Towards understanding the metabolism of *in vitro Sutherlandia frutescens* (L.) R. Br. cultures

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Declaration

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Summary

Sutherlandia frutescens (L.) R. Br., also regarded as *Lessertia frutescens,* is a leguminous, perennial shrub indigenous to South Africa. Extracts prepared from the leaves have traditionally been used for the treatment of various diseases. Reports have also indicated that *S. frutescens* provides certain health benefits to cancer and HIV/AIDS patients. Analysis of extracts indicated the presence of several compounds (bitter triterpenoid glycosides, several flavonoids, amino acids, small amounts of saponins (no alkaloids though), asparagine, L-arginine, canavanine, gamma-aminobutyric acid (GABA) and pinitol) which contribute to the medicinal properties of this plant.

The first part of this study involved testing the effect of six treatments (light, dark, soaking of seeds, physical scarification, chemical scarification and flaming of seeds) on the *in vitro* germination of *Sutherlandia* seeds to elucidate the factors which control seed germination. Those treatments which removed the seed coat were most successful for germination with physical scarification being the most efficient method, resulting in 98.6% of the seeds germinating after 21 days. Although the organogenesis of *Sutherlandia* explants (cotyledons and hypocotyls) *in vitro* were investigated (results not included in this thesis), omitting plant growth regulators (PGR) in the cultivation medium was best for shoot multiplication. However, this PGR-free system successfully provided a continuous supply of plant material for further studies. It would be possible to successfully adopt it for commercial production of plants to assist with cultivation of *Sutherlandia* as a field crop. Another advantage of this system is spontaneous rooting with 85% of the *in vitro* microshoots rooting in PGR-free medium. These rooted plants were acclimated in the glasshouse using vented lids to harden off the shoots and this method resulted in 100% survival of plants.

The second part of this study investigated the induction of hairy root cultures of *S. frutescens* using *Agrobacterium*-mediated transformation. The efficiency of three *Agrobacterium* strains (A4T, LBA9402 and C58C1) to transform different *S. frutescens* explants (cotyledons and hypocotyls) was analyzed. All three strains were equally efficient at inducing hairy roots in both hypocotyls and cotyledons. However, transformation of *S. frutescens* was dependent on the type of explant used with the hypocotyls being more efficiently transformed than the cotyledons. Overall the transformation of both the hypocotyl (93%) and cotyledon (47%) was highest when the strain A4T was used. Four hairy root clones were selected and their cultivation in a liquid system was optimized by investigating their growth in four different types of media (Gamborg B5 (Gamborg *et al*., 1968), White's (White,

1934; White, 1954), MS (Murashige and Skoog, 1962) and half strength MS medium). All the growth of hairy root clones was best in the B5 and MS medium, with White's medium being the least effective cultivation medium. Molecular analysis of hairy roots was used to prove the transgenic status of these four putative transgenic clones. This was achieved using polymerase chain reaction (PCR) amplification of *rol* A (320 bp), B (780 bp) and C (600 bp) genes to determine the presence of the T_L-DNA in the plant genome. During Southern hybridization a radioactively labeled *rol* A probe was used to determine the copy number of the *rol* A gene. The three *rol* genes were present in all four hairy root clones.

The third part of this study focused on the effect of three abiotic stress factors (nitrogen availability, salinity and drought) on the synthesis of four metabolites (gamma-aminobutyric acid (GABA), asparagine, arginine and canavanine). The effect of nitrogen availability on metabolite synthesis and the morphology was determined using *in vitro* shoot cultures as well as the hairy root clone C58C1-g. Nitrogen availability studies were conducted by cultivating the microshoots or root tips on modified MS medium. The MS medium contained either the normal amount of nitrogen (1.9 g L^{-1} KNO_3 and 1.65 g L^{-1} NH_4NO_3) in the MS medium (1x nitrogen), half the normal nitrogen concentration in MS medium (0.5x nitrogen) or twice the normal nitrogen concentration in MS medium (2x nitrogen). The arginine and asparagine levels in the roots and shoots and the canavanine level in the shoots were directly correlated with the amount of nitrogen in the medium (as the nitrogen level increased, the metabolite levels increased). The GABA level in the shoots was inversely correlated with the amount of nitrogen in the medium. Several reasons may explain these metabolic changes including the assimilation of extra nitrogen into asparagine, canavanine and arginine in the shoots. The reduced GABA levels may indicate the preferential flux of the free GABA into other nitrogen assimilatory pathways such as protein synthesis as well as its rapid utilization to replenish the tricarboxilic acid cycle intermediates.

The effect of water (induced by including 3% (w/v) PEG in the medium) and salt stress (induced by including either 50 or 100 mM NaCl in the medium) was only investigated in the shoot cultures as the root cultures lacked the synthesis of canavanine. Water stress did not significantly alter the metabolite levels, but resulted in a significant decrease in the growth (fresh weight and total shoot length) and the rooting response of these microshoots. Salt stress only resulted in a significant increase in arginine levels with increasing salinity and also caused a reduction in the rooting and growth response. Lowered plant vigour may be the first visual sign of water stress. Addition of NaCl may lead to ion toxicity and requires osmotic

adjustment resulting in changes at the metabolic level concomitant to physiological growth changes.

Finally, the anti-bacterial activity and the phytochemistry of transgenic root cultures and untransformed *in vitro* and *ex vitro* plant material was examined. Only the extracts prepared from the wild harvested leaf material exhibited moderate anti-bacterial activity (1.25 mg ml^{-1}) against all the bacteria (*Escherichia coli, Klebsiella pneumoniae, Bacillus subtilis and Staphylococcus aureus)* tested. Changes to the secondary metabolism of hairy roots were investigated using TLC and LC-MS analysis. Several of the compounds in the hairy root extracts were present in higher levels than in the control root extracts. Transformation also increased the complexity of the phytochemical pattern of the hairy roots, either due the synthesis of novel compounds or upregulated synthesis of existing metabolic pathways. The production of hairy roots and the establishment in a liquid system during this study was an important step towards upscaling these cultures to a bioreactor. In future these roots can assist in developing cultures which produce a high yield of the desired metabolites.

Opsomming

Sutherlandia frutescens (L.) R. Br., ook bekend as *Lessertia frutescens* is ‘n peulagtige meerjarige struik, inheems tot Suid Afrika. Ekstrakte wat van die blare voorberei word, is tradisioneel gebruik vir die behandeling van verskeie siektes. Berigte het ook daarop gedui, dat *S. frutescens* sekere gesondheidsvoordele vir kanker en HIV/VIGS pasiënte inhou. ‘n Ontleding van die ekstrakte, dui op die teenwoordigheid van verskeie verbindings (bitter triterpenoïed glikosiede, verskeie flavonoïede, aminosure, klein hoeveelhede saponiene (alhoewel geen alkaloïede), asparagien, L-arginien, canavanien, gamma-aminobottersuur (GABS) en pinitol) wat tot die medisinale eienskappe van hierdie plant bydrae.

Die eerste deel van die studie het die effek van ses behandelings (lig, donker, week van sade, fisiese skarifikasie, chemiese skarifikasie en die vlam van sade) op die *in vitro* ontkieming van *Sutherlandia* sade getoets met die doel om die faktore wat saadontkieming beheer, te identifiseer. Die beste behandeling vir saadontkieming was dié behandelings wat die saadhuid verwyder het. Die mees effektiewe metode van saadhuidverwydering was die fisiese skarifikasie van sade, wat gelei het tot ‘n 98.6% ontkieming van sade na 21 dae. Alhoewel *in vitro* organogenese van *Sutherlandia* eksplante (kotiel en hipokotiel) ondersoek was (resultate nie ingesluit in die tesis nie), was plant groei reguleerders (PGR) uitgesluit in die groeimedium om stingelvermeerdering te bevorder. Nie te min was die PGR-vrye sisteem suksesvol om ‘n voortdurende bron van plant material vir verder studies te verskaf. Dit sou egter moontlik wees om die PGR-vrye sisteem suksesvol te kon aanpas vir die kommersiële produksie van plante met die doel om *Sutherlandia* as ‘n landbougewas te bevorder. ‘n Verdere voordeel van dié sisteem, is die spontane wortelvorming, met 85% van die *in vitro* mikrostingels wat wortels in die PGR-vrye medium produseer het. Hierdie bewortelde plante was in die glashuis geakklimatiseer met behulp van geventileerde deksels (vir stingel afharding) en het tot ‘n 100% oorlewing gelei.

