3X volume 100% (v/v) EtOH] and left at -20 ^{o}C for 2-3 days. The precipitated DNA was collected by centrifugation at 10 000 rpm at 4 ^{o}C for 20 min. This was followed by a 70% (v/v)

EtOH wash step to remove any precipitated salt. The DNA pellet was air-dried and re-dissolved in minimal ultra-pure water depending on the size of the pellet.

(ii) Some of the DNA samples were contaminated with polysaccharides. A clean-up of these contaminants was critical prior to any downstream molecular analyses. The precipitated polysaccharides were removed from the DNA by re-suspending the viscous precipitate in 1 ml of 100% EtOH and a 1/10 volume of 5 M NaCl. The mixture was left at -20 $^{\circ}$C for 4 hours and the DNA was pelleted by centrifugation at 10 000 rpm. The DNA pellet was washed in 70% (v/v) EtOH and centrifuged briefly to remove EtOH. The DNA pellet was air-dried on the laminar flow bench for 20 min. The pellet was dissolved in 50 μl ddH$_2$O (nuclease-free). The DNA samples were quantified by UV absorbance spectrophotometer (Heλios, ThermoSpectronic USA) at 260 and 280 nm wavelengths. The quality and yield of the isolated DNA samples were analysed by electrophoresis on 1% (w/v) agarose with Tris-borate-EDTA (TBE) buffer [90 mM Tris pH 8.0, 90 mM boric acid, 2 mM EDTA] mixed with 1 μg ml^{-1} ethidium bromide (EtBr). The DNA was visualised in the UV transilluminator (APPLIED BIOSYSTEMS, USA).

4.3.2 PCR amplification of *rol* and *ags* genes in the root DNA

Subsequent to optimisation of the DNA extraction protocol, downstream molecular analyses were carried out on both the transgenic roots and plasmid DNA. In order to validate transgenesis of the transformed *S. africana-lutea* roots, genes from both the T$_L$-DNA and T$_R$-DNA of *A. rhizogenes* T-DNA were used for PCR amplifications. Gene-specific primers that amplify a segment complimentary to the *rol* A, B, C (on the T$_L$-DNA region) and *ags* gene (on the T$_R$-DNA region) were used. The sequences of all four primers are illustrated in Table 4-1. A PCR reaction without the DNA was used as a negative control whereas the PCR amplification of the plasmid DNA was used as a positive.

The PCR amplifications were optimised by troubleshooting the optimal DNA, MgCl$_2$ and primer concentrations. Furthermore, the PCR additive dimethyl sulfoxide (DMSO) was eliminated from the reactions. From these modifications of the PCR, the amplifications were

either negative or unspecific. Therefore, the following step on the optimisation of the PCRs was to change the annealing temperature (T_a) of different primers. The T_a of different primers was dropped 5 ^{o}C below the T_m (Equation 1).

Equation 1: $T_m = 4(G + C) + 2(A + T)$

The optimal T_a of different primers is tabulated in Table 4-1. The starting lowest T_a was 38 ^{o}C, this temperature was increased by one degree (^{o}C) at a time until specific positive amplifications were produced.
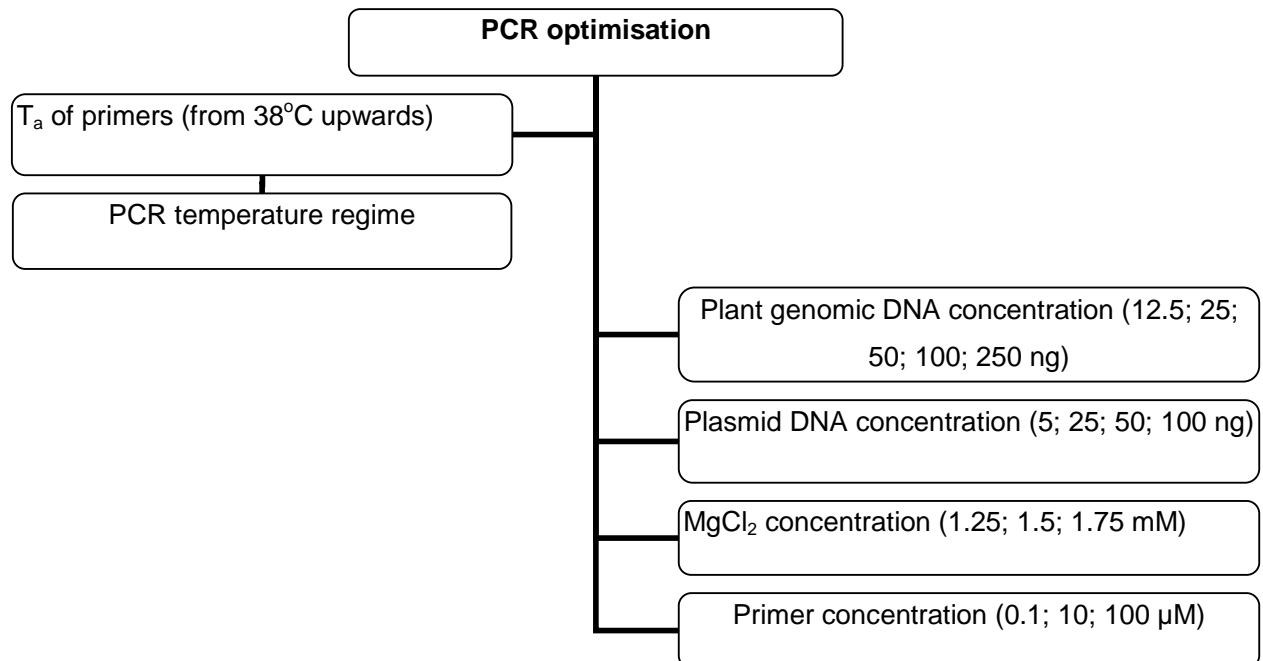
Figure 4-1 Troubleshooting the PCR amplification of *rol* and *ags* genes in the *S. africana-lutea* hairy root DNA

The optimal amount of DNA used for amplification was 50 ng after several optimisation studies. Optimal concentrations of other PCR reagents are in Table 4-2. After several attempts, the optimal PCR cycling conditions consisted of initial denaturation of template at 94 ^{o}C for 1 min for the first cycle. This cycle was followed by 35 cycles of denaturation at 94 ^{o}C for 1 min, annealing at specific temperature (see Table 4-1) for 45 sec and a 2:30 min extension at 72 ^{o}C. The final extension was also at 72°C but for 10 min. The 96-Well GeneAmp$^{®}$ PCR System 9700 (APPLIED BIOSYSTEMS, USA) was used for all PCRs. The amplicons were analysed through separation by electrophoresis on a 1% (w/v) agarose gel in TBE buffer. The agarose gels were stained with 1 µg ml^{-1} EtBr. The amplicons were visualised in the UV transilluminator (APPLIED BIOSYSTEMS, USA). The sizes of all *rol* genes were determined using Roche XIV molecular marker (Roche) whereas for the *ags* gene, Pst λ ladder (generated by digesting Lambda DNA with *Pst* I) was used. The expected sizes of the amplicons for different primers are tabulated in Table 4-1

Table 4-1 Primers used for the PCR amplification of the *rol* and *ags* genes incorporated in the *S. africana-lutea* hairy root DNA

Primer	Sequence	Size (kb)	T$_a$
rol A	5'- CAG AAT GGA ATT AGC CGC ACT A -3'	0.3	54°C
	5'- CGT ATT AAT CCC GTA GGT TTG TTT -3'		
rol B$^{#}$	5'- ATG GAT CCC AAA TTG CTA TTC CTT CCA CGA -3'	0.4	54°C
	5'- TTA GGC TTC TTT CTT CAG GTT TAC TGC AGC -3'		
rol C	5'- CAT TAG CCG ATT GCA AAC TTG -3'	0.6	54°C
	5'- ATG GCT GAC GAC CTG -3'		
ags	5'- CGG AAA TTG TGG CTG TTG TGG AC -3'	1.6	59°C
	5'- AAT CGT TCA GAG AGC GTC CGA AGT -3'		

$^{#}$ The primer set was synthesised by ROCHE (Germany). All other three primers sets were synthesised by Integrated DNA Technologies Inc. (IDT) (USA)

Table 4-2 An optimised PCR reaction for the amplification of the *rol* and *ags* genes

Reagent	Concentration
Primer I (forward)	1 µM
Primer II (reverse)	1 µM
***dNTPs**	0.2 mM
***MgCl$_2$**	1.5 mM
***Taq Buffer (NH$_2$SO$_4$)**	1X
***Taq polymerase**	2.5 u
DNA	50 ng
Ultra-pure water	
(to make total volume 50 µl)	

* Reagents by Fermentas (USA) **Note:** 5% (v/v) DMSO was added in the *ags* PCR reaction and omitted in all the *rol* PCR reactions

4.3.3 Probe preparation and labelling

In order to prepare a probe for Southern blots, the plasmid DNA was isolated from *A. rhizogenes* (A4T) strain by the alkaline lysis method as outlined by Li *et al.* (1995). The isolated plasmid DNA was used as a PCR template to amplify the *rol* and *ags* genes located on the plasmid as outlined in Section 4.3.2 using all primers in Table 4-1. The resultant amplicons were purified using a PCR Purification Kit (QIAGEN, Germany). Pure PCR product(s) were radioactively labelled via two methods:

- PCR-labelling was conducted with 1 mM [α-^{32}P] dCTP 50 µCi, 3000 Ci/mmol 0.025 µl of 50 mM Tricine (pH 7.6) (PROMEGA, USA) and amplified using the same PCR cycle outlined in Section 4.3.2 and Table 4-3; or

- Random prime labelling with a Prime-a-Gene® Labelling System (PROMEGA) was conducted according to manufacturer's instruction manual (333 nM of [α-^{32}P] dCTP 50 µCi, 3000 Ci/mmol).

Table 4-3 The radioactive PCR-labelling reaction for the *rol* gene probe

Reagent	Concentration
Primer I (forward)	1 µM
Primer II (reverse)	1 µM
dATP	0.2 mM
dGTP	0.2 mM
dTTP	0.2 mM
[α-^{32}P] dCTP	0.2 mM
MgCl$_2$	0.2 mM
Taq Buffer (NH$_2$SO$_4$)	1X
Taq polymerase	2.5 u
DNA	25 ng
Ultra-pure water (make up total volume 50 µl)	

4.3.4 Southern hybridisation analysis

4.3.4.1 Restriction digestion

The genomic DNA from hairy root clones (30 µg) isolated as outlined in Section 4.3.1.6, was digested with the restriction enzyme *Eco*R1 (Fermentas) (5X restriction buffer; 0.1 M Spermidine; 5 units restriction enzyme in a 37 °C waterbath overnight. The plasmid DNA (10 µg) was also subjected to the same restriction digest reaction (positive control). The undigested plant DNA was used as a negative control. All the restriction digest reactions were electrophoresed on 0.8 % w/v) agarose gel at 60 V for 2 hours 30 min.

4.3.4.2 Southern transfer

Prior to the transfer of the DNA from the gel onto the Hybond^{TM}-N+ membrane (AMERSHAM, UK), the DNA was depurinated in 250 ml of 0.25 M hydrochloric acid (HCl) and swirled on a tabletop shaker for 15 min. This was repeated for a further 15 min with

fresh 0.25 M HCl. The gel was rinsed briefly in distilled water and then soaked in 250 ml rinsing solution [0.3 M NaOH /3 M NaCl] for 15 min and repeated again in fresh rinsing solution for another 15 min. Lastly, the gel was soaked in 250 ml transfer solution [0.5 M Tris-Cl (pH 7.0) / 3 M NaCl] for 15 min and this step was repeated with fresh transfer solution. The gel was left in the transfer solution whilst the membrane, the handiwipes and the filter papers were being cut to the dimensions of the gel which were then soaked in the transfer solution in a separate container. The DNA was transferred from the gel onto the membrane using the rapid downward transfer method (Koetsier *et al*. 1993). The transfer was complete after 2-3 hours. To confirm the success of the transfer, the gel was viewed under UV light. The membrane was baked in a vacuum oven for an hour at 65 ^{o}C to ensure that the transferred nucleic acids adhered to the membrane.

4.3.4.3 Pre-hybridisation and hybridisation

The membrane was pre-hybridised in Rapid Hyb buffer (GE Healthcare, UK) according to the manufacturer's instructions at 65 ^{o}C in a hybridisation oven (AMERSHAM, UK) with continuous constant rotation. Meanwhile, the probe was denatured by heating at 95 ^{o}C for 2 min in a 96-Well GeneAmp$^®$ PCR System 9700 (APPLIED BIOSYSTEMS, USA). Thereafter, 50 µl of the denatured and labelled probe (as outlined in Section 4.3.3) was added directly into the pre-hybridisation buffer already in the hybirisation tube to initiate hybridisation. The hybridisation of the probe to the membrane was conducted overnight at 65 ^{o}C with continuous constant rotation.

After hybridisation, the unhybridised nucleic acids were removed by a series of post-hybridisation washes. The first wash [2X SSC/0.5% (w/v) SDS] was conducted at room temperature for 15 min and repeated again with 100 ml fresh solution for another 15 min. This was followed by a second post hybridisation wash [1X SSC/0.5% (w/v) SDS] at 65 ^{o}C for 15 min, and this was also repeated for another 15 min in 100 ml fresh solution.

4.3.4.4 Detection of hybridised probe

The membrane was put in Saran Wrap and excess post-hybridisation buffer was pressed off the membrane prior to sealing the wrap. The blot-dried membrane was exposed to

autoradiography film (PACKARD, USA) and developed for 72 hours on a Hyperscreen[TM] (AMERSHAM) to show the hybridised sequence.

4.4 RESULTS AND DISCUSSION

4.4.1 Genomic DNA isolation

In order to study the transgenic *S. africana-lutea* roots at molecular level, it was essential to establish a method for isolation and purification of PCR-quality genomic DNA. This was a challenging, laborious and time-consuming task as *S. africana-lutea* hairy roots are rich in inhibitory compounds such as polyphenols. The polysaccharides were also inhibitory, which is usually the case with the isolation of nucleic acids from most plant tissues (Lodhi *et al.* 1994; Michiels *et al.* 2003). Some of the published optimisation strategies of the existing DNA isolation protocols focus on the ingredients and pH of the extraction buffer (Weising *et al.* 2005). Therefore, the type of the extraction buffer and the different strategies for elimination of inhibitory compounds from the *S. africana-lutea* transgenic root tissue were studied in order to isolate high quality DNA from these tissues.

It was clear that the different extraction buffers tested influenced the quality and the concentration of the isolated DNA from *S. africana-lutea* hairy root clones. Both the urea-based extraction buffer and Buffers A and B produced intact DNA (Figure 4-2). However the concentration of DNA varied for each extraction buffer (Table 4-4).