Die tweede deel van die studie het die induksie van *S. frutescens* harige wortelkulture met behulp van *Agrobacterium*-bemiddelde transformasie ondersoek. Die effektiwiteit van drie *Agrobacterium* stamme (A4T, C58C1 en LBA9402) om verskillende *S. frutescens* eksplante (kotiel en hipokotiele) te transformeer, was geanaliseer. Al drie stamme was ewe effektief om harige wortels op beide hipokotiel en kotiele te induseer. *S. frutescens* transformasie blyk egter tog van die tipe eksplant afhanklik te wees, aangesien die hipokotiele meer effektief as die kotiele getransformeer kon word. Met inagneming van beide die hipokotiel (93%) en kotiel

(47%), was transformasie optimaal met die gebruik van die A4T stam. Vier harige wortelklone was geselekteer en hulle produksie in 'n vloeibare sisteem was geoptimiseer deur hulle groei in vier verskillende tipe media (Gamborg B5 (Gamborg *et al*., 1968), White's (White, 1934; White, 1954), MS (Murashige and Skoog, 1962) en half-sterkte MS medium) te ondersoek. B5 en MS medium was beskou as die beste vir alle die harige wortelklone se groei, terwyl White's medium die minste doeltreffende groeimedium was. Molekulêre analise van die harige wortels was gebruik ten einde die transgeniese status van die vier vermoedelike transgeniese klone te bewys. Dit was behaal deur polimerase kettingreaksie amplifisering (PKR) van die *rol* A, B en C gene ten einde die teenwoordigheid van die T_L-DNS in die plant genoom aan te toon. Tydens Southern hibridisasie was 'n radioaktief gemerkte peiler gebruik om die aantal *rol* A geen kopieë te bepaal. Die drie *rol* gene was teenwoordig in al vier harige wortelklone.

Die derde deel van die studie het gefokus op die effek van drie abiotiese stress faktore (stikstof beskikbaarheid, sout- en droogte stres) op die produksie van vier metaboliete (GABS, asparagien, canavanien en arginien). Die effek van stikstof beskikbaarheid op die metaboliet produksie asook die morfologie was bestudeer deur gebruik te maak van *in vitro* mikrostingels asook die harige wortel kloon C58C1-g. Stikstof beskikbaarheidstudies was uitgevoer deur die mikrostingels of wortelpunte in 'n gewysigde MS medium te groei. Die MS medium was aangepas om die normale hoeveelheid stikstof (1.9 g L^{-1} KNO_3 en 1.65 g L^{-1} NH_4NO_3) in MS medium (1x stikstof), of die helfte van die normale stikstof konsentrasie (0.5x stikstof) of twee keer die normale stikstof konsentrasie in MS medium (2x stikstof) te bevat. Die arginien en asparagien vlakke in die wortels en stingels, asook die canavanien vlak in die stingels was positief gekorreleerd aan die stikstof konsentrasie in die medium. Die GABS vlak in die stingels was egter omgekeerd eweredig aan die stikstof konsentrasie in die medium. Verskeie redes kan aangevoer word om die metaboliet veranderinge te verduidelik, insluitende die assimilasie van addisionele stikstof in asparagien, canavanien en arginien in die stingels. Die verlaagde GABS vlakke kan dui op die voorkeur van vrye GABS vloei na ander stikstof-assimilerende metaboliese paaie soos proteïen sintese, asook die snelle benutting van GABS ten einde die Trikarboksielsuursiklus intermediêre produkte aan te vul.

Die effek van droogte (geïnduseer deur die byvoeging van 3% (m/v) PEG tot die medium) en sout stres (geïnduseer deur 50 of 100 mM NaCl byvoeging tot die medium) was slegs in die stingel kulture ondersoek weens die afwesigheid van canavanien produksie in die wortel kulture. Water stres het nie 'n betekenisvolle verandering in die metaboliet vlakke meegebring nie, maar dit het wel tot 'n beduidende afname in groei (vars massa en totale stingel lengte) en bewortelingsreaksie in die mikrostingels gelei. Sout stres het slegs tot 'n betekenisvolle

toename in arginien vlakke asook ‘n afname in die wortelvorming en groeireaksie tydens die toenemende sout vlakke gelei. ‘n Verlaging in plant groeikragtigheid mag ‘n eerste visuele teken van water stres wees. Die toevoeging van NaCl tot die medium kan tot ioontoksisiteit lei en plante reageer deur middel van osmotiese aanpassing wat tot veranderinge in die metaboliet vlakke asook veranderinge in fisiologiese groei, lei.

Die finale deel van die studie het die anti-bakteriële aktiwiteit en die fitochemie van die transgeniese wortel kulture asook die ongetransformeerde *in vitro* en *ex vitro* plant materiaal ondersoek. Slegs die ekstrakte verkry vanaf blaar materiaal geoes uit die natuur, het matige anti-bakteriële aktiwiteit (1.25 mg ml^{-1}) teen al die bakterië (*Escherichia coli, Klebsiella pneumoniae, Bacillus subtilis en Staphylococcus aureus)* wat ondersoek is, getoon. Aanpassings in die sekondêre metabolisme van die harige wortels is deur middel van dunlaag chromatografie (DLC) en vloeibare chromatografie-massa spektroskopiese (VC-MS) analise ondersoek. Verskeie verbindings was in hoër vlakke in die harige wortels teenwoordig, as in die kontrole wortel ekstrakte. Transformasie het ook die kompleksiteit van die harige wortels se fitochemiese patroon verhoog, moontlik weens die produksie van nuwe verbindings of weens die opregulasie van bestaande metaboliese paaie. Die produksie van harige wortels en die vestiging daarvan in ‘n vloeibare sisteem tydens hierdie studie word beskou as ‘n belangrike stap na die opskalering van die kulture na bioreaktore. Hierdie wortels kan toekomstig tot die ontwikkeling van kulture met ‘n hoë produksie van gewenste metaboliete lei.

Papers from this thesis

Colling J, Stander MA, Valentine AJ, Kossmann J, Makunga NP. Influence of abiotic stresses on canavanine and other medicinal amino acids on *in vitro* regenerated *Sutherlandia frutescens* shoots using liquid chromatography mass spectroscopy. (Manuscript in preparation for submission to Plant Cell Environment).

Colling J, Kossmann J, Makunga NP. *Agrobacterium*-mediated induction of hairy roots in the medicinal legume, *Sutherlandia frutescens*. (Manuscript in preparation for submission to Plant Cell Reports)

Conference contributions from this thesis

Makunga NP, **Colling J**, Horsthemke HR, Ramogola WPN and Van Staden J (2007). Adopting biotechnological strategies – plants from South Africa as targets. PSE Congress: Plants for Human Health in the Post-Genome Era (Helsinki, Finland)

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

Colling J, Kossmann J, Makunga NP (2008). Canavanine accumulation in organ cultures of *Sutherlandia frutescens* (L.) R. Br. 4th World Congress on Medicinal and Aromatic Plants, Cape Town (Poster prize).

Colling J, Kossmann J, Makunga NP (2009). Piecing together *Sutherlandia frutescens* (L.) R. Br. metabolism using *in vitro* tools. 35th Annual conference of the South African Association of Botanists (SAAB), Stellenbosch.

Colling J, Makunga NP, Stander M (2009). Metabolomic profiling of *in vitro* cultures of *Sutherlandia frutescens* (L.) R. Br. 35th Annual conference of the South African Association of Botanists (SAAB), Stellenbosch (Poster prize).

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Abbreviations

A_{260nm}	Absorbance at 260 nm
ABA	Abscisic acid
Acetyl-CoA	Acetyl-Coenzyme A
Ags	Agropine synthase gene
AOX	Alternative oxidase
Asp-AT	Aspartate aminotransferase
AS	Asparagine synthetase
ASL	Argininosuccinate lyase
ASS	Argininosuccinate synthase
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
BA	6-benzyladenine (PGR)
bp	Base pairs (nucleic acid)
˚C	Degrees Celsius
C: N	Carbon to nitrogen ratio
[ ]	Concentration
Ca^{2+}	Calcium ion
CAM	Crassulacean acid metabolism
CAT	Catalase
cDNA-AFLP	cDNA-amplified fragment length polymorphism
Ci/mmol	Curie per millimolarity
Cl^-	Chloride ion
cm	Centimeter (10^{-2} m)
+ control	Positive control

- control	Negative control
CO_2	Carbon dioxide
CTAB	Cetyl Trimethyl Ammonium Bromide
CTP - P_{32}	Radioactively labeled deoxycytidine 5'-triphosphate
Cu	Copper
2,4-D	2,4-dichlorophenoxy acetic acid
DCM	Dichloromethane
dH_2O	Distilled water
ddH_2O	Deionized water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP's	Deoxyribonucleotide triphosphates
DW	Dry weight
EDTA	Ethylene diamine tetra-acetic acid
EtBr	Ethidium bromide
EtOH	Ethanol
EV	*Ex vitro*
'Ex vitro'	Cultivated in the natural conditions
'Ex situ'	Cultivation outside the natural habitat
$FADH_2$	Flavin adenine dinucleotide
Fd	Ferredoxin
FDA	Food and Drug Administration
FNR	Flavoprotein-NADP reductase
FT-MS	Fourier transform mass spectroscopy
B5	Gamborg B5 (1968) basal salts
g	Gram
GA_3	Gibberellic acid
GABA	Gamma-aminobutyric acid

GABA-T	Gamma-aminobutyric acid amino-transferase
GAD	Glutamate decarboxylase
GC-MS	Gas chromatography mass spectrometry
GDH	Glutamate dehydrogenase
g L^{-1}	Gram per liter
g ml^{-1}	Gram per milliliter
GOGAT	Glutamate synthase
Gram–	Gram-negative
Gram+	Gram-positive
GS	Glutamine synthetase
GUS	β-glucuronidase
h	Hour
H^+	Hydrogen ion
OH^-	Hydroxide ion
HIV	Human immuno-deficiency virus
HPLC	High performance liquid chromatography
HR	Hairy root
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
IAA	Indole-3-acetic acid (PGR)
IBA	Indole-3-butyric acid
iNOS	Inducible nitric oxide synthase
INT	p-iodonitrotetrazolium violet
IV	*In vitro*
In vitro	“in glass”
K^+	Potassium ion
K^+/Na^+	Potassium to nitrogen ratio

kg	Kilogram
KNO_3	Potassium nitrate
kPA	Kilopascal
L	Liter
LC-MS	Liquid chromatography mass spectrometry
LS	Linsmaier and Skoog (1965) medium
M	Molar
MAP	Medicinal and aromatic plants
MeOH	Methanol
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
MH	Müller-Hinton
0.5xMS	Half-strength MS medium
MS	Murashige and Skoog (1962) medium
µg	Microgram (10^{-6} g)
µg ml^{-1}	Microgram per milliliter
µM	Micromolar (10^{-6} M)
µl	Microliter (10^{-6} L)
µmol m^{-1} s^{-1}	Micromole per meter per second
mg	Milligram (10^{-3} g)
mg g^{-1}	Milligram per gram
mg ml^{-1}	Milligram per milliliter
mg L^{-1}	Milligram per liter
MIC	Minimum inhibitory concentration
Min	Minute
ml	Milliliter (10^{-3} L)
mM	Millimolar (10^{-3} M)
mm	Millimeter (10^{-3} m)

>	More than
Mn	Manganese
MPa	Megapascal
M_r	Molecular mass
MS	Mass spectrometry
MRM	Multiple reaction monitoring
n	Sample size
N	Nitrogen
NAA	1-naphthalene acetic acid (PGR)
N/A	No activity
Na^+	Sodium ion
NaCl	Sodium chloride
NAD^+	Oxidized β-nicotinamide adenine dinucleotide
NADH	Reduced β-nicotinamide adenine dinucleotide
$NAD(P)H^+$	Oxidized β-nicotinamide adenine dinucleotide phosphate
NH_4^+	Ammonium ion
NH_4NO_3	Ammonium nitrate
nm	Nanometer (10^{-9} m)
NMR	Nuclear magnetic resonance
NH_4OH	Ammonium hydroxide
NaOCl	Sodium hypochlorite
NaOH	Sodium hydroxide
NiR	Nitrite reductase
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NP/PEG	Natural products-polyethylene glycol reagent
no.	Number

NR	Nitrate reductase
OAA	Oxaloacetate
O_2^-	Superoxide anion
OCT	Ornithine transcarbamoylase
OD_{600}	Optical density at 600 nm
ORF	Open reading frame
O_2	Oxygen
%	Percentage
±	Plus / minus
P	Phosphate
Pc	Plastocyanin
PC	Phillips and Collins (1979) medium
PCR	Polymerase chain reaction
PEG	Polyethylene Glycol
PEP	Phosphoenol pyruvate
Pi	Inorganic phosphate
PPi	Inorganic pyrophosphate
PITC	Phenylisothiocyanate
PGR	Plant growth regulator
PPFD	Photosynthetic photon flux density
ppm	Parts per million
PVP	Polyvinyl pyrrolidone
PQ	Plastoquinone
¼MS	Quarter-strength MS medium
®	Registered
Ri	Root inducing
Rol	Root locus
ROS	Reactive oxygen species

RNA	Ribonucleic acid
rpm	Revolutions per minute
R_t	Retention time
RT-PCR	Reverse transcriptase polymerase chain reaction
Rubisco	Ribulose-1,5-bisphosphate carboxylase-oxygenase
SANBI	South African National Biodiversity Institute
s	Second
SE	Standard error
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
Sp.	species
SSC	Saline sodium citrate
Ssp.	subspecies
Syn.	Synonym
Ta	Primer annealing temperature
TBE	Tris-borate EDTA
TC	Tissue culture
TCA	Tricarboxylic acid cycle
T-DNA	Transfer DNA
TE buffer	Tris-EDTA buffer
TLC	Thin layer chromatography
T_L	Left T-DNA border
TM	Trade mark
T_R	Right T-DNA border
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Tris-HCl	Tris-hydrochloride
U	Units (enzyme)
µCi	Microcurie

UV	Ultra-violet
V	Volts
Vir	Virulence
v/v	Volume per volume
WHO	World Health Organization
w/v	Weight per volume
Zn	Zinc
x	times

CHAPTER 1:

General introduction

1.1 MEDICINAL PLANTS OF THE WORLD

In the earliest days, humans were reliant on the use of plants as a source of medicine, for the flavouring of foods, for fragrances and dyes (Kumar and Gupta, 2008). As science progressed and the active or desirable metabolites in these plants were identified they were gradually replaced by chemically synthesized drugs and compounds (Kumar and Gupta, 2008). In recent years, a new found interest in the use of herbal drugs has awakened worldwide, resulting in the establishment of an herbal drug market worth approximately $62 billion in the US alone (Kumar and Gupta, 2008). Julsing *et al*. (2007) listed the top 10 most popular medicinal plants which are the highest sold products of all medicinal plants. At the top of this list is *Hypericum perforatum* (St John's Wort) used to treat anxiety, depression and insomnia, followed by *Echinacea purpurea* (purple coneflower) which stimulates the immune system and third on the list is *Ginkgo biloba,* used for dementia, Alzheimer's disease and tinnitus.

Although the popularity of herbal medicine in developed countries appears to be increasing, the World Health Organization (WHO) has estimated that 80% of the population in developing countries solely relies on the use of medicinal plants for their basic healthcare needs. This is due to a lack of a formal healthcare system in many of the countries in the developing world (Canter *et al*., 2005; Brown *et al*., 2008). In some of these developing countries these plants also provide a source of income for the poorer communities. Despite the fact that such a large percentage of the global population in both the first and third world countries use medicinal plants, currently only 10% of the medicinal plant species that are being used are cultivated. This implies that most of the plant material is still being collected from the wild (Julsing *et al*., 2007). This is very unfortunate as the over-exploitation and incorrect harvesting of wild plants such as the collection of bulbs, rhizomes and bark can damage the natural populations and eventually result in species extinction and ruin ecosystems (Jäger and Van Staden, 2000; Julsing *et al*., 2007). Several problems are also persistent when solely relying on the use of medicinal plants as a source for healthcare products. This can be attributed to the reduced concentration of the active metabolites and the lack of supply of plant material.

Pressure on wild populations can be alleviated if more investments were made in the cultivation of highly sought after plants (Jäger and Van Staden, 2000). Unfortunately this is currently not taking place since food crops still receive preference above the cultivation of medicinal crops. Progress in this field is further hampered due to difficulties in the establishment of medicinal plants as crops due to a lack of knowledge on their cultivation methods (Kumar and Gupta, 2008). Successful establishment of crops would provide several benefits such as controlling the level of active metabolites and reducing toxic compound levels (Canter *et al*., 2005). Cultivation also opens avenues for conducting scientific research as it provides a source of plant material which can be used to select and breed high-yielding cultivars. In the past, traditional breeding techniques which involve artificial selection of those plants with the most fertile and vigorous genotypes or mutation breeding were used to obtain high yielding lines. However, tradition breeding methods are not always favourable as they can result in the production of sterile plants and these processes can be time consuming due to a long generation time. Other problems involve the perennial nature of plants and difficulties with enhancing secondary metabolite synthesis due to the complex nature of the secondary metabolic pathways (Kumar and Gupta, 2008). Conventional breeding is now being supplemented and in some instances replaced with more rapid techniques which involve the use of a biotechnological approach.

An example of a biotechnological approach includes the use of micropropagation which allows the cultivation of the plants in a controllable system. Subsequently, these cultures can be used for the synthesis and extraction of secondary metabolites (Kumar and Gupta, 2008). However, more advanced biotechnological techniques also allow for the manipulation of biosynthetic pathways by inserting the relevant genes or the use of transcription factors to change pathways for enhanced synthesis of desirable metabolites. One of the prerequisites for changing the synthesis of metabolites is knowledge of their biosynthetic pathways and how they are controlled (Kumar and Gupta, 2008). This can be disadvantageous as our knowledge on secondary metabolism is still limited, particularly with respect to regulatory genetic mechanisms. Research on the biosynthetic pathways has revealed that primary and secondary metabolism is connected. Some of the primary metabolites provide the basic carbon skeleton (precursors) for the synthesis of the secondary metabolites and it is the incorporation of the skeletons in different combinations which results in the large diversity of secondary metabolites observed in medicinal plants (Kumar and Gupta, 2008).

1.2 MEDICINAL PLANTS IN SOUTH AFRICA

It was estimated that approximately 3 000 medicinal plants are being used in southern Africa (Van Wyk, 2008). The trade of medicinal plants in South Africa contributes to both the informal economy as well as the more formal natural products industry (Makunga *et al*., 2008). It is currently estimated that approximately 35 000 to 70 000 tonnes of material derived from both renewable (leaves, stems, flowers and fruits) and non-renewable sources (bark, bulbs and rhizomes) are sold through the informal market which serves a community of between 50 and 100 million people (Mander and Le Breton, 2006; Makunga *et al*., 2008). The majority of these people, who either consume the products themselves or sell them, come from disadvantaged or poor, socio-economic backgrounds (Mander and Le Breton, 2006; Makunga *et al*., 2008). Currently only 38 medicinal plants have been commercialized in some way and can be bought as teas, tinctures, tablets, capsule and ointments (Van Wyk, 2008). Some of these plants are currently being commercially cultivated and these include buchu (*Agathosma betulina*), rooibos (*Aspalathus linearis*) and devil's claw (*Harpagophytum procumbens*). However, the plant material and products are mostly sold to the overseas markets (Jäger and Van Staden, 2000).