Reagents in the extraction buffers have different roles in the elimination of inhibitory compounds during the DNA extraction. The DNA extraction process is complex which involves the interaction between the reagents in the buffer and the nucleic acids. Ensuring that the plant tissue does not thaw before addition of the extraction buffer protects the DNA from degradation by cellular enzymes that are usually activated when the tissue thaws (Weising *et al.* 2005). The ground *S. africana-lutea* transgenic root tissue were kept in a frozen state by transferring the powder into a liquid N_2 pre-chilled polypropylene tube prior to addition of extraction buffer.

The first modification of the basic DNA extraction protocol was cell disruption using heat treatment (see Section 4.3.1.2) because according to Pateraki and Kanellis (2004) heat cell-lysis yields ten times more DNA than non-heat lysed cells. Heat cell-lysis produced variable DNA yield for different *S. africana-lutea* root clones (Table 4-4).
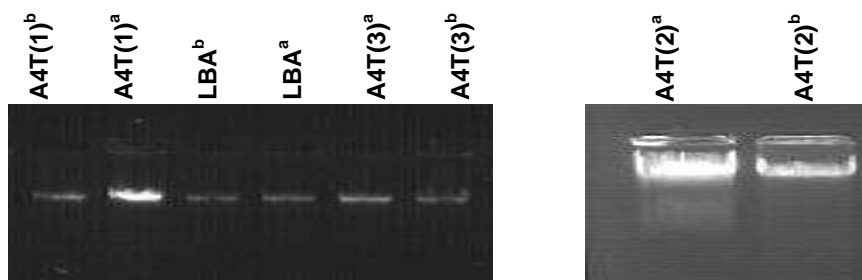
Figure 4-2 Genomic DNA isolated from *S. africana-lutea* hairy root clones with [a]urea buffer and [b]Buffers A and B only. Note: Each well was loaded with 10 µl of DNA

Table 4-4 Quality and concentration of the genomic DNA from *S. africana-lutea* root clones with different extraction protocols (Sections 4.3.1.2 and 4.3.1.3 respectively)

	Urea buffer (65 $^{\circ}$C)		Buffers A and B (37 $^{\circ}$C)	
	$A_{260/280}$	DNA (ng µl^{-1})	$A_{260/280}$	DNA (ng µl^{-1})
A4T(1)	1.67	1250	1.94	1330
A4T(2)	1.5	130	1.78	1450
A4T(3)	1.81	1680	1.26	1960
LBA	1.76	3950	2.31	380

All the DNA samples extracted using the basic protocol and modifications in Sections 4.3.1.2 to 4.3.1.4 yielded non-amplifiable DNA (Table 4-5). Therefore, in subsequent modification of

the extraction protocol, the Tris-saturated phenol was eliminated from the protein precipitation step as it was suspected that the residual phenol in the DNA inhibited PCR amplification. Even after the elimination of phenol from the extraction protocol, the isolated DNA was still not amplifiable. However, the use of the precipitation buffer AP2 from Mini Kit along with Buffers A and B (self-made) yielded amplifiable DNA even though the amplification was not reliable (See Table 4-5). Therefore, in the DNA extraction steps that followed, the focus was on the removal of inhibitory secondary compounds which might had been preventing PCR amplifications.

Phytochemical studies of *Salvia* species have shown that this genus is rich in alkaloids, terpenes, phenols and polyphenols (see Section 2.1.3). Phenols and polyphenols are of particular interest during the DNA extractions (Weising *et al.* 2005). According to Weising *et al.* (2005), the oxidation products are powerful oxidising agents that can denature the DNA structure and cause the browning of the DNA pellet (Table 4-5). These oxidising compounds may even block the DNA making it inaccessible for some enzymes (Weising *et al.* 2005) such as polymerases and endonucleases.

The detrimental effects of polyphenols and oxidation products are overcome by addition of various reagents in the extraction buffer. For instance, PVPP (or water-soluble polyvinylpyrrolidone; PVP) and BSA are added into the extraction buffer to bind polyphenols that may contaminate the DNA (Angeles *et al.* 2005). It is reported that the addition of the soluble PVP increases the yield of nucleic acids by 25% (Pateraki and Kanellis 2004). The PVP forms complex hydrogen bonds with phenolics and co-precipitates with cell debris upon lysis (Michiels *et al.* 2003). The phenolic-PVP complex is then separated from the DNA by centrifugation (Lodhi *et al.* 1994) forming an interface between organic and aqueous phases in the presence of chloroform (Michiels *et al.* 2003). Polyphenol adsorbents are usually at concentration range of 1 to 6% (Weising *et al.* 2005). Although 5% (w/v) BSA and 1.5% (w/v) PVPP were added in Buffer A, the colour of the DNA pellet was still brown. The brown colour indicated the presence of residual oxidised polyphenols despite the addition of 16 mM ß-mercaptoethanol in both Buffers A and B. ß-mercaptoethanol is a reducing agent that protects the DNA from direct or indirect damage by oxidation products by inhibiting the oxidation of polyphenols (Weising *et al.* 2005). However, Angeles *et al.* (2005) have reported the successful removal of oxidised polyphenols from the coconut DNA. In the study by

Angeles *et al.* (2005), PVPP was added directly to the ground tissue whereas in this study, PVPP was added as a reagent in the extraction buffer. This could explain the difference between the two studies, probably meaning that PVPP binds polyphenols better when added directly to the ground tissue.

Table 4-5 The fidelity of DNA samples extracted with different protocols to downstream molecular analyses

Method	PCR	Restriction digestion	Comments
Section 4.3.1.1: Basic protocol	-	nt	
4.3.1.2: Urea at 65°C	-	nt	
Section 4.3.1.3: Buffer A and B only	-	nt	
Section 4.3.1.4: Buffer A and B with Mini Kit	-	*	Brown pellet, insoluble most of the time.
Section 4.3.1.5: Buffer A and B with Mini Kit (AP2 without phenol)	+	*	
Section 4.3.1.6 (i): Maxi Kit	++	yes	White fluffy pellet
Section 4.3.1.6(ii): Maxi Kit and salt/EtOH precipitation	+++	yes	Occasional polysaccharide contamination. Otherwise white fluffy pellet most of the time

+ unreliable amplification (only once); **++** frequent amplification (more that twice); **+++** consistent reproducible amplification; **-** no amplification; **nt** not tested; **yes** endonuclease digestible ***** quantity of DNA too low to be restricted (≤ 2µg)

In addition to constant contamination of the *S. africana-lutea* transgenic root DNA with secondary compounds, occasional co-precipitation of polysaccharides was also a problem (Table 4-5). This was not surprising as Weising *et al*. (2005) stated that the DNA precipitation steps with isopropanol or EtOH do not always remove all contaminants as RNA and most of polysaccharides tend to co-precipitate with the DNA (Weising *et al*. 2005). The precipitated polysaccharides were removed by high salt concentration and EtOH solution (Section 4.3.1.6). Sodium salts remove polysaccharides that interfere with the DNA

extraction (Lodhi *et al.* 1994). The presence of polysaccharides often makes the samples viscous making the DNA unamplifiable during PCR and impossible to restrict with endonucleases (Lodhi *et al*. 1994). The polysaccharides interfere with the polymerases and restriction endoucleases (Lodhi *et al.* 1994; Michiels *et al*. 2003). Sodium chloride keeps polysaccharides in solution during the EtOH-precipitation of the DNA (Weising *et al*. 2005). Furthermore, the addition of salts to the extraction buffer prevents the activity of phenol oxidase (Gegenheimer 1990) thus protecting the DNA integrity.

It was evident that the high salt/EtOH re-precipitation of the eluted DNA from the Maxi Kit had a significant improvement in both the purity and quantity of the DNA from all *S. africana-lutea* root clones (Table 4-6). All the DNA samples re-precipitated were highly amplifiable and the amplifications were reproducible (Table 4-5).

Table 4-6 The effect of salt/EtOH precipitation on the quality and the concentration of the hairy root DNA eluted with Qiagen Kit

	A4T(1)	A4T(2)	A4T(3)	LBA
Purity	1.8	1.69	1.46	3.14
DNA (ng µl^{-1})	10.9	8.6	12.4	3.4
After salt/EtOH precipitation				
Purity	1.66	1.73	1.75	1.78
DNA (ng µl^{-1})	41.1	118.7	99.5	12.5

4.4.2 Optimisation of the PCR amplification

Optimisation of the PCR amplification of the *rol* genes from the Ri plasmid genomic DNA was also quite difficult, as numerous parameters had to be optimised prior to successful specific amplifications. Initially, the main problem was the template quality. The low quality genomic DNA contaminated with phenolics and other compounds inhibited the amplification

of the *rol* genes from the *S. africana-lutea* root DNA. This was overcome by various steps as illustrated in Figure 4-1 and discussed in Section 4.3.2.

Although a good quality DNA template was eventually extracted, non-amplification or deleterious amplification PCRs were still a problem. The T_a for each *rol* primer pair was dropped to as low as 38 ^{o}C and the PCRs were still unsuccessful. DMSO is known to improve the specificity and yield of the PCR at 5% (v/v) (Weising *et al*. 2005). On the contrary, elimination of DMSO had a significant improvement in the PCR amplification of the *rol* genes from the *S. africana-lutea* hairy root DNA. However, the amplification was unspecific (Figure 4-3A). It was only after the positive PCRs that the optimal concentration of $MgCl_2$ and T_a were determined with relative ease (without DMSO). The T_m of primer-template drops by 5 ^{o}C factor in the presence of DMSO (Fermentas pamphlet). Therefore, the T_a of *rol* primers had to be determined experimentally without consideration of Equation 1 (Fermentas). Interestingly, the presence of DMSO did not affect the PCR amplification of the *ags* genes from the *S. africana-lutea* hairy root DNA. However, the yield of *ags* amplicons from the A4T(3) and LBA clones were lower even though DMSO is known to improve PCR yield (Figure 4-4A).

In a further attempt to optimise the specificity of the PCR amplification of the *rol* and *ags* genes incorporated in the *S. africana-lutea* hairy root DNA, optimal concentration of $MgCl_2$ was determined. Out of the three concentrations of $MgCl_2$, 1.5 mM was optimal as the PCR at this concentration was specific (see white oval on Figure 4-3B). According to Newton and Graham (1997), the concentration range of 1 to 1.5 mM $MgCl_2$ is usually optimal for *Taq* polymerase. Mg^{2+} ions are important for optimal amplification of the template DNA by the DNA polymerase (Newton and Graham 1997; Metzenberg 2007). The Mg^{2+} ions form complexes with the polymerase, dNTPs, primers and the template DNA during amplification (Fermentas; Newton and Graham 1997). It is evident that the concentration of 1.25 mM $MgCl_2$ was too low resulting in no amplification whereas 1.75 mM was too high (unspecific amplification) for the PCR. Insufficient concentration of Mg^{2+} results in low yield of PCR product whereas high $MgCl_2$ might increase the yield (Dale and von Schantz 2002) thus result in unspecificity of PCR (Fermentas; Newton and Graham 1997; Dale and von Schantz 2002; Metzenberg 2007) as illustrated in Figure 4-3A. High concentrations of Mg^{2+} might even decrease the fidelity of the polymerase (Metzenberg 2007) thus producing no

amplification. The final optimal PCR reaction for amplification of *S. africana-lutea* roots with *rol* and *ags* primers is in Table 4-2 and the cycling conditions stated in Section 4.3.2.

In the study for optimisation of the DNA concentration for PCR, the hairy root DNA concentrations ranging from 12.5 to 250 ng had equal amplification efficiency with *rol* A primer pair (Figure 4-3C). Equal efficiency of the PCR of template implies that the DNA extraction described in Section 4.3.1.6 produced genomic DNA of high quality and purity (Pateraki and Kanellis 2004).

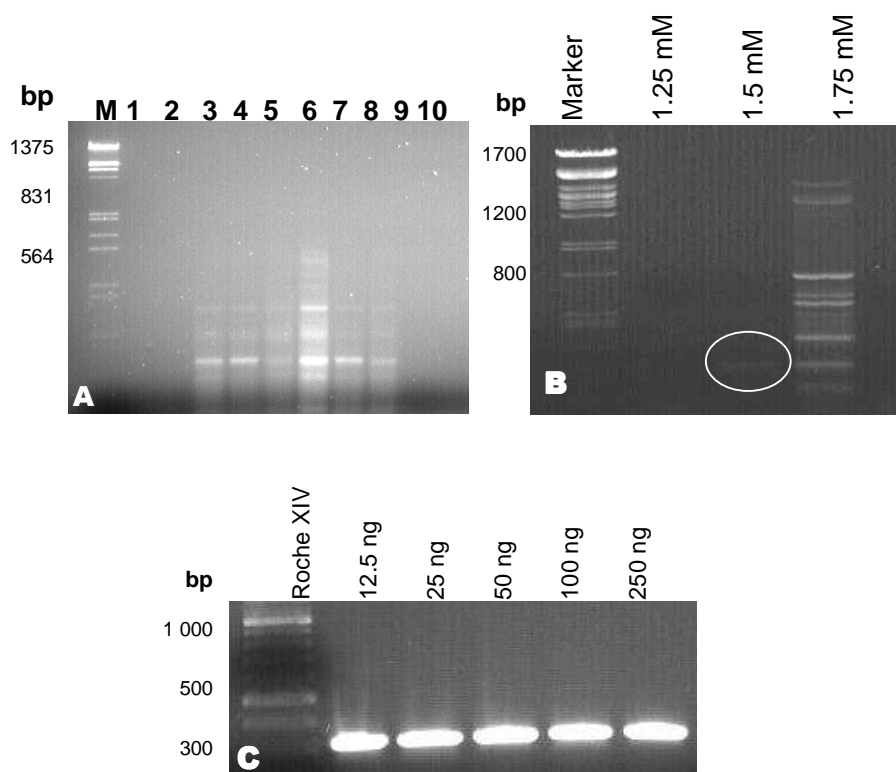**Figure 4-3 Optimisation of the PCR amplification of the *rol* genes in the *S. africana-lutea* root DNA A)** Unspecific PCR amplification. M= Roche III Molecular Marker; Lane 1= -ve control; Lane 2=LBA; Lane 3=A4T(1); Lane 4=A4T(1); Lane 5= A4T(3); Lane 6=+ve; Lane 7= A4T(3); Lane 8=A4T(2); Lane 9= A4T(2); Lane 10= LBA **B)** MgCl$_2$ optimisation **C)** Determination of the optimal hairy root DNA concentration.