The review by Van Wyk (2008) which focused on some of the medicinal plants in South Africa revealed a general increase in the number of scientific reports and patents since 1995. One of the commercially important medicinal plants listed is *Sutherlandia frutescens* (syn. *Lessertia frutescens*) more commonly known as the 'cancer bush'. The historical uses of this plant were documented as early as 1895 by Smith and 1962 by Watt and Breyer-Brandwijk. In the period from 2001 – 2005, *S. frutescens* was cited 27 times in scientific papers and only twice for patents. Although fewer papers (12) cited this herb from 2006 – 2008, the number of citings for patents has now increased to nine (Van Wyk, 2008). The interest in this herb can possibly be ascribed to the large number of applications described in the literature and the increased popularity can be linked to benefits described by cancer and HIV/AIDS patients.

The description that *Sutherlandia* is an 'intricate species complex with countless regional forms, genotypes and chemotypes' (Van Wyk, 2008) has made research of this species an interesting subject to investigate by using a biotechnological approach in this study. This biotechnological strategy which firstly involved the micropropagation of *Sutherlandia frutescens* would aid with the problems related to wild harvesting of material by supplementing *ex situ* cultivation or providing an *ex situ* resource of acclimated plants. Secondly, through

Agrobacterium mediated transformation, a system which produces interesting biochemical changes, would inform on the impact of transgenesis in *S. frutescens*. Both micropropagules (non-transgenic) and hairy root cultures (transgenic) were used as a scientific tool which would add to our overall understanding of metabolism.

1.3 LITERATURE CITED

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CHAPTER 2:

Literature review

2.1 *SUTHERLANDIA FRUTESCENS*, A MEDICINAL HERB FROM SOUTH AFRICA

2.1.1 Classification of the *Sutherlandia* lineage

The genus *Sutherlandia* belongs to the family Fabaceae (Leguminosa, tribe Galegeae) and like other legumes the roots of these plants has nitrogen-fixing nodules (Chadwick *et al*., 2007; Xaba, 2007). The genus *Sutherlandia* is also closely related to the genus *Astragalus* which has about 2 500 species (Chen, 2007). There has been great taxonomic dispute amongst the scientific community with regards to the number of *Sutherlandia* species. Even so, these plants are all indigenous to South Africa (Chadwick *et al*., 2007). The different species can be distinguished from each other by their habitat, the shape of their pods, the orientation of the fruit characteristics and the pubescence of the leaflets (Mosche *et al*., 1998). Initially Phillips and Dyer (1934) described six closely related species a) *S*. *frutescens* b) *S*. *montana* c) *S*. *microphylla* d) *S*. *tomentosa* e) *S*. *humilis* and f) *S*. *speciosa* (Xaba, 2007; Van Wyk and Albrecht, 2008). These species are widely distributed across southern Africa (Fig 2.1.1).

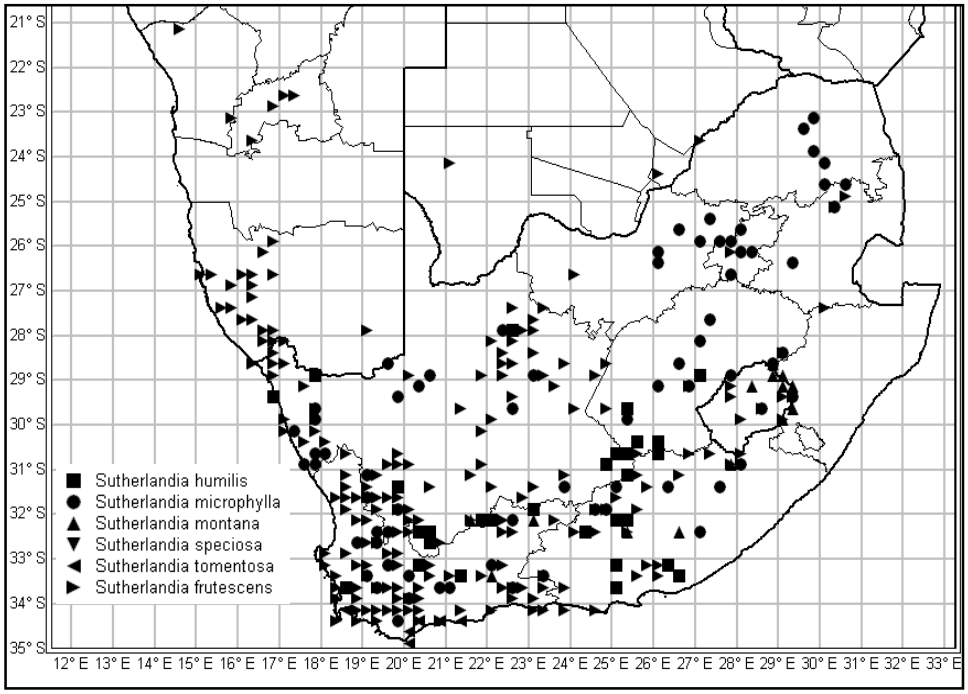


Figure 2.1.1: The distribution of the various *Sutherlandia* species in southern Africa (reproduced with kind permission from SANBI, PRECIS)

In 1998, Mosche and co-workers used enzyme electrophoresis studies to indicate that some of these species are actually subspecies and that only two main species exist, *S*. *frutescens* and *S*. *tomentosa*. Recent work by Goldblatt and Manning (2000) suggested that *Sutherlandia* should be included in the genus *Lessertia* DC. due to the assumption that *Sutherlandia* only represents an adaptation to bird pollination, however, this remains to be confirmed by morphological and genetic analysis (Van Wyk and Albrecht, 2008). The transfer of *Sutherlandia* to *Lessertia* required that the two species mentioned previously, be renamed to *Lessertia frutescens* and *Lessertia canescens* (classification changes are summarized in Figure 2.1.2). In this thesis, I will however continue the use of the more commonly known and accepted name, *Sutherlandia*.

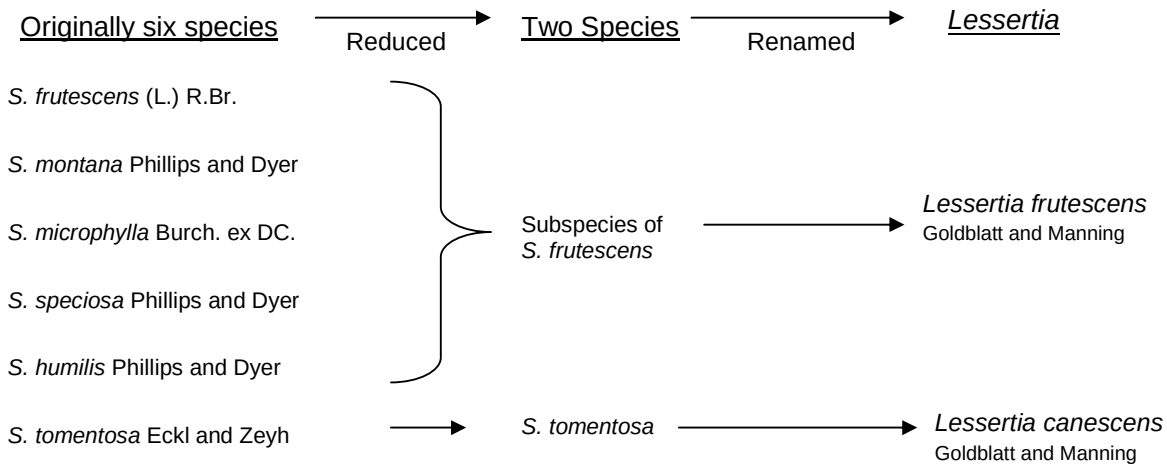


Figure 2.1.2: The classification of the *Sutherlandia* species lineage

2.1.2 *Sutherlandia frutescens* distribution and vernacular names

Of all the species, *Sutherlandia frutescens* is most variable and widespread, occurring throughout southern Africa (Fig 2.1.3) including the drier parts of the Western Cape, Eastern Cape, KwaZulu-Natal, Lesotho, Namibia and the southeastern corner of Botswana (Xaba, 2007; Van Wyk and Albrecht, 2008). According to Xaba and Notten (2003) these plants grow particularly in arid areas where the ground has been disturbed.

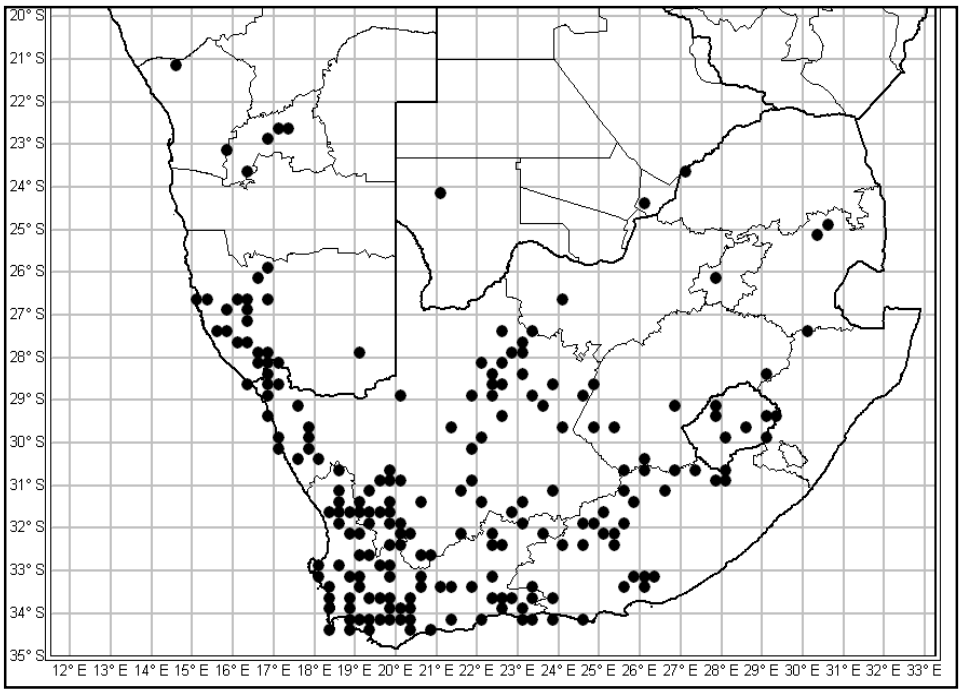


Figure 2.1.3: The distribution of *Sutherlandia frutescens* in southern Africa (reproduced with kind permission from SANBI, PRECIS)

Due to the large cultural diverse group of people using this herb, several interesting vernacular names can be found for this plant (Scott and Springfield, 2004; Tai *et al*., 2004, Van Wyk and Albrecht, 2008). Examples include English names such as cancer bush, turkey flower or balloon pea; in Afrikaans it is known as kankerbos, gansies, jantjie-bêrend, klappers, kalkoentjiebos, bitterbos, keurtjies; in German, blasenstrauch or krebsbusch; in Sesotho, musa-pelo or motlepelo meaning “which brings the heart back to life” and in Zulu and Xhosa it is known as insiswa (“dispersal of darkness”) or unwele (Grandi *et al*., 2005; Van Wyk and Albrecht, 2008). The San know this plant as “pethora” meaning “this which modifies the evolution of the disease” (Grandi *et al*., 2005).

2.1.3 General botany of *Sutherlandia frutescens*

Sutherlandia frutescens is a perennial, short-lived shrub which can grow up to 2.5 meters high and which produces red to orange flowers (Figure 2.1.4A and B) during the flowering season which lasts from Spring to Summer (Xaba, 2007). The bitter tasting leaves of this plant are compound, pinnate and have a silvery green-grey colour (Figure 2.1.4B). After flowering, black seeds are produced in the green to red swollen, bladder-like pods (Figure 2.1.4C and D), which grow about 5 cm long and become papery upon drying (2.1.4E). The plants growing

near coastal regions are shorter and have light grey leaves, whereas the plants growing in the inland regions are taller and have darker grey leaves (Scott and Springfield, 2004; Xaba, 2007). During a survey of medicinal plants used in the Karoo region, Van Wyk *et al*. (2008) reported that the shorter plant (*S. frutescens*) is considered to be female and is used for men's problems, whilst the taller plant (*S. microphylla*) is considered to be male and is used for women's problems.

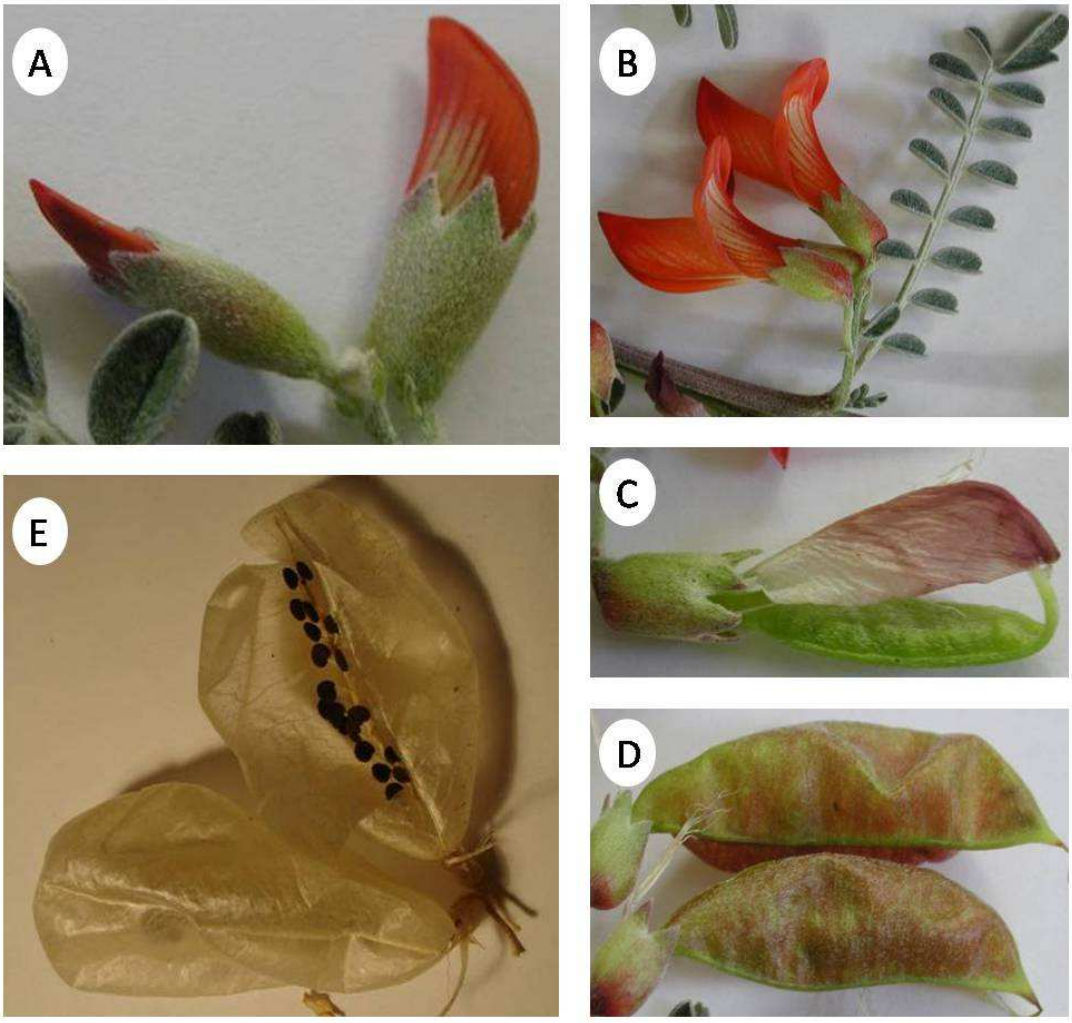


Figure 2.1.4: The cycle of flower to seed development. A) Development of flowers; B) a compound leaf of *S. frutescens*; C) initial development of a seed pod; D) matured seed pods containing the seeds inside, and, E) dried seed pods with seeds

2.1.4 Traditional medicinal uses of *S. frutescens*

For many years the Khoi-San, Nama, Sotho, Zulu and Xhosa people used mainly the leaves, although branches and roots were also pulverized and boiled to prepare an extract which is drunk or topically applied to treat various diseases (Tai *et al*., 2004; Xaba, 2007; Van Wyk and Albrecht, 2008). The diseases and uses which are mentioned varied from aches, diseases of the eye, chest complaints, stomach, intestinal and digestive problems, as a febrifuge (treatment of fevers), for uterine ailments and urinary tract infections. Extracts were also used as a cough and cold remedy, as a restorative tonic, to treat internal cancers, stress, influenza, diabetes, varicose veins, piles, inflammation, liver problems, burns, backache, rheumatism and the cleansing of open wounds in cattle (Mosche *et al*., 1998; Tai *et al*., 2004; Chadwick *et al*., 2007; Xaba, 2007). The early Dutch settlers also used the extracts to treat chicken pox and its common name 'cancer bush', was originally derived from its popular use for the treatment and prevention of cancer (Van Wyk and Albrecht, 2008). Due to the high levels of free and protein-bound amino acids, this plant is also used as fodder for cattle (Van Wyk and Albrecht, 2008).

Today, *Sutherlandia* extracts are also reported to assist with reducing muscle wasting (cachexia) in cancer patients. Claims have also been made that *Sutherlandia* extracts can improve the CD4 counts and reduce the viral load of HIV as well as improve the mood and appetite of HIV/AIDS patients (Chaffy and Stokes, 2002; Van Wyk and Albrecht, 2008). Considering this wide spectrum of reported activity, it is no wonder that *S. frutescens* is known as an adaptogenic[1] plant (Van Wyk and Albrecht, 2008). In an attempt to understand the reported health properties of this plant, a few studies have been conducted to identify the metabolites present in the extracts as this may provide some insight into the pharmacological activity of this plant.