4.4.3 Validation of trangenesis of the hairy roots

Transgenesis of *S. africana-lutea* hairy root clones was confirmed by PCR amplification showing the presence of the *ags* gene (Figure 4-4A) from the T_R-DNA, whereas the presence of genes from the T_L-DNA was confirmed by both *rol* A (Figure 4-4B) and *rol* C (Figure 4-4C). *S. africana-lutea* hairy root clones showed characteristics of transgenic roots such as abundant adventitious growth in PGR-free liquid medium (**Error! Reference source not found.**-2), relatively high production of secondary metabolites commonly produced by transgenic roots. The transgenic characteristics of the *S. africana-lutea* hairy roots are due to the presence of genes from the *rol* loci on the T_L-DNA. White *et al.* (1985) stated that the *rol* loci on the T_L-DNA are the most important virulence factors with *rol B* playing the integral role in pathogenicity. Although *rol* B gene was amplified successfully from the Ri plasmid DNA, the *S. africana-lutea* root clones seemed to be lacking the *rol* B gene as no amplicons were detected (Figure 4-4D). This is not surprising as Spéna *et al.* (1987) reported the induction of hairy roots by both *rol* A and C genes in tobacco leaf discs. *Rol* genes can act synergistically in pairs to produce a stronger effect on the induction of hairy roots than single genes (Spéna *et al.* 1987). Also, according to Nilsson and Olsson (1997), other genes on the T_R-DNA of the agropine strains play additional roles in establishing full hairy root symptoms. These genes encode for auxin biosynthesis (Nilsson and Olsson 1997), thus the improvement in the proliferation of hairy roots. Therefore, transgenesis of *S. africana-lutea* hairy root cultures in this study could be due to a concerted effect between the *ags*, *rol* A and C genes.

Another explanation of the absence of the *rol* B gene in the *S. africana-lutea* hairy root clones is probably due to the frequent spontaneous deletions within the Ri T-DNA of *A. rhizogenes* agropine strains. The prolonged root culture of potato hairy root culture has been reported to be the probable cause of the deletions (Hanisch ten Carte *et al.* 1990). This could be the case since the *S. africana-lutea* hairy root clones used in this study have been in culture since 2005. However, whether the change in both T_L-DNA and/or T_R-DNA are due to the properties of the Ri T-DNA integration or to the period and type of the *in vitro* root culture still remains to be clarified (Batra *et al.* 2004).

In the other PCR analysis results, the A4T(2) root clone showed the absence of the T_R-DNA (*ags* gene) (Figure 4-4A). The lack of T_R-DNA in agropine strain transformants is a common result. According to Nilsson and Olsson (1997), most of the hairy root clones induced by agropine strains in most cases contain only the T_L-DNA. Batra *et al.* (2004) stated that lack of either T_L-DNA or T_R-DNA is indicative of fragmented integration of the Ri T-DNA. However, detailed analysis of the integration process still needs to be done in order to understand the mechanism of fragmented Ri T-DNA integration in hairy root clones (Batra *et al.* 2004).
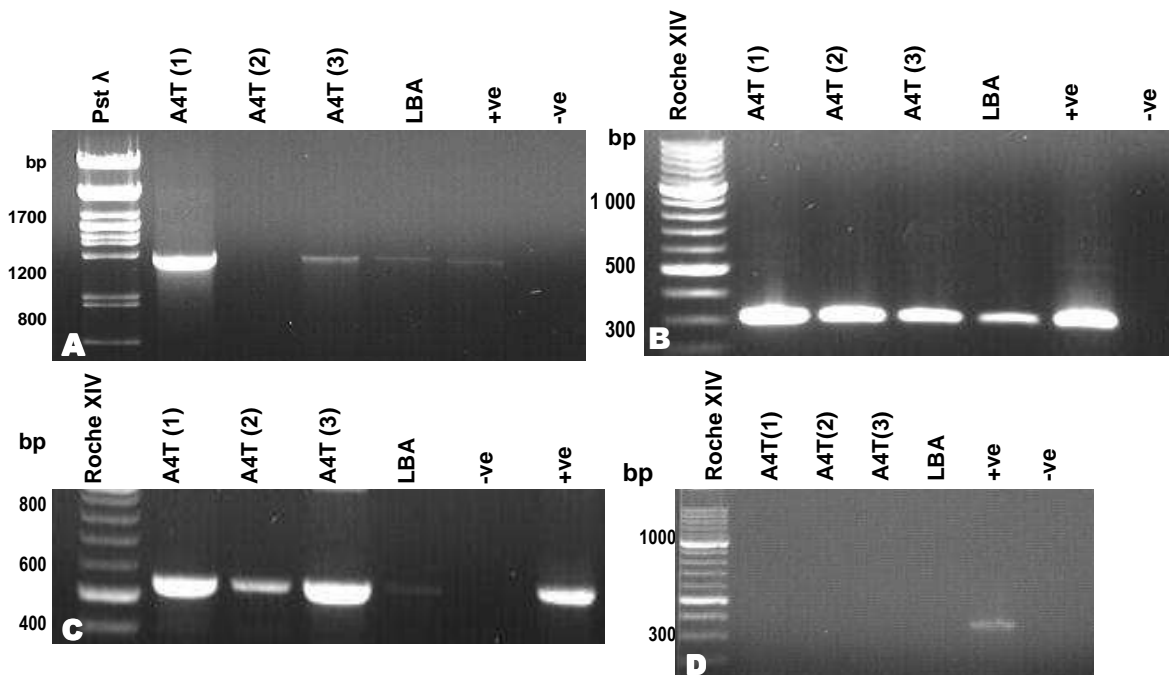
Figure 4-4 PCR amplifications of the *rol* and *ags* genes in *S. africana-lutea* root and Ri plasmid DNA. **A)** *ags* amplification (1600 bp) **B)** *rol* A amplification (300 bp) **C)** *rol* C amplification (600 bp) **D)** rol B amplification (300 bp)

4.4.4 Probe preparation

The *rol* A amplicon was used as a probe for Southern hybridisation since its yield was the highest in comparison to the other PCR-purified amplicons (Figure 4-5B).

4.4.5 Southern hybridisation

The Southern blot detected the *rol* A gene in the *S. africana-lutea* hairy root clones however, due to visible smears, it is difficult to tell whether it is a single or multiple copies (Figure 4-5C).
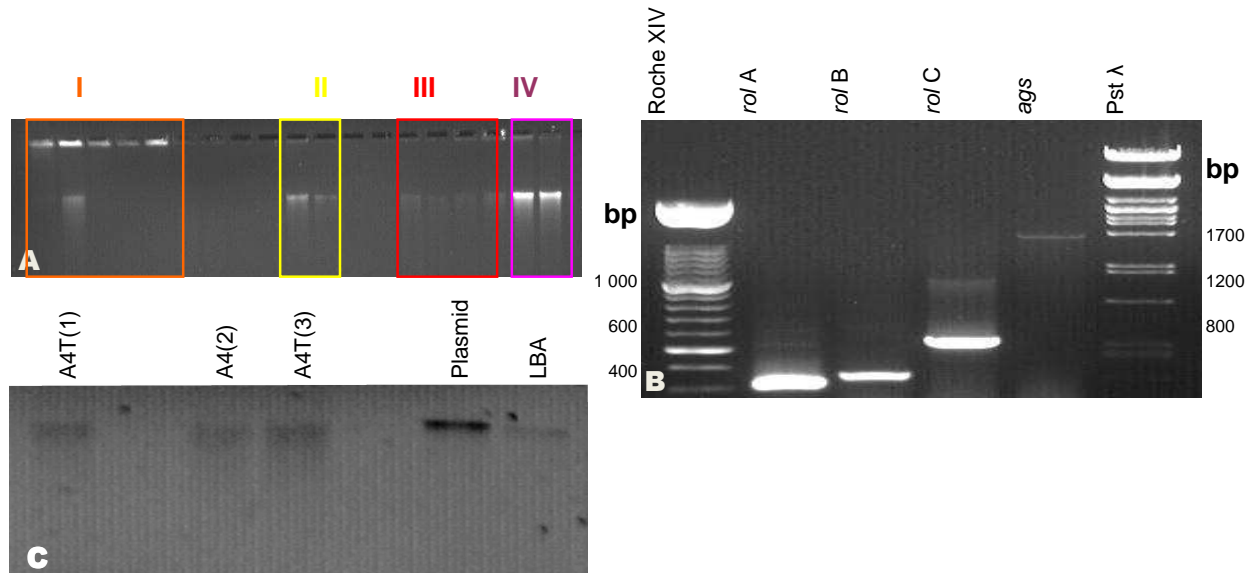
Figure 4-5 Southern hybridisation of *S. africana-lutea* root DNA with *rol* A probe
A) Restriction digestion of hairy root DNA I= A4T(2); II= A4T(3); III= LBA; IV= A4T(1); **B)**
PCR-Purified probes **C)** Southern blot (using *rol* A probe)

To conclude this chapter, the best DNA extraction protocol for *S. africana-lutea* hairy roots is by Maxi Kit as it has proven to successfully remove metabolites that interfere with downstream molecular analyses of transgenic *S. africana-lutea* root DNA. The successful PCR amplifications and restriction digestion proved the credibility of this protocol. The Southern hybridisations elucidated the integration of transgenes into the *S.africana-lutea* hairy root genome.

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CHAPTER 5

PHYTOCHEMISTRY AND PHARMACOLOGY OF *S. AFRICANA-LUTEA*[1] CLONES

5.1 STRATEGIES FOR THE DISCOVERY OF BIOACTIVE PHYTOCHEMICALS

One of the aims for phytochemical research is the discovery of new biologically-active compounds for medicinal use. As discussed previously, research on the South African Salvias is still limited even though these plants are extensively used in traditional medicine particularly in areas where they are occurring in abundance. Even so, research has been undertaken using wild-harvested plant material. Details on the bioactivity of the key compounds found in exotic and indigenous sages were previously discussed as part of the literature review (Sections 2.1.2 and 2.1.3).

Since S. *africana-lutea* is mainly used traditionally for various microbial infections (reviewed by Jäger and van Staden 2000), this study focussed on highlighting the antimicrobial properties of this plant and giving the overall profile of the bioactive compounds. Qualitative and quantitative bioassays have been used to determine the antimicrobial activity of salvias. A disc diffusion method (qualitative bioassay) was used to analyse the methanolic extracts and essential oils of *S. africana-lutea* and *S. africana-caerulea* (Fisher 2005). The disc diffusion method is open to subjectivity (Swenson *et al*. 1989) because the interpretation of results depends largely on the technique and judgment (Marsh and Goode 1994). The micro-dilution assay is therefore commonly used for quantifying the antimicrobial activity of sages. The micro-dilution assay involves serial dilutions of the extract, followed by addition of the test micro-organism to determine the minimum inhibitory concentration (MIC) for the test microbe (Eloff 1998). The MIC is the lowest concentration at which the extract inhibits visible microbial growth (Hewitt and Vincent 1989; Eloff 1998).

The chemical profiling of medicinal plants involves quantitative and qualitative analyses of metabolites. The overall profiling of an organism's metabolites produced in the living cells is known as metabolomics (Verpoorte *et al.* 2005). According to Verpoorte *et al.* (2005), there is no single method that can quantify all metabolites. Therefore, various methods are used in metabolomics which are chromatography based (e.g. gas chromatography, thin-layer chromatography), molecular weight based (mass spectrometry) and physical characteristics based methods (nuclear magnetic resonance) each with its own advantages and disadvantages (Verpoorte *et al.* 2005). For instance, nuclear magnetic resonance (NMR) is the best tool for initial overall macroscopic analysis for plant metabolomes. In subsequent metabolomics stages, chromatography and molecular weight based methods can be used for more targeted identification and quantification (Verpoorte *et al.* 2005). An example of a more targeted metabolomics approach is the use of methods which combines both chromatography and molecular weight based methods such as gas chromatography (GC) coupled with mass spectroscopy (GC-MS). Most of metabolomic studies on *Salvia* used more targeted approaches for profiling the presence of volatile and non-volatile components. The GC-MS has been used for analyses of the volatile compounds in the essential oils of South African Salvias (Kamatou *et al.* 2006[ab]; 2007[b]; 2008[a]). GC-MS is a powerful analytical tool in phytochemical analyses used for the identification of constituents for essential oil (Binks *et al.* 1969) and other volatile compounds. GC-MS can detect other compounds including sugars, sugar alcohols, organic acids, amino acids, fatty acids and a wide range of secondary compounds (Fiehn *et al.* 2000). *Salvia* species are also rich in non-volatile compounds commonly referred to as phenolics. Non-volatile samples to be analysed by GC-MS can be rendered volatile by an additional step of derivatisation (Maloney 2004). Most phenolic compounds common to *Salvia* species are the caffeic acid derivatives. This means that caffeic acid is the building block of most phenolic compounds isolated from *Salvia* species (Kamatou *et al.* 2008[b]). Some of phenolic compounds were detected in salvias using HPLC, UV and mass spectroscopy (Kamatou *et al.* 2007[b]). On the other hand, NMR analysis (both one- and two-dimensional) were used for structural elucidation of three bioactive abietane diterpenes carnosol, rosmadial and carnosic acid isolated from S. *africana-lutea* (Hussein *et al.* 2007).

Medicinal plants are not only directly important to human health but also to the production of agricultural crops which consequently affect human health. Scientists have focused mainly on the increase of food production to feed the growing world population by genetically

modifying crops to have better resistance against pests and plant diseases caused by bacteria, viruses and fungi (Kotan *et al.* 2008). Despite the continual improvement of crop resistance, chemicals are still widely used to control plant diseases (Kotan *et al.* 2008). The use of chemicals for eradicating plant diseases is associated with phytopathogens developing resistance (Daferera *et al.* 2003; Bajpai *et al.* 2008). As a result, higher concentrations of these chemicals have to be applied to overcome resistance of phytopathogens (Daferera *et al.* 2003; Bajpai *et al.* 2008). High concentrations of these chemical pose a risk of leaving toxic residues on agricultural products (Bajpai *et al.* 2008) especially on the fruits and vegetables which are usually consumed soon after harvest (Daferera *et al.* 2003). Furthermore, some of these chemicals pollute the environment especially groundwater (Jouany 2007) due to their slow biodegradation. Some of these are not biodegradable (Barnard *et al.* 1997). As a result, there has been considerable interest in finding alternative ways of controlling plant diseases using plant extracts, essential oils and other secondary metabolites (Jouany 2007; Bajpai *et al.* 2008; Kotan *et al.* 2008) which are safer to consumers and the environment (Daferera *et al.* 2003).