2.1.5 The phytochemistry of *S. frutescens* extracts

Phytochemical profiles of extracts prepared from the leaves indicated the presence of a complex mixture of compounds such as bitter triterpenoid glycosides, several flavonoids, amino acids and small amounts of saponins; however, no alkaloids seem to accumulate in the plants (Van Wyk *et al*., 1997). Several of these compounds have been studied individually in an attempt to determine their biological properties both in the plant and the human body. A closer inspection of the type of compounds present in the extracts included the following:

[1] Adaptogenic – something 'that helps the human body to mobilize its own immunological and physiological resources to combat diseases and physical and mental stress' (Ojewole, 2008)

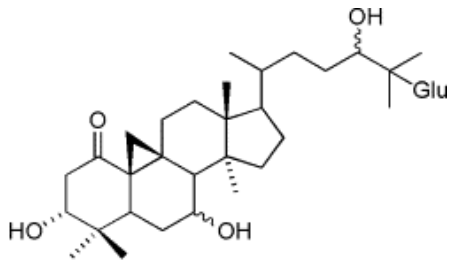
a) Flavonoids

Sutherlandia leaves are reported to contain at least six flavonoids and these are likely flavonol glycosides (Van Wyk and Albrecht, 2008).

b) Triterpenoid saponins

The presence of triterpenoids in the extracts is reported to be responsible for the bitter taste and the *'amarum'* effect detected by users of this herb (Van Wyk and Wink, 2004). These bitter compounds promote the production of saliva, gastric juices and bile which aid digestion and stimulate the appetite (Van Wyk and Wink, 2004).

In 1996, Gabrielse isolated and identified the first triterpene in *S. frutescens* (Van Wyk and Albrecht, 2008). This cycloartane-type triterpene glycoside compound which is hydroxylated at C-24 and has a 3-oxo group (Figure 2.1.5) is known as SU1 (Van Wyk and Albrecht, 2008). Previously cycloartanes with this conformation exhibited powerful inhibitory effects in an *in vivo* mouse skin carcinogenesis test (Van Wyk and Albrecht, 2008). This compound, SU1, is now also known as Sutherlandioside B (see Figure 2.1.6 no.2 below) (Olivier *et al*., 2009).



SU1

Figure 2.1.5: Triterpenoid saponin SU1 (Van Wyk and Albrecht, 2008)

Recently Fu *et al*. (2008) purified and identified four cycloartane glycosides known as Sutherlandioside A (SU2), B (SU1), C and D (Figure 2.1.6) from *S. frutescence* leaves. Upon investigation of the anti-microbial activity of these four compounds, the results indicated that at the highest concentration (20 µg ml^{-1}) they did not display anti-microbial activity against *Candida albican*s, *Cryptococcus neoformans*, *Aspergillus fumigates*, *Staphyloccous aureus*, methicillin-resistant *S. aureus*, *Pseudomonas aeruginosa* and *Mycobacterium intracellulare*. At 40 µg ml^{-1} the compounds did not exhibit anti-malarial activity against *Plasmodium faciparum* (D6 and W2) clones either (Fu *et al*., 2008).

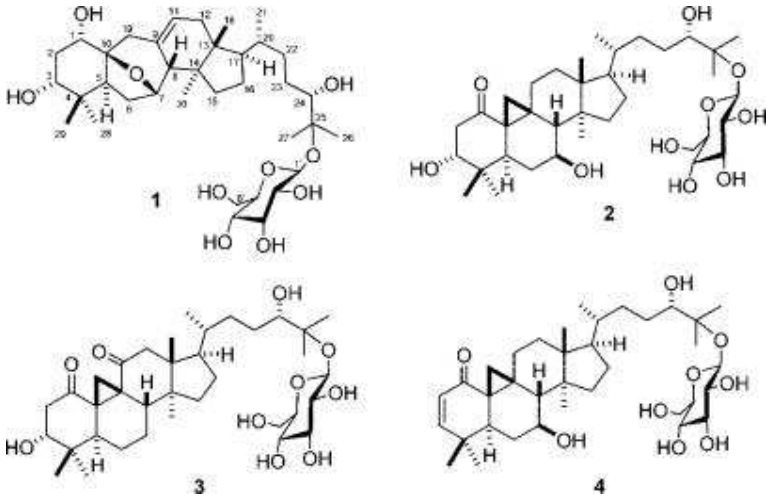


Figure 2.1.6: Chemical structures of Sutherlandioside A-D (1-4 respectively) (Fu *et al*., 2008)

Mosche (1998) reported limited variation in the triterpene patterns within a *Sutherlandia* population, but large differences could be detected between populations (Van Wyk and Albrecht, 2008). So far, 56 different triterpene glycosides have been detected and the mixture of cycloartane-type triterpenoid glycosides varies geographically in South Africa (Van Wyk and Albrecht, 2008).

c) Other secondary metabolites

Other secondary compounds identified include hexadecanoic acid, γ-sitosterol, stigmast-4-en-3-one and at least three other long chain fatty acids which have been detected in the extracts (Tai *et al*., 2004). Olivier *et al*. (unpublished data) have also detected unidentified polysaccharides which total 35% of the dry weight in an aqueous extract (Van Wyk and Albrecht, 2008).

d) Pinitol/ ino-inicytol

S. frutescens extracts contained up to 14 mg pinitol g^{-1} dry weight (Van Wyk and Albrecht, 2008). Pinitol (Figure 2.1.7) is a sugar alcohol and its role as an anti-diabetic compound has been investigated (Van Wyk, 2008). In humans, this compound may assist with diabetes by lowering blood sugar levels and making the glucose more available to the cells (Bates *et al*., 2000). Pinitol is speculated to be the compound which is responsible for preventing wasting and inflammation in HIV/AIDS patients (Van Wyk and Albrecht, 2008). Furthermore, pinitol

can also alter the retention of creatinine by the muscle cells (Van Wyk and Albrecht, 2008). In plants, pinitol (a cyclitol) can act as a compatible solute during stress (see Section 2.5 on water and salt stress).

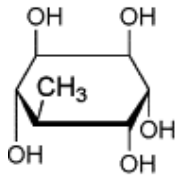


Figure 2.1.7: Chemical structure of pinitol (Van Wyk and Albrecht, 2008)

e) Proline

Proline (Figure 2.1.8) is an amino acid. It has a non-polar side chain and it is the only amino acid which has a secondary amino group (Voet and Voet, 2004). In humans, this amino acid is considered as one of the non-essential amino acids as it can be synthesized by the human body (Voet and Voet, 2004). In plants, proline is another example of a compatible solute which can be synthesized in response to stress (Ashraf and Foolad, 2007). In *S. frutescens*, proline was detected in a level of 0.7 – 7.5 mg g^{-1} (Van Wyk and Albrecht, 2008).

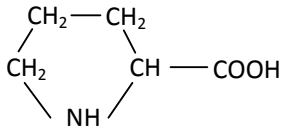


Figure 2.1.8: The chemical structure of proline (Taiz and Zeiger, 2002)

The other metabolites that have been found in *S. frutescens* (Figure 2.1.9) are amino acids (asparagine, arginine) and non-protein amino acids (gamma-aminobutyric acid (GABA) and canavanine). Their synthesis and role in plants and humans are reviewed in Section 2.4.2 which deals with nitrogen assimilation.

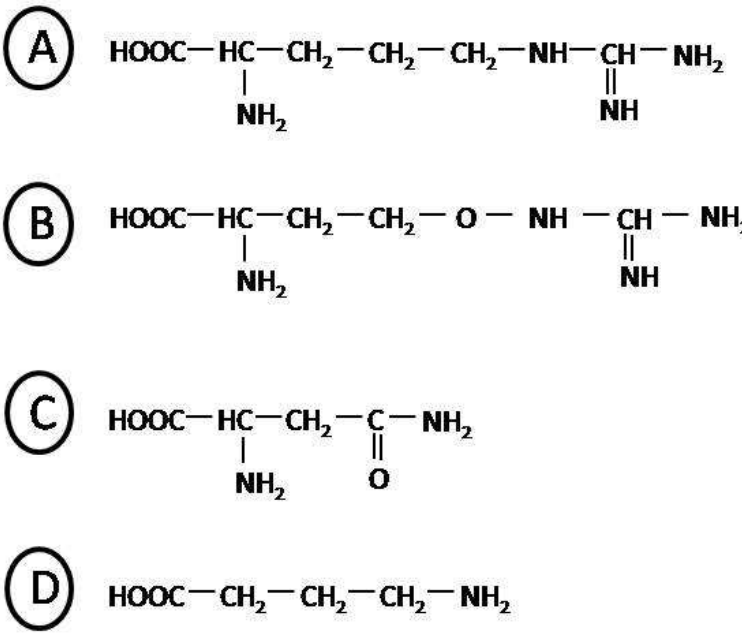


Figure 2.1.9: The chemical structure of amino acids and the non-protein amino acids in *S. frutescens* extracts: A) arginine; B) canavanine; C) asparagine, and, D) GABA. The chemical structures for arginine, canavanine and asparagine are re-drawn from Taiz and Zeiger (2002) whereas the structure for GABA is according to Van Wyk and Albrecht (2008).

Considering the wide variety of primary and secondary metabolites present in *S. frutescens* extracts, Tai *et al*. (2004) suggested that the pharmacological activity of *S. frutescens* result from the synergistic effects of all the compounds and that these plants exert their activity in a complex mechanisms (Stander *et al*., 2007). To date, several pharmacological studies have been conducted in an attempt to understand the mechanisms involved and to possibly identify the key metabolites responsible for these mechanisms.

2.1.6 The pharmacological activity of extracts

Attempts to elucidate the pharmacological activity of this plant resulted in a broad compilation of scientific articles (Table 2.1.1) which provide insights into the health-promoting activity of *S. frutescens.* An excellent review on *S. frutescens* on the pharmacological activity was recently prepared by Van Wyk and Albrecht (2008).

Some of the pharmacological activities investigated over the years included the anti-proliferating, anti-cancer and anti-mutagenic activity of *S. frutescens* extracts and the related

mechanisms involved. A novel compound responsible for the anti-cancer properties of this plant has not yet been identified; however several compounds with known anti-cancer properties have been identified and isolated in *S. frutescens* extracts. These include canavanine and some triterpenoids (Section 2.1.5b) which are structurally related to cycloartane-type triterpenoids which were previously found to have cancer chemopreventive activity (Kikuchi *et al*., 2007; Van Wyk and Albrecht, 2008). The anti-cancer properties of the extracts indicate that different cell lines appear to exhibit differences in their sensitivity to *Sutherlandia* extracts as they did not respond similarly to the extracts. Alternatively, a difference in the extraction procedure or the natural variability in plants can contribute to differences observed in this activity (Steenkamp and Gouws, 2006).

Secondly, the activity against HIV/AIDS virus was also investigated. Previous reports indicate that consumption of the herbal extract was beneficial to the health of HIV/AIDS patients (refer back to Section 2.1.4). Another plant from this family, *Astragalus membranaceus* is also reported to have immune-stimulating properties (Van Wyk and Albrecht, 2008). This property in *S. frutescens* was reported to be due to the activity of canavanine, which can inhibit nitric oxide synthase (NOS) and can be used to treat septic shock syndrome which is associated with advanced stages of HIV/AIDS (Van Wyk and Albrecht, 2008).

Sutherlandia also appears to benefit the health of diabetic patients which was attributed to the presence of pinitol (Section 2.1.5d) and L-canavanine (Section 2.4.2.2) (Sia, 2004; Van Wyk and Albrecht, 2008). Chadwick *et al*. (2007) found that *S. frutescens* water extracts promoted glucose uptake by the muscle and adipose tissue cells. This was ascribed to either increased insulin sensitivity at the cellular levels or alternatively the extracts substituted insulin itself and therefore reduced the demand on the β-cells. Additionally the extracts also reduced the amount of glucose taken up from the intestine which decreased the glucose concentration in the blood system (Chadwick *et al*., 2007).

The stress alleviating properties of *Sutherlandia* extracts was partly attributed to *S. frutescens*' ability to act as an adaptogenic plant, by causing non-specific increased resistance such as a small change in physiology and its wide spectrum of activity which results in a 'normalizing' effect (Van Wyk and Albrecht, 2008). Prevoo *et al*. (2004; 2008) also investigated this stress relieving property and focused mainly on the effect on corticosterone levels as these are associated with chronic stress.

Several of the other health promoting activities (wound healing) was attributed to the anti-microbial, anti-oxidant (Fernandes *et al*., 2004; Tai *et al*., 2004) and anti-inflammatory (Tai *et al*., 2004) properties of the extracts. The anti-oxidant activity was speculated to be due to the presence of phenolics such as tannins and flavonoids in the extracts (Fernandes *et al*., 2004).

Recent work also described the anti-convulsant activity of *S. frutescens* aqueous extracts in mice and indicated that these extracts can be used as a supplemental remedy to manage and/or control partial and generalized seizures (Ojewole, 2008). Although the mechanism for this activity is still unknown, it was speculated that the presence of GABA may have a role in the anti-convulsant activity (Ojewole, 2008). Other examples of the pharmacological properties include the anti-thrombotic activity and alleviation of heart burn (Table 2.1.1).

Table 2.1.1: Scientific papers citing the pharmacological activity of *Sutherlandia frutescens*

Pharmacological activity investigated	**References**
Anti-proliferating/ anti-cancer/Anti-mutagenic / cytotoxic	Na *et al*., 2004; Tai *et al*., 2004; Chinkwo, 2005; Kundu *et al*., 2005; Reid *et al*., 2006; Steenkamp and Gouws, 2006; Chen, 2007; Stander *et al*., 2007; Stander *et al*., 2009
HIV studies	Harnett *et al*., 2005; Mills *et al*., 2005; Brown *et al*., 2008
Anti-diabetic	Bates *et al*., 2000; Ojewole, 2004; Sia, 2004; Chadwick *et al*., 2007
Anti-inflammation properties, pain, assisting with the healing of wounds	Fernandes *et al*., 2004; Ojewole, 2004; Tai *et al*., 2004; Chen, 2007
Anti-microbial activity	Katerere and Eloff, 2005
Stress alleviation	Prevoo *et al*., 2004; Smith and Myburgh, 2004; Prevoo *et al*., 2008
Anti-thrombotic activity and alleviating heart burn	Van Wyk and Albrecht, 2008
Anti-oxidant	Fernandes *et al*., 2004; Tai *et al*., 2004; Katerere and Eloff, 2005
Anti-convulsant (epilepsy)	Ojewole, 2008

The list shown here may not be comprehensive

2.1.7 Safety and toxicity of extracts

The first safety and toxicity study of *S. frutescens* herbal products was conducted in 2002 by Seier and co-workers. During this study, the recommended daily dose of 9.0 mg kg^{-1} body weight was administered in levels of 0, 1, 3 and 9 times this value to vervet monkeys for a period of three months. The conclusion from the haematological, clinical-biochemical, physiological and other behavioural variables indicated that *Sutherlandia* was clinically not significantly toxic nor did it cause side effects in the test animals.

In 2004, that study (Seier *et al*., 2002) was followed by an investigation by Ojewole (2004) of the acute toxicity of aqueous *Sutherlandia* extracts which were administered intraperitoneally to fasted Balb C albino mice (Van Wyk and Albrecht, 2008). The lethal dose (LD_{50}) was calculated as 1280 ± 71 mg of extract kg^{-1} body weight day^{-1} and the recommended therapeutic dose is 9 mg kg^{-1} day^{-1} (Mills *et al*., 2005; Van Wyk and Albrecht, 2008). Today commercial products containing 300 mg extract $tablet^{-1}$ are recommended to be taken twice per day (Van Wyk and Albrecht, 2008). From these studies it was concluded that crude *Sutherlandia* extracts were safe for ingestion by mammals.

In 2007, Johnson *et al*. conducted a phase I clinical trial using 25 healthy adults at the Karl Bremer Hospital, Bellville, South Africa. During this trail, two 400 mg leaf powder capsules were consumed daily for a period of three months. The results indicated that this dosage was tolerated without side effects (clinically significant changes in physical, vital, blood and biomarker indices). The treatment group displayed a statistically significant increase in appetite and a lower respiration rate, a higher platelet count, total protein and albumin levels. However these changes remained within the normal physiological range. The only side effects reported by patients using *Sutherlandia* products were dryness of the mouth, occasional mild diarrhea or mild diuresis and dizziness in cachectic patients (Mills *et al*., 2005).

Sia (2004); Mills *et al*. (2005) and Brown *et al*. (2008) warned that care should be taken when *Sutherlandia* products are consumed simultaneously with anti-retroviral medication or with insulin or other diabetic medicine, as an adverse effect on the adsorption and effectivity of these allopathic medicines in the human body can occur.

2.2 SECONDARY METABOLITES

Secondary compounds are usually low molecular weight compounds such as alkaloids, anthraquinones, lignans, anthocyanins, flavonoids, saponins and terpenes synthesized by plants (Kolewe *et al*., 2008). Although they are not essential for sustaining life, they may improve a plant's chances of survival (Kolewe *et al*., 2008). However, it is the presence of these secondary metabolites which has made the use of medicinal plants valuable for the treatment of diseases. Secondary compounds from medicinal plants have pharmacological properties such as; analgesic, anti-tussive, anti-hypertensive, cardio-tonic, anti-neoplasic and anti-malarial to name a few (Oksman-Caldentey and Hiltunen, 1996; Gómez-Galera *et al*., 2007). However, these compounds are also used for flavouring, fragrance, pesticides and as a source of pigments (Oksman-Caldentey and Hiltunen, 1996). Industries have used medicinal (and other) plants to develop a wide spectrum of pharmaceuticals, nutraceuticals, cosmetics, perfumes, dyes and new flavours due to the wide array of secondary metabolites synthesized by plants (Bourgaud *et al*., 2001; Hu and Du, 2006). Unfortunately commercial production of these products may often be restricted due to the low yields of these metabolites in the plants (Kolewe *et al*., 2008).