Most of the herbal plants used for health benefits have recently been used in controlling phytopathogens. For instance, antifungal activity (phytofungi) of essential oils and plant extracts from some members of the Lamiaceae family such as rosemary (Bajpai *et al.* 2007; Daferera *et al.* 2003), peppermint (López *et al.* 2004; Bajpai *et al.* 2007), basil (Bajpai *et al.* 2007), oregano (Daferera *et al.* 2003; López *et al.* 2004), thyme, dictamnus, lavender and sage (*S. fruticosa*) (Daferera *et al.* 2003) have been documented. Nonetheless, most of the microbial studies of *Salvia* species emphasised the antifungal properties of these plants against human fungal pathogens. For instance, research focused mainly on the clinical *Candida* species such as *C. albicans* (Kuźma *et al.* 2007; Pinto *et al.* 2007), *C. krusei*, *C. tropicalis*, *C. glabrata* (Pinto *et al.* 2007) and sometimes some filamentous fungi such as *Aspergillus flavus* and *Fusarium monoliforme* (Pinto *et al.* 2007). The antifungal properties of *S. africana-lutea* against two phytopathogenic fungi were also determined in this particular study using *F. verticilliodes* and *F. proliferatum*. *Fusarium* species can have detrimental effects on the crop yield (Jouany 2007). *Fusarium verticilliodes* leads to substantial yield losses in crops such as maize, sorghum, sugarcane, wheat, cotton in almost every crop production season because it causes stem rot in these crops (Fandohan *et al.* 2004). *Fusarium proliferatum* on the other hand is well known for causing damping-off and root rot on crops such as maize and rice (Abdalla *et al.* 2000). *Fusarium* species not only lead to

loss of crop yield, but they are also a danger to the consumers' health. The fungal pathogens tend to produce carcinogens called mycotoxins once they have infected the crop grains either in the field or during grain storage (Dambolena *et al.* 2008). High levels of mycotoxins in grains are a threat to both humans and animals that may consume the contaminated grains and grain products (Dambolena *et al.* 2008). The long-term health effects of mycotoxins include the development of cancers (Samapundo *et al.* 2007). The social and economical implications caused by fungi on the agronomic production have thus led to attempts in developing new antifungal agents that are safer for both consumption and the environment (Bajpai *et al.* 2008). At present, the research on the phytochemical and biological activity on South African sages is limited and no studies have focussed on analysing the effect of *Salvia* species on *Fusarium* species.

In this chapter, the metabolite profile and bioactivity of the wild growing *S. africana-lutea* was compared with that of the *in vitro* cultures (both transgenic roots and non-transgenic plantlets). This research was undertaken to determine the impact of biotechnological manipulations on the overall metabolite profile and antimicrobial bioactivity of *S. africana-lutea*. GC-MS analysis was utilised to compare the general chemistry of different organ cultures. This technique also facilitated the preliminary identification of major compounds that accumulate in the transgenic hairy root clones. Using the micro-dilution bioassay, the antimicrobial activity of the S. *africana-lutea* cultures was investigated. An *in vitro* culture system of medicinal plants is essential as it can be used as an alternative source of important secondary compounds with potential of up-scaling for commercial production.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of plant extracts (Non-volatile compounds)

Different hairy root clones and foliage tissue (both *in vitro* and *ex vitro*) were dried in a 50 ^{o}C oven for two days. The oven-dried plant material was ground to a fine powder using a pestle and mortar. The extraction of compounds was carried out by sonicating the tissue in methanol:dichloromethane (MetOH:DCM) (1:1 v/v) for two hours at room temperature. The extracts were filtered using Whatman filter paper No. 1. The extraction process was repeated twice. The filtered extracts were dried under vacuum at 40 ^{o}C in a rotary

evaporator (BUCHI) to yield a concentrated residue. The dried residues were kept dry by covering the flasks with tin foil and stored at room temperature in the dark. Each dried residue was dissolved in 1 ml MetOH:DCM (1:1 v/v) to give a final concentration of 50 mg ml^{-1}.

5.2.2 Extraction of essential oil (Volatile compounds)

Fresh wild growing foliage tissue of *S. africana-lutea* was harvested from Stellenbosch University campus along the Polymer Science building during springtime on 30th August 2007 (Figure 2-2A). Freshly harvested leaves of wild growing *S. africana-lutea* (26.4 g) were separated from the wooden branches. A Clevenger-type apparatus was used to produce a hydrodistillate of the essential oils by heating approximately 250 ml distilled water at 40 ^{o}C. The hydrodistillation was carried out for four hours. The hydrodistilled essential oil was collected in an airtight vial and HYDRANAL®-Molecular sieve 0.3 nm (RIEDEL DE HAËN®) was added into the collected oil to absorb any moisture during storage. The distilled essential oil was stored at 4 ^{o}C in the dark prior to GC-MS analysis.

5.2.3 TLC analysis

To determine the secondary metabolite fingerprint of each extract, 20 µl of each extract (50 mg ml^{-1}) was applied as a band on a 0.25 mm Silica gel 60 F_{254} TLC plate (MERCK) and developed in a saturated glass chamber in an ascending one-dimensional mode using polar and non-polar eluents. Two eluents were used, namely, 1) a polar eluent consisting of ethylacetate:methanol:water (100:13.5:10 v/v) and 2) a non-polar solvent of toluene:ethylacetate (93:7 v/v). Chromatograms were visualized under 254 nm and 366 nm UV light prior to spraying with anisaldehyde-R stain (465 ml ethanol, 5 ml glacial acetic acid and 13 ml concentrated sulphuric acid). The sprayed TLC plates were incubated immediately at 100 ^{o}C for 5 min to develop the stain. Secondary metabolite profiles of transgenic root and foliage tissues were compared after the development of the TLC chromatograms.

5.2.4 Antibacterial bioassays

Two Gram-positive (Gram +ve) bacteria, *Bacillus subtilis* (ATCC 6051) and *Staphylococcus aureus* (ATCC 12600) and two Gram-negative bacteria (Gram –ve), *Escherichia coli* (ATCC 11775) and *Klebsiella pneumoniae* (ATCC 13883), kindly donated by Professor Johannes van Staden from the University of KwaZulu-Natal (Pietermaritzburg) were used for antibacterial assays. Bacterial cultures were grown in cooled autoclaved (one atmosphere, 121 ^{o}C for 20 min) Mueller-Hinton (MH) broth overnight in a 37°C incubator shaking at 100 revolutions per minute (rpm).

The MetOH hairy root and leaf extracts (50 mg ml^{-1}) of *S. africana-lutea* were serially diluted in 96-well micro-titre plates using a micro-dilution assay according to Eloff (1998). Each well was filled with 100 µl sterile water. A two-fold serial dilution was prepared by diluting 100 µl of extract. After a serial dilution, 100 µl of undiluted overnight bacterial culture was added to each well. The covered microplates were incubated overnight at 37 ^{o}C. As an indication of bacterial growth, 40 µl of 10 mg ml^{-1} *p*-iodonitrotetrazolium violet (INT) (SIGMA$^{®}$) dissolved in sterile water was added to each well the following day and incubated at 37 ^{o}C for 30 min. The colourless tetrazolium salt in INT is reduced to a red colour by biologically-active organisms such as bacteria. Therefore, a clear solution showed inhibition whereas a red solution was a detection of bacterial growth. The MIC was recorded as the lowest concentration of the extract that inhibited bacterial growth (Eloff 1998). The following controls were also included in the bioassay:

- A positive control containing serially diluted streptomycin (10 mg ml^{-1}) and the pathogen. This was to ensure that the test pathogen was not an antibiotic-resistant strain and to compare the activity of the pathogen in the antibiotic solution relative to *S. africana-lutea* extracts.
- A negative control containing the pathogen and the solvent (MetOH) to ensure that the solvents were not inhibiting pathogen growth, and
- A pure bacterial culture to guarantee that laboratory conditions that bacteria were inoculated into were not inhibitory.

5.2.5 Antifungal bioassays

To determine the antifungal activity of *S. africana-lutea* foliage and transgenic root extracts, the PROMEC Unit (Programme on Mycotoxins and Experimental Carcinogenesis Microbiology) at the Medical Research Council (MRC) in Tygerberg, Cape Town was visited to carry out these assays using the two plant pathogenic fungi kindly provided by Doctor David Katerere. Susceptibility of these two plant pathogenic fungi namely, *Fusarium verticilliodes* (MRC 8267) and *F. proliferatum* (MRC 7140) to the plant extracts was tested. The fungi were suspended in 0.05% (v/v) Tween 20 and standardized to 0.5% (v/v) McFarland. The fungal suspension (12.5 ml) was inoculated into 37.5 ml autoclaved (1 atm, 121 ^{o}C for 20 min) potato dextrose broth (PDB) and incubated at 25 ^{o}C for two days.

In contrast to the antibacterial assays, more dilute extracts (10 mg ml^{-1}) prepared by weighing 20 mg of plant extract and dissolving in 2 ml acetone were used for the antifungal bioassays. Each extract was serially diluted as described in Section 5.2.4. The fungal suspension (100 µl) was added in each well after serial dilution of extracts. Before incubation of the microplates at 25 ^{o}C, 40 µl of 0.2 mg ml^{-1} INT (SIGMA$^®$) was added in each well as an indicator for fungal growth. Microplates were incubated for four days and the fungal growth was recorded every 24 hours. The MIC was recorded as the lowest concentration of the extract that inhibited fungal growth (Eloff 1998). The following controls were also included in the bioassay:

- A positive control contained a clinical fungicide Amphotericin B (SIGMA) and fungus. In another positive control, an agricultural fungicide Cantus (SIGMA) was added to the fungi. Fungicides were included in the bioassay to compare the resistance of test fungi against fungicides and *S. africana-lutea* extracts.
- The negative control containing fungi and acetone was included to ensure that the acetone (solvent) was not inhibiting fungal growth.
- A pure fungal culture to monitor fungal growth was utilised to ensure that the laboratory conditions were not inhibitory for fungal growth.

For all the micro-dilution bioassays (antibacterial and antifungal tests), the experiments were done in triplicate. If one or more of the three wells were not at the same level, the assay was

repeated until the triplicate value was obtained. Since the micro-dilution bioassay is a susceptibility test, no statistical analysis was performed.

5.2.6 Bioautographic assay

In addition to the micro-dilution assays, a bioautographic assay was conducted on the TLC plates developed as described in Section 5.2.3. The developed plates were dried in the fumehood to ensure that the eluent (solvent) had evaporated completely. Both the direct and indirect bioautographic assays were conducted.

- The direct method was carried out according to Stafford *et al.* (2005). Briefly, the overnight bacterial culture of each strain was centrifuged in a sterile 15 ml microfuge tube at 3000 rpm for 10 min. The bacterial pellet was diluted with sterile MH broth. The TLC plates were sprayed evenly with diluted bacterial suspension under the fumehood and incubated at 100% relative humidity at 37 $^{\circ}$C for 24 hours. Although disarmed bacterial strains were used for the bioassays, a surgical mask was worn for protection from possible potential contamination.

- The indirect method of Chomnawang *et al.* (2005) was followed and developed TLC plates were overlaid with agar containing an aliquot of bacterial culture which had been grown overnight 37 $^{\circ}$C. The bacterial culture was centrifuged at 3000 rpm for 10 min and diluted with sterile MH broth. A millilitre of this diluted overnight bacterial culture was transferred to 20 ml of the cooled MH broth with 0.2% (w/v) agar. This mixture was mixed by gently swirling the Erlenmeyer flask. Thereafter the bacterial agar culture was overlaid on the developed TLC plates and incubated at a 100% relative humidity at 37 $^{\circ}$C overnight in the dark.

All the bacterial inoculations and overlaying of the TLC plates were carried out on the bacterial laminar flow bench. The TLC plates were run in duplicate.

5.2.7 GC-MS analysis

For the GC-MS analysis, 100 µl of hairy roots and leaf (*in vitro* and *ex vitro*) MetOH extracts were first vacuum dried in a SpeedVac® Plus SC 110A (SALVANT). To render these vacuum-dried extracts volatile, they were derivatised by adding 80 µl of methoxyamine HCl in pyridine (20 mg ml^{-1}) and vortexed to dissolve the dried residue thoroughly. The extracts were incubated at 30 ^{o}C for 90 min on a shaker. Next, 140 µl of N-Methyl-N-(Trimethylsilys)-trifluoroacetamide (MSTFA) was added and the samples were incubated further on a shaker at 37 ^{o}C for 30 min. The derivatised samples were left at room temperature for two hours before injection into the GC-MS.

5.2.7.1 Hairy root and foliage extracts

Derivatised authentic standards (as described in Section 5.2.7) in Table 5-1 were run along with the sample extracts. The components of the derivatised sample extracts were identified by comparing their relative retention times and mass spectra with those of the derivatised GC-MS standards.

For analysis, one µl of derivatised sample was injected with a split-less injection into the GC-MS system with the flow rate of 1 ml min^{-1}. The GC-MS system consisted of an AS 2000 autosampler, trace GC and a quadropole trace MS (THERMOFINNIGAN). The gas chromatography was done on a 30 m Rtx®-5Sil MS column (RESTEK) with Integra Guard with an inner diameter of 0.25 mm and 0.25 mm film thickness. The injection temperature was 230 ^{o}C and the ion source temperature was 200 ^{o}C. The following temperature programme was used: 5 min at 70 ^{o}C, followed by the first ramp of 1 ^{o}C min^{-1} to 76 ^{o}C and a second ramp of 4 ^{o}C min^{-1} to 320 ^{o}C. Before the injection of the next sample, the temperature of the system was then equilibrated to 70 ^{o}C. The mass spectra were recorded at two scans per second with the scanning range of 30-600 *m/z*. The Xcalibur^{TM} software bundle version 1.2 was used to assess the chromatograms and the mass spectra (Finningan Corporation 1998-2000).

5.2.7.2 Essential oil

The analysis of the essential oil was similar to derivatised root and foliage extracts except that there was no derivatisation prior to the GC-MS analysis. Instead, one µl of the essential oil was dissolved in GC-standard DCM (MERCK). The DCM-dissolved oil mixture (one µl) was injected into the GC-MS system at 40 $^{\circ}$C. The temperature programme for the analysis of the essential oil was also different in that it consisted of only one ramp of 2 $^{\circ}$C min^{-1} to 300 $^{\circ}$C. Derivatised GC-MS standards (Table 5-1) were run along the essential oil samples and their retention times and mass spectra were used for profiling of *S. africana-lutea* essential oil components.

Table 5-1 Authentic standards derivatised with MSTFA for GC-MS analysis

Standard name	Quantity	Manufacturer
α-myrcene	1 µl	Fluka
(IR)-(+)-α-pinene	1 µl	SΛFC™
β-caryophyllene	1 µl	SΛFC™
3-carene	1 µl	Fluka
Caffeic acid	1 mg	SIGMA®
Camphene	1 mg	ALDRICH®
(IR)-(+)-Camphor	1 mg	ALDRICH®
Caryophyllene oxide	1 mg	SΛFC™
R-(+)-limonene	1 µl	Fluka
7,8 Dihydroxy-6-methoxycoumarin	1 mg	ALDRICH®
Rosmarinic acid	1 µl	CALBIOCHEM®
(-)-α-Thujone	1 µl	Fluka
1,4 Cineole	1 µl	Fluka

5.2.8 NMR analysis

The NMR analysis was undertaken as part of the metabolite profiling of *S. africana-lutea*. The ^{1}H NMR spectra were run on either a 300 MHz Varian VNMRS spectrometer equipped with Oxford magnet (7.0T) operating at 300 MHz for ^{1}H, or a Varian Unity Inova spectrometer equipped with a Oxford magnet (11.74T) operating at 400 MHz for [1]H. In both instances a 5 mm dual broadband pulsed field gradient (PFG) probe with a probe temperature of 25 ^{o}C was used. A standard pulse sequence was applied for the ^{1}H experiments with a 1 second relaxation delay and 4 seconds acquisition time.

5.3 RESULTS AND DISCUSSION

5.3.1 TLC analysis

Polar and non-polar solvents produced different secondary metabolite profiles from both transgenic root and foliage extracts on TLC plates (Figure 5-1). For instance, the polar eluent separated more compounds especially at the top of the chromatogram (indicated by an arrow) which some of them were not present on the non-polar eluent chromatogram. This is because the separations of the components of the extract depend on the polarity of the solvent with more polar components separated by polar eluents and vice versa for non-polar eluent (Hahn-Deinstrop 2000). Distinct TLC metabolite profiles of polar and non-polar eluents showed that *S. africana-lutea* plants synthesise both polar and non-polar secondary compounds.
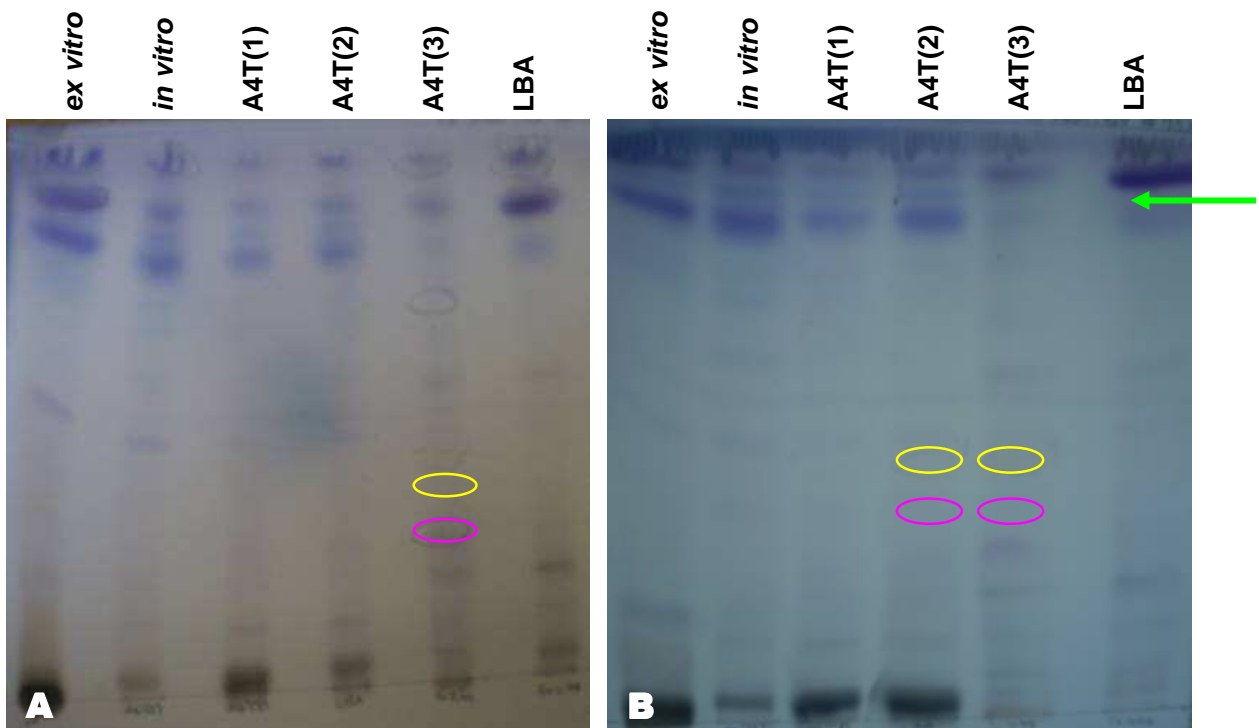
Figure 5-1 Transgenic root and leaf extracts from *in vitro* and *ex vitro Salvia africana-lutea* plant material analysed using thin layer chromatography A) A non-polar solvent of toluene: ethyl-acetate (93:7 v/v) and B) a polar eluent consisting of ethyl-acetate: methanol: water (100:13.5:10 v/v)

5.3.2 Antibacterial bioassays

The results of this particular study indicated that the use of the *in vitro* propagules produces antibacterial compound(s) especially against *B. subtilis* (Table 5-2). In this particular case, the microenvironment during tissue culture strongly influenced the chemical composition in tissue cultured plantlets resulting in extracts produced from these plants being more biologically active. The influence of different environmental conditions on secondary metabolism is well-documented for example by Kamatou *et al.* (2008[a]). The antibacterial activity of the *in vitro* S*. africana-lutea* leaf extracts is important as these leaves can possibly be used as the alternative foliage material in decoctions for treatment of various bacterial infections mentioned in Section 2.1.2. Distinct antibacterial fractions were not detected (no

inhibition zones) by the bioautographic assay. This was because insignificant MICs against bacterial strains were recorded for all *S. africana-lutea* extracts (Table 5-2).

Table 5-2 Antibacterial MIC (mg ml^{-1}) of *S. africana-lutea* extracts (after 24 hours)

Extract	*B. subtilis*	*S. aureus*	*K. pneumoniae*	*E. coli*
A4T(1)	3.125	3.125	3.125	3.125
A4T(2)	ND	ND	6.25	ND
A4T(3)	6.25	ND	ND	ND
LBA9402	3.125	3.125	3.125	3.125
In vitro leaves	0.39	3.125	3.125	6.25
Ex vitro leaves	ND	ND	6.25	3.125
Streptomycin	3.125	3.125	0.78	0.39

NOTE: ND= Not Detected; values shaded indicate the lowest MIC.

The total volume of the hydrodistilled essential oil from fresh wild growing *S. africana-lutea* foliage tissue was 0.37 µl g^{-1}. The *ex vitro* leaf extracts had relatively poor antibacterial activity (Table 5-2) which is contradictory to results documented by Kamatou *et al.* (2008[a]). According to Kamatou *et al.* (2008[a]), the *S. africana-lutea* foliage tissue harvested in springtime had the best antibacterial activity in comparison to leaf extracts from other seasons. The best bioactivity of leaf material collected during springtime in this particular case could be because plants produce significant amounts of essential oils in springtime to attract pollinators (Kamatou *et al.* 2008[a]). The relatively poor antibacterial activity of wild *S. africana-lutea* leaves used in this study could have been influenced by the seasonality. Plant material collected in different seasons may contain novel compounds with varying bioactivities (Eloff 1999) causing fluctuating bioactivities. The low bioactivity of *ex vitro* leaves was not surprising because many studies reported fluctuations in bioactivity of the same plant collected in different seasons and geographical locations (McGaw *et al.* 2002). For instance, foliage tissue used in this particular research was collected from Stellenbosch University gardens (Western Cape) while Kamatou *et al.* (2008[a]) collected leaves from the South Western Cape. Watering regimes of the Stellenbosch garden could have influenced the chemistry of these plants. Therefore, for commercial reasons tissue culture would be

ideal to produce plants with a 'predictable' chemical composition of metabolites as the *in vitro* environment is controlled.

5.3.3 Antifungal bioassays

Most of the *S. africana-lutea* extracts inhibited *F. verticillliodes* at relatively low concentrations ranging from 0.02 to 0.64 mg ml^{-1} with A4T(3) giving the MIC of 0.08 mg ml^{-1} against both *F. verticillliodes* and *F. proliferatum* over the four day incubation period (Table 5-3). The most notable finding here is that all hairy root clone extracts inhibited *F. verticillliodes*. This is a significant finding particularly for the agricultural sector as there has been a paradigm shift globally towards the use of eco-friendly, natural plant-based extracts to control fungal pathogens (Bajpai *et al*. 2008). Furthermore, there has been an increasing demand from consumers for natural products that are safe and effective (Bajpai *et al*. 2008) in controlling pests and plant pathogens.

Table 5-3 Antifungal MIC (mg ml^{-1}) of *S. africana-lutea* extracts (after 96 hours)

Extract	*F. verticillliodes* (MRC 8267)	*F. proliferatum* (MRC 7140)
A4T(1)	0.04	0.16
A4T(2)	0.02	0.64
A4T(3)	0.08	0.08
LBA9402	0.64	ND
In vitro leaves	ND	0.32
Ex vitro leaves	0.02	0.64
Amphotericin B	0.64	0.08
Cantus	0.16	0.08

NOTE: ND= Not Detected; values shaded indicate those that are comparable to the standard and/or have lower MICs

According to Bajpai *et al.* (2008), plant-derived extracts especially the essential oils are generally considered non-phytotoxic and potentially active against pathogenic fungi. The phenolic compounds mainly active against microbes in the essential oils are the terpenes. For instance, the monoterpenes and sesquiterpenes along with the monoterpene and sesquiterpene hydrocarbons are attributed for the antimicrobial activity of most plants' essential oils (Bajpai *et al.* 2008). The oxygenated monoterpenes present in relatively high quantities in the *S. hydrangea* essential oil, have been proven to have stronger antifungal activity than essential oils rich in only monoterpene hydrocarbons (Kotan *et al.* 2008). The essential oil of *S. hydrangea* was potent against 15 phytopathogenic *Fusarium* species including *F. verticilliodes* (Kotan *et al.* 2008).

In another study, the authentic cyclic terpenes (limonene, menthol, methone and thymol) inhibited the growth of *F. verticilliodes* however; the bioactivity was dependent on the nature and concentration of the terpenes (Dambolena *et al.* 2008). Similarly, the increase in concentration of either vanillic and caffeic acid (natural phenolics) decreased the growth rate of both *F. verticilliodes* and *F. proliferatum* (Samapundo *et al.* 2007).

This research has shown that *S. africana-lutea* is not only an important herb to human health but also for the first time has been shown to be a potential source of natural antifungal compounds which would be more acceptable for use in both pre- and post-harvest fungal control. Therefore, these extracts can be used as 'green' agricultural fungicides instead of the synthetic industrial fungicides. The natural protective effect of plant extracts against plant pathogens can be exploited using the *in vitro* systems used in this research. Nevertheless, more studies should be carried out to identify the active components of the extracts and their modulatory activity (Jouany 2007). The identification of the compounds responsible for this activity requires attention but is beyond the scope of this particular thesis.

5.3.4 Secondary metabolite profiles

The overall objective of metabolite profiling was to gain an overall picture on the composition of the extracts and to compare metabolite profiles of the different root clones with the foliage. This was essential for gaining a better understanding of the key compounds present in the *S.*

africana-lutea root clones and leaves. Two different approaches, NMR and GC-MS were used. The NMR detected the major groups of compounds such as sugars, saponins and terpenoids. On the other hand, the GC-MS Xcalibur™ library was use to identify compounds belonging to the reducing sugars, quinines, alkaloids, diterpernes, triterpenes, phenolics and steroid chemical groups. Majority of the GC-MS identified compounds were precursor or intermediate compounds for the biosynthesis of different pharmaceuticals, agricultural chemicals and other important industrial products (Table 5-4). It must be noted that all compounds listed on Table 5-4 were the ones with the closest library match (between 85-100%).

The application of plant biotechnological techniques (plant tissue culture and transformation) had a noteworthy change in the phytochemistry of *S. africana-lutea*. The hairy roots and leaf extracts had distinct metabolite profiles. Each of the hairy root clones had a unique secondary metabolite profile (Figure 5-2A-D). For instance, the A4T(1) (Figure 5-2A) was the most complex with A4T(2) (Figure 5-2B) being the least complex. Although the metabolite profile of A4T(2) was relatively simple, the relative abundance of the detected compounds (mostly sugar compounds) was high in comparison to other clones (Figure 5-2B). On the other hand, the complex A4T(1) profile comprised of various compounds such as pyridine, 3,4 dihydronaphthalene and glucofuranoside amongst many others. Despite the distinctiveness of the hairy root clone profiles, there were some similarities in some parts of the profile pattern amongst the different root clones (indicated by arrows). The compounds that eluted between 23 and 24 min were common to all four clones. Some parts of the A4T(1) and A4T(3) profiles showed some relatedness with A4T(2) and LBA 9402 being partially similar (Figure 5-2A-D). The profiles of both A4T(2) and LBA 9402 were similar at approximately 12 and 46-47 min retention times (see arrows on Figures 5-2B and D). The common compounds to A4T(2) and LBA 9402 were sorbose, α-D glucopyranose and fructose oxime. In the case of A4T(1) and A4T(3) the profile pattern was similar between 29-30 min with common compounds being mannose, D-gluconic acid, Cholestan-2,3-dione-2,3-dioxime and [1] Benzothiepino [4,5-c] pyridine (see arrows on Figures 5-2A and C). Only one compound 2-azathianthrene a phenolic anti-oxidant (Jeong *et al.* 2004) was present in all hairy root clones.

The metabolite profile of tissue culture-derived leaf extracts (Figure 5-3A) and the wild growing *S. africana-lutea* leaves (Figure 5-3B) were equally complex. Some of the compounds produced in the *in vitro* cultures were not detected in the *ex vitro* leaves (Table 5-4). Only 2, 4-Imidazolidinedione was unique to the wild growing leaves while most compounds were unique to tissue cultured leaves. Therefore, it seems that the *in vitro* environment induces *de novo* biosynthesis of secondary compounds in tissue-cultured tissues. This could explain the good antibacterial activity of *in vitro* leaves against *B. subtilis* (Section 5.3.2).

The common compounds to both the leaf and root extracts were various simple sugar oximes (Table 5-4). *S. africana-lutea* extracts (both *in vitro* and *ex vitro*) had various bioactive compounds that justify the anti-microbial activities in Sections 5.3.2 and 5.3.3. For instance, germanicol, a triterpenoid with antimicrobial properties found in sages (Anaya *et al.* 1989; Veličković *et al.* 2002) was detected in A4T(2) and A4T(3) clones. Another compound thiocyanic acid, an intermediate used for the production of antibiotics and fungicide pharmaceuticals was detected in the tissue-cultured leaves. Furthermore, glucopyranose and quinolines were identified in the A4T(2) and *in vitro* leaves' extracts respectively. Glucopyranose and quinolines are used as fungicides and antibacterial agents respectively (Del Valle 2004). Accumulation of these antimicrobial compounds may explain the *in vitro* bioactivity of *S. africana-lutea* extracts. Other identified secondary compounds on Table 5-4 are used for various therapeutic applications similar to those reported in other sages worldwide as discussed in Section 2.1.2. For instance, caffeic acid, an anti-oxidant was detected only in the tissue-cultured plants and LBA 9402 hairy root clone. Caffeic acid is highly synthesised in most *Salvia* species (Lu and Foo 2002). Caffeic acid monomer forms dimers, trimers and other complex sage phenolic compounds (Jiang *et al.* 2005). Also, sorbose which is a monosaccharide used for commercial production of Vitamin C (an antioxidant) (Hoshino *et al.* 2003; Sugisawa *et al.* 2005) was detected in all extracts except A4T(2).

In addition, the *S. africana-lutea* extracts comprised of compounds important for the treatment of female diseases (e.g. mannose), mental and nervous conditions (e.g. pipecolic acid, [1] benzothiepino-[4,5-c]- pyridine) and insecticidal agents (e.g. pyridine, β-glucopyranose) (Table 5-4) justifying the various traditional uses of this herb in the Western

Cape (Watt and Breyer-Brandwijk 1962). These various uses have previously been reported for some exotic sages (Baricevic and Bartol 2000). Moreover, anti-cancer compounds such as butanoic acid (syn. butyric acid) and 1-methyl-3-D-1,2,4-triazole were detected. The anti-cancer properties of *S. africana-lutea* have been reported by Kamatou *et al.* (2008[c]) against colon and breast cancer. Kamatou *et al.* (2008[c]) reported that *S. africana-lutea* was amongst the *Salvia* extracts that had the best cytotoxic activity againt the colon cancer cell line. Correspondingly, the salts of butanoic acid (found in LBA 9402 and *in vitro* leaves) inhibit proliferation of human colon cancer cells (Lupton 2004) whereas the 1-methyl-3-D-1,2,4-triazole (only in A4T(3)) inhibits post-menopausal breast cancer (Dowsett 1997). The detection of anti-cancer compounds could explain the potential anti-cancer properties of *S. africana-lutea* and other South African sages reported by Kamatou *et al.* (2008[c]). Furthermore, Kamatou *et al.* (2008[c]) reported some anti-malarial activity of some South African sages. In contrast to the bioactive betulafolientriol oxide isolated from *S. radula* (Kamatou *et al.* 2008[c]), the identified anti-malarial compounds of *S. africana-lutea* were gluconic acid (found in A4T(2), A4T(3) and LBA 9402) and dehydroaporphine (found in LBA 9402 and *in vitro* leaves) (Chang *et al.* 1998; Kanokmedhakul *et al.* 2003; Neves and Vitolo 2007). Some sages have been reported to have anti-inflammatory properties and likewise the *S. africana-lutea* extracts contained compounds such as glucofuranoside (A4T(1) and LBA 9402) and malonic acid (only A4T(3)) which are anti-inflammatory (Yang *et al.* 2002). Lastly, arabinofuranose an anti-TB compound (Seepersaud and Al-Abed 2000; Pathak *et al.* 2004) was detected in the LBA 9402 and *in vitro* leaf extracts. Kamatou *et al.* (2007[a]) also reported the potential anti-TB properties of S*. africana-lutea.* To summarise, various pharmacological uses of different compounds detected in the *S. africana-lutea* extracts in this research are similar to those reported in both local and exotic sages (Section 2.1.2). In conclusion, the GC-MS analysis of the extracts illustrated that the tissue culture microenvironment and transformation altered the chemical profile of *S. africana-lutea*.

The GC-MS profile of the *S. africana-lutea* essential oil (Figure 5-4A) did not match the profile of the authentic standards (Figure 5-4B). During the write up of this thesis, the Stellenbosch Central Analytical Facility (CAF) did not have the library for essential oil volatiles.
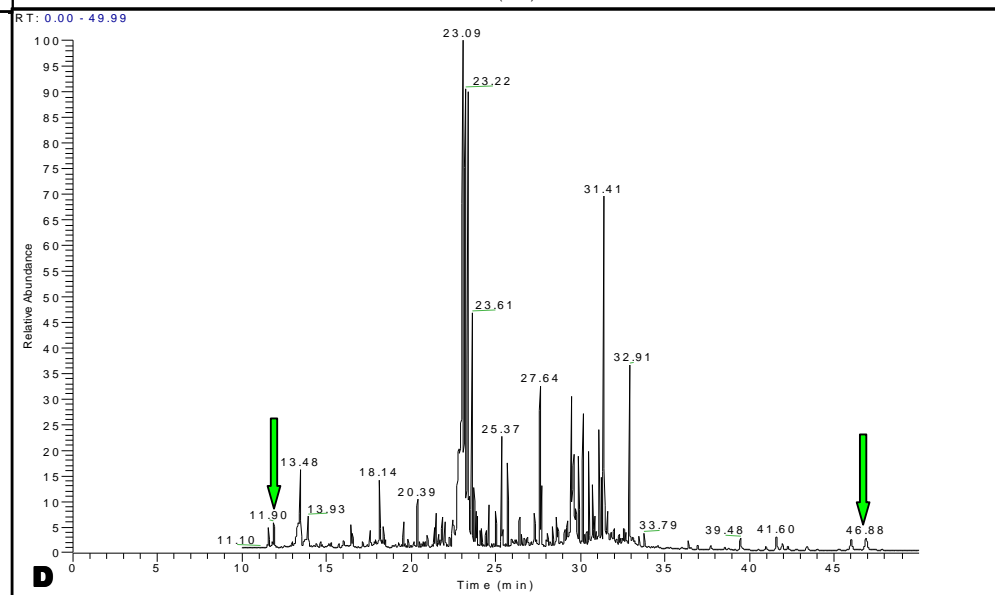
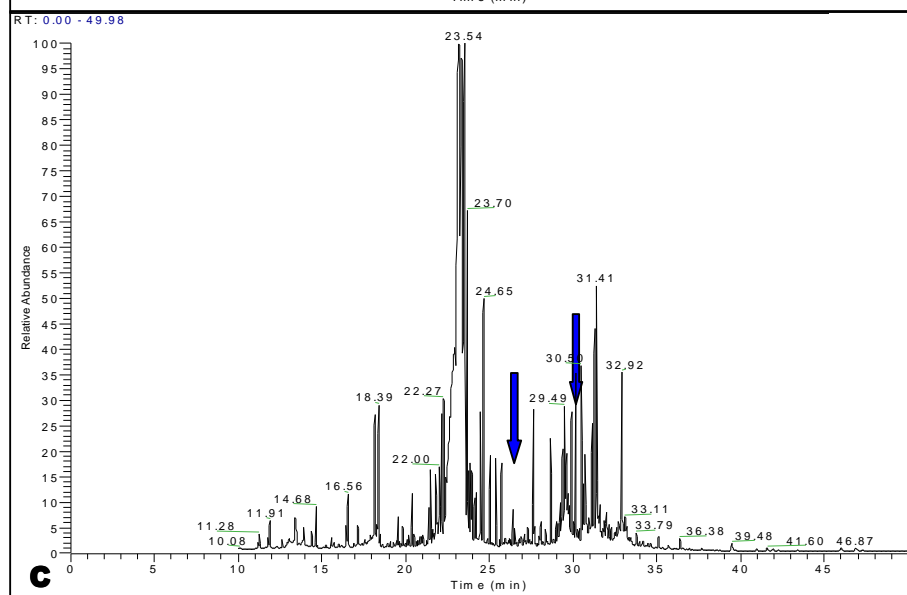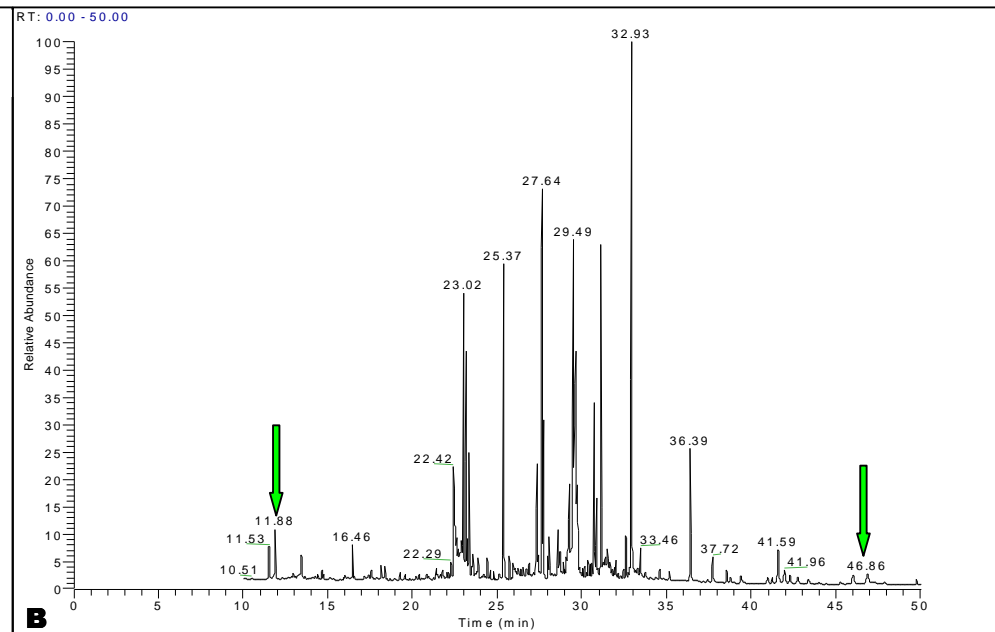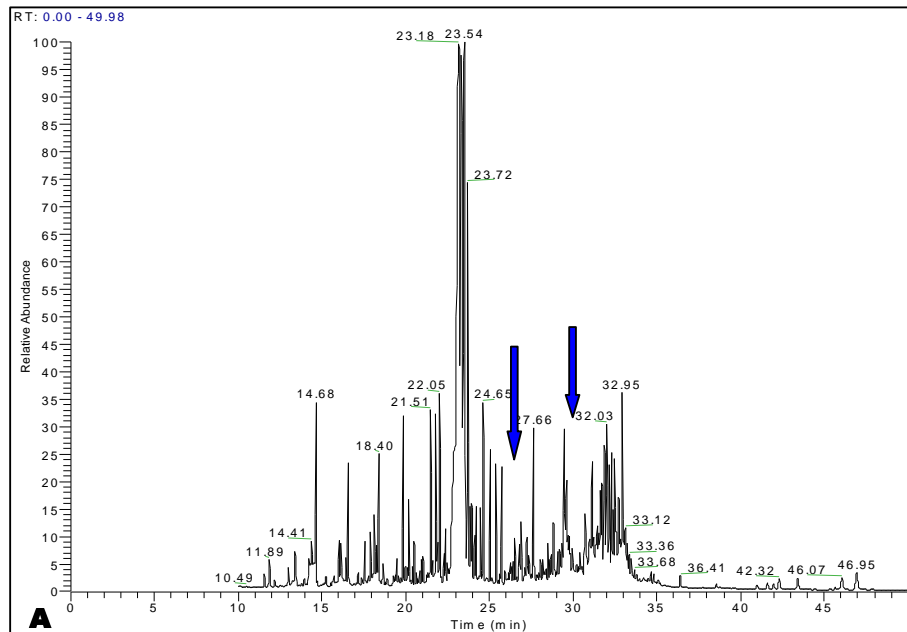**Figure 5-2 GC-MS chromatograms of A) A4T(1) hairy root clone B) A4T(2) hairy root clone C) A4T(3) hairy root clone D) LBA 9402 hairy root clone**

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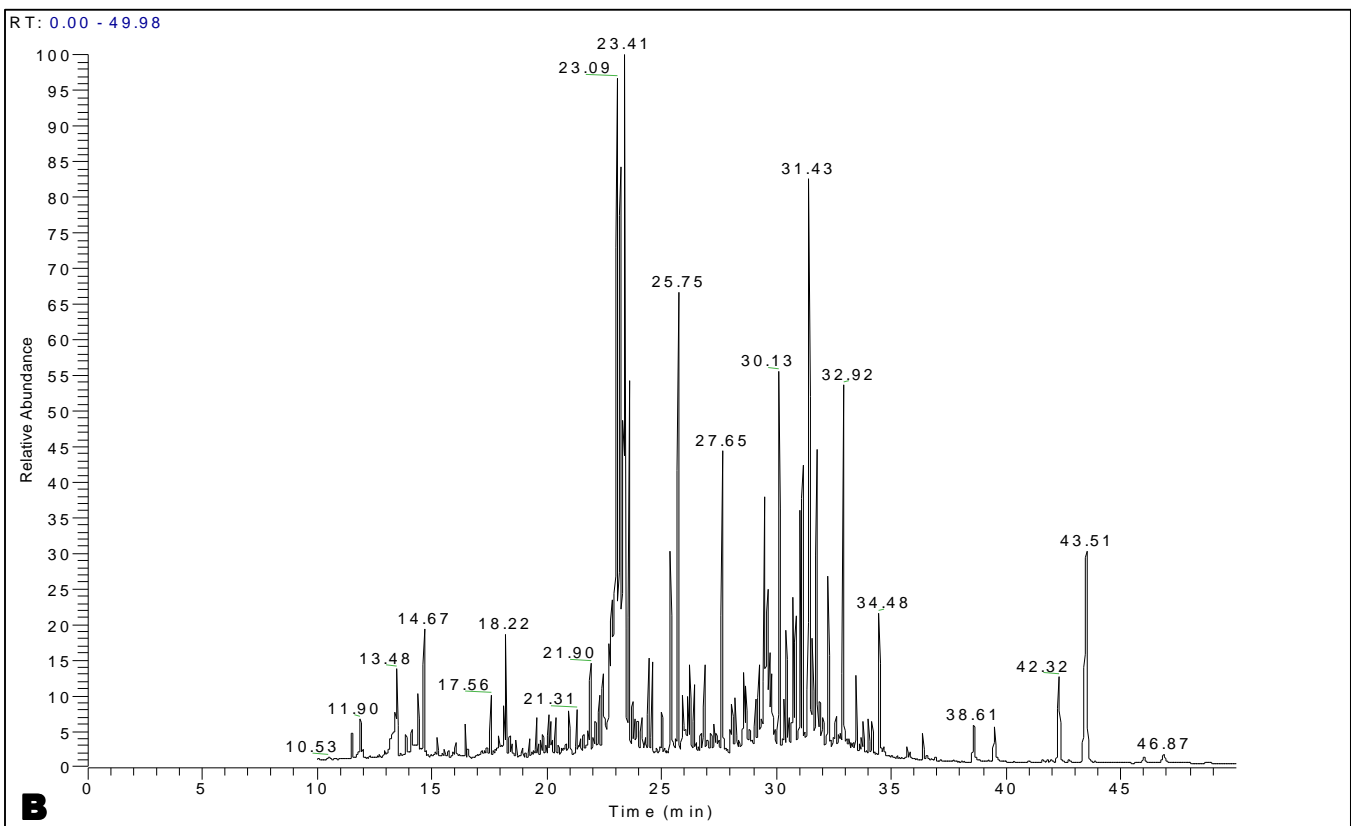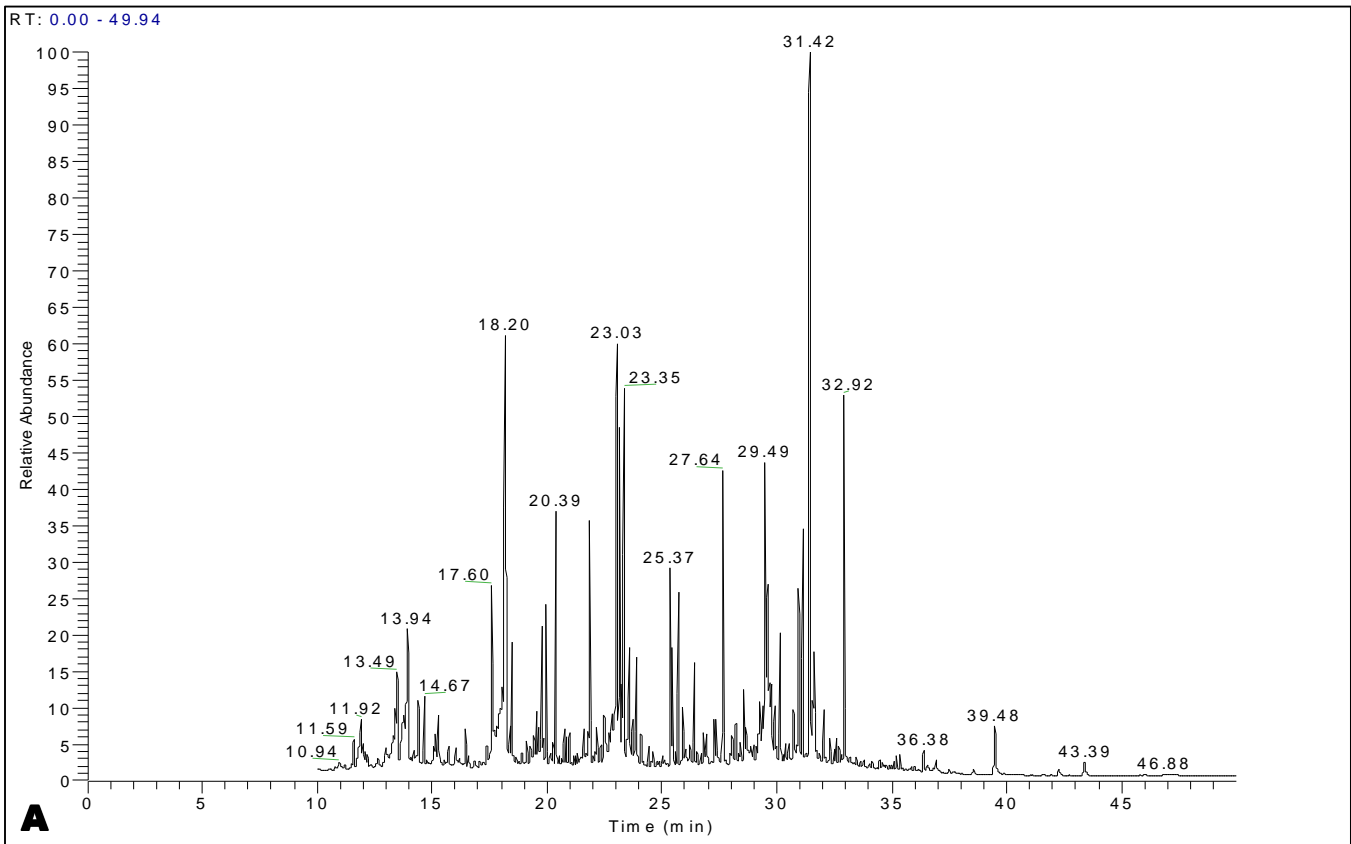
Figure 5-3 The GC-MS chromatogram of A) *in vitro* **leaf extract and B)** *ex vitro* **leaf extract**
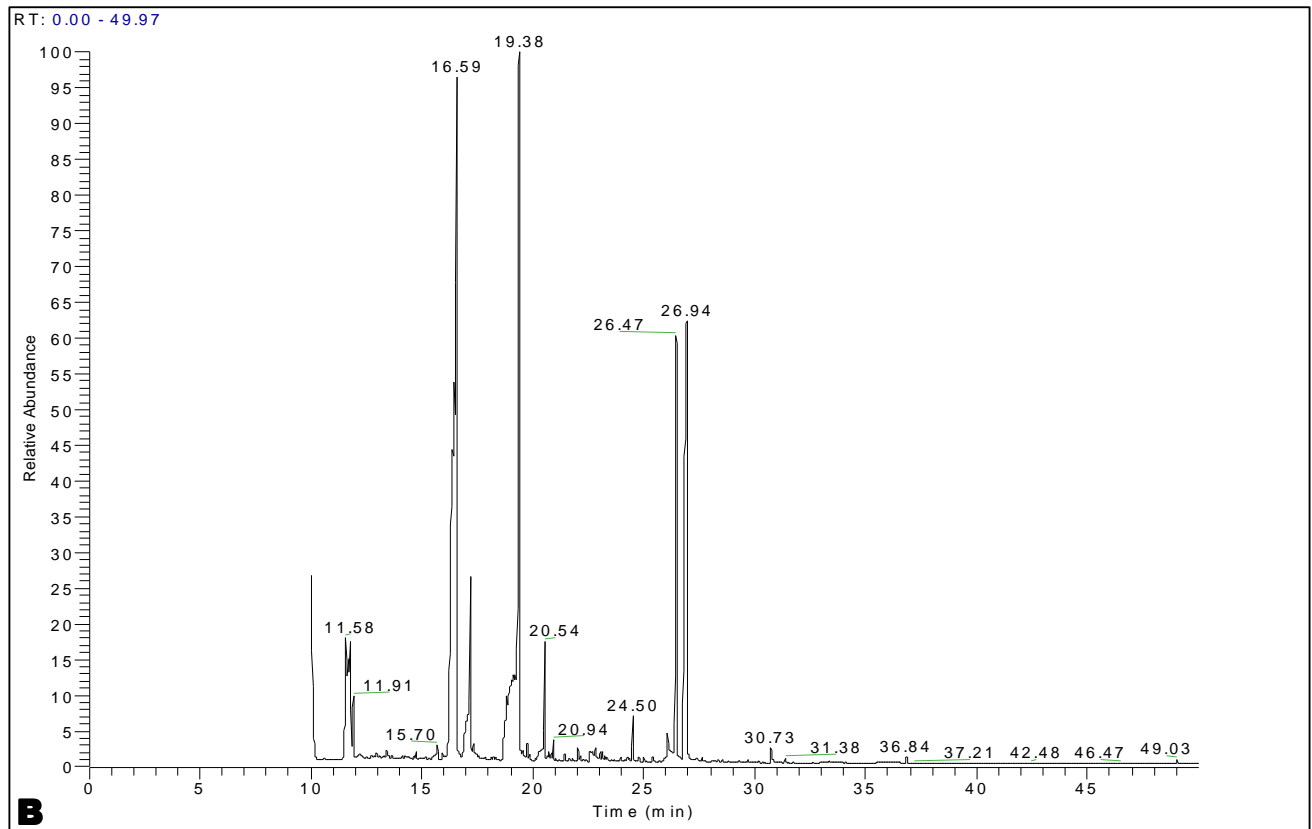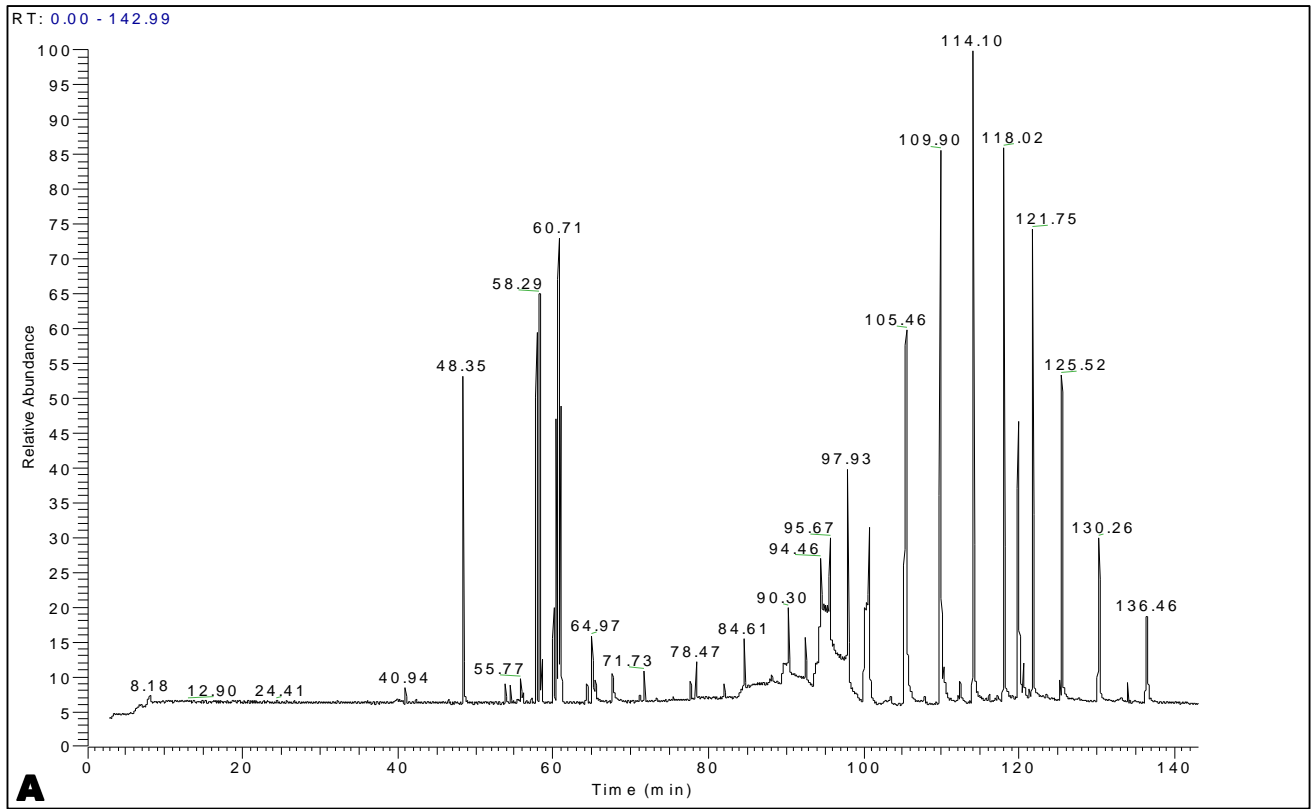
Figure 5-4 The GC-MS chromatogram of A) The hydrodistilled essential oil from *ex vitro S. africana-lutea* leaves and B) The authentic standards listed on Table 5-1.

Table 5-4 GC-MS library identification of some chemical compounds found in *S. africana-lutea* (XCalibur™)

Compound	Rt	A4T(1)	A4T(2)	A4T(3)	LBA	*In vitro*	*Ex vitro*	Uses	Reference(s)
Pyridine	5.7	+	-	-	-	-	-	An intermediate in synthesis insecticides, herbicides, pharmaceuticals, food flavourings, disinfectants. Pyridine is also used as ethyl alcohol for fungicides.	http://en.wikipedia.org/wiki/Pyridine http://www.freepatentsonline.com/5164507.html
3,4-dihydronaphthalene	25.7	+	-	-	-	-	-	Its derivatives have excellent pharmacological activities such as vasodilator and increasing cerebral blood flow	http://www.freepatentsonline.com/4148897.html
Glucofuranoside	30.6	+	-	-	+	-	-	Its derivatives are useful for treating animals and mammals with inflammatory and/or autoimmune disorders such as psoriasis, atopic dermatitis, rheumatoid arthritis, osteoarthritis, scleroderma and systemic lupus erythematosus. Derivatives of glucofuranose are used in the synthesis of pharmaceuticals, antibiotics	http://www.freepatentsonline.com/4996195.html http://www.nzp.co.nz/products.php?cid=1&pid=19
D-Fructose	31.2	+	+	+	+	+	+	A monosaccharide found naturally in fruits. It has low glycaemic index in comparison to sucrose and glucose therefore can be used as a sweetener for diabetics	Curry (1989)

Name								Uses	References
D-Glucitol (syn. Sorbitol)	32.8	+	-	-	+	-	-	These sugar alcohols are used as a sugar substitute (as "sugar-free") in baked products, jam and confectionery suitable for diabetics. Also used as a non-stimulant laxative in either oral suspension or suppository.	Fruijtier-Pölloth (2005)
								They are also used in the pharmaceutical industry as a protectant of active protein ingredient against processing stresses involved during production of solid protein formulations.	Hulse et al. (2008)
Mannitol	33.5	-	+	+	-	-	-	Sorbitol is also used as a thickener in some cosmetics	
								Mannitol is an osmotic diuretic	
2-azathianthrene	31.4	+	+	+	+	-	-	Phenolic with antioxidant activity	Jeong et al. (2004)
Sorbose	31.58	+	-	+	+	+	+	Is the intermediate in the commercial production of vitamin C	Hoshino et al. (2003)
									Sugisawa et al. (2005)

Compound	Value							Description	Reference
α-D-Glucopyranose	31.9	+	-	+	+	-	+	Is a building block for cyclic oligosaccharides (CD). CDs are used in agriculture, pharmaceutical, food industries etc. For instance, they are used in agricultural chemicals such as herbicides, insecticides, fungicides, repellents, growth regulators and pheromones.	
β-Glucopyranose	34.8	-	+	-	-	-	-	Pharmaceutically, they enhance drug delivery through biological membranes by making hydrophobic drugs in solution. Furthermore, improved water-solubility of drugs improves their bioactivity thus increasing the pharmacological effect allowing dosage reduction of the respective drug. In food industry, it is used as an additive to preserve flavour. In personal care products, it controls the release of fragrance in perfumes, household products	Del Valle (2004)
Fructose oxime	32.9	+	-	+	+	+	+		
Galactose oxime	33.2	+	+	-	+	+	+	Oximes are used as antidotes for nerve agents by treating organophosphate-pesticide poisoning through reactivating acetylcholinesterase	Eddleston et al. (2002) Kassa (2002)
Cholestan-2,3-dione-2,3-dioxime	44.4	-	+	+	+	-	-		
Glucose-O-methoxime	33.0	-	-	+	+	-	-		
[1] Benzothiepino [4,5-c] pyridine	33.5	-	+	+	+	+	+	These compounds are active ingredients of pharmaceutical compositions which have a depressant effect upon the central	Girgis et al. (2007)

nervous system.

Benzothiepinopyridine isomers are also important pharmacologically as non-steroidal anti-inflammatory drugs, analgesics, inhibit bronchospasms and treat neurodegenerative diseases such as Alzheimer's disease.

Compound	Value							Description	Reference
1H-Indole-2-carboxylic acid	33.9	+	-	-	+	-	-	Indole-2-carboxylic acid derivatives are used for treatment of skin diseases, anti-HIV agents, anti-oxidant and anti-obesity activity. Indole-2-carboxylic acid inhibit release of arachidonic acid in human platelets and the esters of Indole-2-carboxylic acid is COX-2 selective enzyme inhibitor	Goyal and Sangal (2005)
20-methyl-5-α-pregnan-16-one	32.9	-	-	+	-	-	-	Is a phytosteroid. Most of plant steroids are used as food additives and cholesterol-lowering properties.	Ostlund et al. (2003)
5,6,7,8-tetrahydroquinoxaline	5.6	-	+	-	+	-	-	Tetrahydroquinoxalines are important for preventing atherosclerosis by inhibiting the accumulation of bad cholesterol in order to elevate the good cholesterol levels. Some tetrahydroquinoxalines have been reported to have better antioxidant activity than tetrahydroquinolines	Nishiyama et al. (2002)
L-Proline	24.5	-	+	-	-	-	-	L-Proline is used as an osmoprotectant as result used in pharmaceutical and biotechlogical applications. Proline is essential for collagen production thus assist in	Heffernan et al. (2006)

wound healing

Compound	Value							Description	Reference
α-D-Galactopyranose	31.53	-	+	-	-	-	-	Galactopyranose (Gal*p*) and galactofuranose (Gal*f*) form the outer coats, capsules, cell walls etc of many pathogenic micro-organisms essential for the virulence of pathogens. Gal*p* and Gal*f* are antibody generating in the mammalian cells therefore they have been targeted for the production of therapeutic agents especially for the antibiotic resistant pathogens.	Pedersen and Turco (2003) Carlson *et al*. (2006)
β-D-Galactofuranose	26.9	-	-	-	+	+	-		
Mannose	33.0	-	+	-	+	+	-	A sugar monomer which prevents bacterial infections in females' urinary tract and bladder	http://en.wikipedia.org/wiki/Mannose#Cranberry_juice
D-gluconic acid	35.2	-	+	+	+	-	-	Occurs naturally in plants, honey and the gluconic acid salts known as gluconates are widely used in pharmaceutical industry. For instance, gluconate ester such as quiniline gluconate is used for the treatment of malaria	das Neves and Vitolo (2007)
Methyl 8-acetyl-labdanolate	36.8	-	+	-	-	-	-	Methyl 8-acetyl-labdanolate is the precursor for biosynthesis of biologically-active bicyclic diterpenes called labdane. The bioactivities anti-inflammatory, anti-bacterial, anti-protozoal, anti-fungal and anti-cancer.	Urones *et al*. (1995) Girón *et al*. (2008) Matsingou *et al*. (2006)
Germanicol	41.5	-	+	+	-	+	-	A triterpene found in some sage species and latex-producing plants. It is has antimicrobial properties	Anaya *et al*. (1989) Veličković *et al*. (2002)

Compound	Value	1	2	3	4	5	6	Description	Reference
Malonic acid	16.2	-	-	+	-	-	-	An important intermediate in synthesis of vitamins B1 and B6, non-steroidal anti-inflammatory, anti-cancer agents and other pharmaceuticals. Also used for production of some agrochemicals, flavourings and fragrances.	Yang *et al*. (2002)
1-methyl-3-D-1,2,4-Triazole	23.9	-	-	+	-	-	-	Triazole family compounds (non-steroidal) inhibit are aromatase inhibitors which play an important role in the treatment of advanced breast cancer in post-menopausal women. **Note:** Aromatase activity lead to proliferation of cancerous cells in the breast tissue.	Dowsett (1997)
Pipecolic acid	24.6	-	-	+	-	+	-	A precursor for non-ribosomal peptides and polyketide synthases which subsequently synthesise pharmacologically important secondary metabolites used as immunosuppressors, anti-convulsant for neurological disorders such as epilepsy	Ho *et al*. (2001) Naranjo *et al*. (2001)
Phosphoric acid	18.2	-	-	+	+	-	-	Used for the production of biphosphonates which are important for human skeletal complications such as osteoporosis. Phosphoric acid is also used highly used in dentistry and orthodontics as a teeth cleaning solution.	Papapoulos (1999)
Glucosamine	32.6	-	-	+	-	-	-	Glucosamine is a natural amino-monosaccharide. Its derivatives are used for treatment of osteoarthritis	McAlindon *et al*. (2000) Reginster *et al*. (2001)

Name								Description	Reference
Galactosamine	33.6	-	-	+	-	-	-	Galactosamine is an amino sugar which is known to be toxic by causing liver-damage in animals	Sinha *et al*. (2007)
Gulose	33.9	-	-	+	+	-	-	The rare hexose sugar is one of the substrates for the biosynthesis of ascorbic acid (Vitamin C)	Sugisawa *et al*. (2005)
Galactonic acid	35.2	-	-	+	+	-	-	Some yeasts use galactonic acid for biosynthesis of ascorbic acid.	Onofri *et al*. (1997)
Thiocyanic acid	12.7	-	-	-	-	+	-	Its salts are used as intermediates for creating various compounds used as fungicides, antibiotics, pharmaceuticals. For instance, some isothiocyanates are chemopreventative agents which act against the development and proliferation of some cancers.	http://chemicalland21.com/industrialchem/organic/CALCIUM%20THIOCYANATE.htm
L-Alanine	13.2	-	-	-	+	+	-	L-alanine is a non-essential amino-acid important for the build up of the immune system by producing immunoglobulins and antibodies. L-alanine metabolises sugars and organic acids for production of body energy	http://www.anyvitamins.com/alanine-info.htm
L-Valine	16.5	-	-	-	-	+	-	A precursor in the penicillin biosynthetic pathway. Also an essential amino acid which promotes muscle growth and tissue repair. It is also used as pharmaceutical intermediates or nutritional supplements	http://www.anyvitamins.com/healthlist/valine.htm Gilbert and Migeon (1975)
L-Isoleucine	18.7	-	-	-	-	+	-	An essential amino acid which is needed for haemoglobin formation. It also promotes muscle recovery after exercise and stabilises energy levels by helping to	http://www.vitaminstuff.com/amino-acid-isoleucine.html

								Uses	Reference
								regulate blood sugar	
Maleic acid	21.1	-	-	+	+	+	-	They are used in effervescent saline pharmaceutical. Also used in preparations used for removing necrotic tissue on the surface of wounds and ulcers. They used as food additives	Lian *et al*. (1999)
Butanedioc acid (syn. succinic acid)	19.1	-	-	-	-	+	-		
2-Butenedioc acid/ (syn. fumaric acid)	20.0	-	-	+	+	+	-		
Butanoic acid (syn. Butyric acid)	24.7	-	-	-	+	+	-	Is a short chain fatty acid. Butanoic acid salts and esters (butanoates or butyrates) are antineoplastics i.e they inhibit cancerous tumour development (for example; colon and prostate cancer) and are not toxic to humans.	Lupton (2004); Kuefer *et al*. (2007)
7-methoxy-Quinoline	26.2	-	-	-	-	+	-	Derivatives of quinoline are excellent antibacterial activity and are useful antibacterial agents.	http://www.freepatentsonline.com/4886810.html
Arabinofuranose	31.3	-	-	-	+	+	-	Arabinofuranose forms part of the *M. tuberculosis* cell wall which is important for the virulence of this pathogen. Analogues of arabinofuranose are used as inhibitors of *M. tuberculosis.* Inhibition of biosynthetic enzymes of arabinofuranose and other cell wall polysaccharides of *M. tuberculosis* is an attractive approach in the treatment of TB.	Seepersaud and Al-Abed (2000) Pathak *et al*. (2004)
Dehydroaporphine	31.4	-	-	-	+	+	-	An alkaloid with anti-malarial and antiplatelet properties.	Chang *et al*. (1998) Kanokmedhakul *et al*. (2003)

Compound	Value							Description	Reference
Caffeic acid	37.2	-	-	-	+	+	-	A building block of sage polyphenolic compounds which have various biological activities such as antioxidant, antiviral, antitumour, treatment of cerebral and coronary diseases etc	Lu and Foo (2002) Jiang *et al*. (2005)
2,4-Imidazolidinedione	49.2	-	-	-	-	-	+	Used as analgesics	http://www.freepatentsonline.com/6022875.html

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CHAPTER 6
GENERAL CONCLUSIONS AND RECOMMENDATIONS

Although phytochemical and antimicrobial studies have been done on *S. africana-lutea*, these studies were conducted using plant material harvested from the wild. The usage of this aromatic herb could potentially threaten its natural biodiversity in future therefore, ways of conserving the wild stock plants are essential. Hence, the main aim of this study was to apply biotechnological tools on *S. africana-lutea* as a way of conserving this plant. A micropropagation system and transgenic root culture were initiated. The cultures were useful for phytochemical and pharmacological studies without affecting the wild growing *S. africana-lutea* plants.

In terms of mass propagation, numerous propagules should be produced in a short time through a simple regime with easy steps. In this case, both the rooting and acclimation steps were combined in a single step which reduced the acclimation time to four weeks instead of eight weeks. As a result, this current improved *S. africana-lutea* micropropagation is commercially viable with a regeneration rate of 36 plantlets per bottle (each bottle had three plantlets and each plantlet produced four internodes) which totals to 720 plantlets per cycle of four weeks. Furthermore, a high survival rate of 92% was recorded in the greenhouse environment. Also, this new improved micropropagation protocol is relatively simple, less laborious and not time-consuming. All of these factors are crucial for mass propagation at a commercial scale. However, the *in vitro* environment occasionally made the *S. africana-lutea* plantlets hyperhydric. Vented culture bottles reduced excessive moisture in the bottle thus eliminating hyperhydricity. These acclimatised *in vitro* propagated *S. africana-lutea* plantlets can be used as a source of seedlings for field transplantations. *In vitro* plant material proved beneficial as an alternative source for phytochemical and pharmacological studies. Hence, application of this reliable plant tissue culture system could be used for *in vitro* conservation of other highly used *Salvia* species locally.

In terms of efficient conservation purposes, it is crucial that the antimicrobial properties of the *in vitro* cultures are relatively similar or better to those of a natural origin. Given that biotechnological tools have been applied on sages worldwide highlighting their phytochemistry and pharmacology, this research was a pilot study on application of high-tech tools on the South African sages. The *S. africana-lutea* was used as a model plant for highlighting the phytochemistry and antimicrobial properties of this herb. The *in vitro* plants along with transgenic root culture were used to compare their phytochemical and pharmacological properties with those of the wild growing *S. africana-lutea*.

This research was fruitful, as the micropropagation and transgenic root culture have proven to be efficient in the conservation of the *S. africana-lutea*. The microenvironment has altered the chemical profile of *S. africana-lutea* foliage. The *in vitro* leaves had better antibacterial activity than the wild leaves. Furthermore, the chemical profile and pharmacological activites of transgenic roots and non-transgenic plants are clearly different with the root clones being more complex and biologically-active against the fungal phytopathogens. The antifungal activity of *S. africana-lutea* against phytofungal pathogens can be taken to the next level by conducting a bioassay-guided fractionation in order to isolate and identify the antifungal compound(s). This would involve bulking up the root tissue and fractionating the samples. Pure active fractions could then be identified by NMR assay.

Further studies on the specific factor(s) responsible for the altered phytochemical profile are recommended. It is recommended that the abiotic (such as temperature, light, relative humidity) and biotic factors of the microenvironment be studied closely to determine their influence in phytochemical changes. Understanding the influence of these factor(s) on changing the phytochemical profile of *S. africana-lutea* will be of great benefit in the optimisation of *in vitro* production of secondary metabolites from this plant and other sages.

Molecular analyses of the transgenic root clones confirmed the presence of *rol* genes. The presence of *rol* and *ags* genes confirmed transgenesis of the *S. africana-lutea* hairy

root clones via PCR analysis. The Southern blotting proved stable integration of the transgene in the genome. Nonetheless, further studies are necessary to determine the expression of *rol* genes and their influence on the phytochemical profile and pharmacological properties. Such studies would enlighten the understanding of the genetic control on the metabolome(s) produced and elucidating the pathways that have been up-regulated. It was clear that the hairy root cultures of *S. africana-lutea* produced a new set of bioatives, which are not synthesised naturally in the wild growing plants. These subsequently led to variable antimicrobial activity of the transgenic root extracts. These novel compounds are likely to be responsible for the activity against *Fusarium* species tested. The novel compounds that accumulate in these cultures are the targets for the pharmaceutical companies in exploring the production of new natural and more potent drugs. Therefore, the *in vitro* hairy root liquid culture has a great potential for up-scaling in the industrial production of bioactive metabolites.

In conclusion, this study has contributed to ethnopharmacology and phytochemistry by showing that plantlet and root cultures can be used as green factories for the production of bioactive compounds from medicinal plants without affecting the wild growing plants. The use of plant cultures is even more advantageous since the *in vitro* production of the important secondary metabolites is timeless without the seasonality and geographical limitations. The biological activity of the *S. africana-lutea* extracts against both the human and plant pathogens indicates that this aromatic herb is not only potentially important to human health but also to agricultural crops. Good antifungal activity against the *Fusarium* species provides new leads for natural fungicides. These fungicides are important as consumers and farmers of the agricultural crops are shifting to the use of natural and eco-friendly chemicals, safe for consumption.

APPENDIX

A1. PLANT TISSUE CULTURE MEDIA

(i) **Gamborg's B5 medium** (Gamborg 1968)

COMPONENTS	Amount (mg L^{-1})
Macronutrient salts	
$MgSO_4$	122.09
KNO_3	2500
NH_4SO_4	134
$CaCl_2$	113.24
Micronutrient salts	
H_3BO_3	3.0
$MnSO_4.H_2O$	10.0
$ZnSO_4.7H_2O$	2.0
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
KI	0.75
$FeSO_4.7H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3
Organic supplements	
Thiamine-HCl	10.0
Pyridoxine-HCl	1.0
Nicotinic acid	1.0
Myo-inositol	100
Sucrose	30

(ii) **Miller's medium** (adapted from Miller, 1965)

Chemical components	mg L^{-1}
Macronutrient salts	
KH_2PO_4	300
KNO_3	1000
NH_4NO_3	1000
$Ca(NO_3)_2.4H_2O$	500
Micronutrient salts	
$MgSO_4.7H_2O$	71.5
KCl	65
$MnSO_4.3H_2O$	14
KI	0.8
$Cu(NO_3)_2.3H_2O$	0.35
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.1
Organic supplements	
Myo-inositol	100
Nicotinic acid	2
Pyridoxine HCl	0.8
Thiamine HCl	0.8

(iii) **MS medium** (Murashige and Skoog 1962)

COMPONENTS	Amount (mg L^{-1})
Macronutrient salts	
$MgSO_4.7H_2O$	370
KH_2PO_4	170
KNO_3	1900
NH_4NO_3	1650
$CaCl_2.2H_2O$	440
Micronutrient salts	
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
KI	0.83
$FeSO_4.7H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3
Organic supplements	
Thiamine-HCl	0.5
Pyridoxine-HCl	0.5
Nicotinic acid	0.5
Myo-inositol	100
Glycine	2
Sucrose	30

(iv) **½MS** (half concentration of all MS chemical constituents)

(v) **MS-NH$_4$** (all MS chemical constituents **except** the NH_4NO_3)