The problem is that secondary metabolites are often synthesized or accumulated in the vacuoles of specific cells or other differentiated tissues (eg. trichomes) at a specific time during a developmental stage or in response to stress (Oksman-Caldentey and Hiltunen, 1996). Storage of compounds can also be linked to the age of the plant and it may therefore take years before plants can be harvested to obtain the metabolites of interest (Oksman-Caldentey and Hiltunen, 1996). These factors make it difficult to extract, isolate and purify high levels of these metabolites. As described earlier, these metabolites can alternatively be chemically synthesized in the laboratory, but this may be a costly and complicated procedure due to the complex chemical structures of the metabolites (Srivastava and Srivastava, 2007). Therefore many of the secondary metabolites are still obtained through conventional ways by preparing extracts from plants, which suggests that large amounts of plant material often have to be harvested to isolate the compounds of interest.

This approach can be problematic depending on the source from which these plants are obtained. Although some of the plants are successfully cultivated, several others are directly harvested from the wild. Large-scale production of herbal extracts and the increase in commercial demand is resulting in plants being harvested faster than they can regenerate in

the wild. This is cause for concern as this type of unsupervised and destructive harvesting (stripping the bark; removal of the whole plant, underground parts and the reproductive organs) can reduce the population size of those plants in the wild. Furthermore, it can also lead to a loss of genetic diversity, cause local extinctions of certain species, result in habitat destruction and degradation, and ultimately species can eventually become endangered or threatened (Canter *et al*., 2005). Unfortunately, limited legislation and a few conservation efforts are not enough to protect medicinal plants from being exploited (Gómez-Galera *et al*., 2007).

Harvesting from the wild can be reduced if an alternative source for the medicinally important plants is developed. As mentioned previously, a conventional approach would involve investing in the establishment of agricultural practices to domesticate these plants, cultivating them in fields. Although practical, field cultivation is not always ideal due to certain constraints. These include the high costs involved in erecting the infrastructures and high labor costs, limited plant resources and the difficulty in cultivation of certain species due to their requirement for a specific climate, thereby complicating processes in establishing them as crops outside of their natural environment (Oksman-Caldentey and Hiltunen, 1996). The slow growth rates of some plants imply that it can take many years to successfully establish them as crops in the field (Gómez-Galera *et al*., 2007; Srivastava and Srivastava, 2007; Kolewe *et al*., 2008). Further problems arise because domesticated plants are vulnerable to pathogen attack and climate changes which influence the metabolite composition, resulting in varying compound yields (Srivastava and Srivastava, 2007). An alternative approach which would exclude several of these unfavourable conditions involves using a biotechnological strategy.

2.3 BIOTECHNOLOGICAL APPROACHES TO STUDY MEDICINAL PLANTS

2.3.1 Micropropagation

Micropropagation is a rapid multiplication technique which allows the production of large quantities of genetically similar plants (clones) from small segments of starting material (Mithila *et al*., 2001). Firstly, this system is ideal as it can benefit the conservation of rare plants and represents an *in vitro* conservation strategy for *ex situ* purposes. *In vitro* cultivation will not only alleviate the pressure on the wild populations but it will also allow the use of other biotechnological techniques to solve problems related to wild harvesting of medicinal plants. These include the reliable identification of specific plant species and populations, decrease in

the phenotypic and genotypic variability, improving the product yield, the detection of toxic and contaminating compounds and assisting in the development of a uniform, stable, high quality product (Canter *et al*., 2005; Gómez-Galera *et al*., 2007). Furthermore, micropropagation is ideal for research as it provides easy access to plant material and experiments are therefore not limited by geographical distribution, environmental or climatic conditions, seasonal and/or even socio-political instabilities (in cases where these plants of interest grow in developing countries) as well as changes in the import prices which may fluctuate (Oksman-Caldentey and Hiltunen, 1996; Fennell *et al*., 2006). These micropropagated plants are therefore available during any time of the year (Georgiev *et al*., 2007; Srivastava and Srivastava, 2007).

Additionally, through biotechnological manipulation using transgene technology, it is now possible to study metabolic pathways such as those enzymes and compounds involved in the synthesis of secondary metabolites. This information can be used to increase the production of pharmacologically active chemicals. Micropropagation and gene transformation (metabolic engineering) can thus be used to solve the low accumulation of secondary metabolites in cultivated medicinal plants. However, micropropagation is not only useful to multiply and cultivate plants, but it also provides a valuable tool which can be used to investigate the effect of various stresses (biotic and abiotic) on the metabolism (primary and secondary) and the general growth and development of plants.

2.3.1.1 Using *in vitro* cultivation to study stress

In vitro cultivated plants are ideal for this purpose as they provide a system which can easily be controlled by manipulation of the nutritional and environmental cultivation conditions (Oksman-Caldentey and Hiltunen, 1996). Some examples of abiotic stress studies on medicinal and other plants include the investigation of salt stress (Shibli and Al-Juboory, 2002; Shibli *et al*., 2003); water stress; pH modifications (Andreazza *et al*., 2009); temperature (Yu *et al*., 2005); UV light (Binder *et al*., 2009); heavy metal toxicity and nutrient (nitrogen and phosphate) availability. *In vitro* cultivated plants can also be used to investigate biotic stress which includes the use of fungal elicitors and methyl jasmonate (Kim *et al*., 2004).

The micropropagated plants can be used to investigate the effect of stress on the synthesis of secondary metabolites in medicinal plants, as these micropropagated plants often produce similar compounds to those found in the native plants (Kolewe *et al*., 2008). The application of stress can be used to study the observable changes in physiology as well as the mechanisms

which plants utilize to respond to the applied stress. However, micropropagation involves the establishment of different plant cultures in the laboratory which can vary from undifferentiated cell cultures to differentiated organ (shoot and root) cultures.

2.3.2 Plant cell cultures

Cell suspension cultures are initiated when the explant is cultivated in conditions which leads to the proliferation of undifferentiated totipotent[2] cells or callus which, upon transfer to liquid medium and shaken continuously, break up and produce liquid suspension cultures (Kolewe *et al*., 2008). The use of cell suspension cultures for metabolite synthesis would be ideal as they are 1) easy to scale up; 2) renewable; 3) environmentally friendly; 4) allow easier purification than intact tissue; and 5) they can be tightly regulated to meet the Food and Drug Administration (FDA) manufacturing standards (Kolewe *et al*., 2008). The application of cell suspension cultures for commercial production of secondary metabolites requires the selection of a stable cell line with a high growth rate which also has to produce a high yield of the compounds of interest (Oksman-Caldenty and Hiltunen, 1996).

Unfortunately, cell suspension cultures have certain flaws which have prohibited their successful upscaling to bioreactors for metabolite synthesis. These flaws have also hampered progress in commercialization (in industrial application) of the cell culture system for the production of a wider range of plants in a commercial set-up. The first challenge is that the cultivation conditions such as the medium composition (carbon source, minerals, anti-oxidants and phytohormones) have to be optimized for each new species (Kolewe *et al*., 2008). Secondly, cell suspension cultures are genetically unstable and they may therefore lose their ability to accumulate specific metabolites as the plant cells age or they may synthesize the compounds to a lower level (Kolewe *et al*., 2008). Furthermore, cells may completely lack the ability to synthesize the metabolites since the pathways for metabolite synthesis are often linked to certain developmental processes in the plant. These metabolites may therefore only be synthesized or accumulated in specific organs, which is why these pathways are not activated in undifferentiated cells (Kolewe *et al*., 2008). Examples of tissue-specific synthesis of secondary metabolites include the synthesis of asiaticoside (a trisaccharide triterpene) which is specific to the leaves of *Centella asiatica* (Kim *et al*., 2004; Kim *et al*., 2005). These limitations make the use of this system less favourable (Srivastava and Srivastava, 2007).

[2] Totipotent – the ability to regenerate into a fertile plant

However, despite these challenges several cell cultures have been successfully scaled up for commercial production of interesting compounds. Currently, there are 14 plant cell culture systems which are being used for the commercial production of secondary compounds. Two examples include the synthesis of paclitaxel (an anti-tumour compound harvested from *Taxus brevifolia*) by Phyton Biotech and Shikonin (an anti-tumour, anti-HIV, anti-inflammatory compound) harvested from *Lithospermum erythrorhizon* by Mitsui Petrochemical industries (Kolewe *et al*., 2008). An alternative approach for obtaining secondary metabolites from *in vitro* cultures includes the use of differentiated tissue represented by organ cultures for secondary metabolites synthesis (Oksman-Caldenty and Hiltunen, 1996).

2.3.3 Organ cultures

Organ cultures include the use of shoots and roots in culture, which can be obtained by manipulation of the medium plant growth regulator (cytokinin: auxin) balance. The advantage of *in vitro* organ cultures is that the compounds in these tissues often occur in similar patterns and levels as in the intact plants (Oksman-Caldenty and Hiltunen, 1996).

2.3.3.1 Shoot and root cultures

Shoot apex cultures provide multiple, genetically stable, cloned shoots which are derived from apices or axillary buds (Cano *et al*., 1998). Due to the anatomical organization and ability to root and develop into whole plants, these *in vitro* shoots represent “mini-replicas” of a plant (Cano *et al*., 1998).

Although root cultures have been established, they are not preferable cultures to work with as they may often grow too slowly and the secondary metabolite yield and diversity can differ from intact roots. This root culture system therefore provides little potential use for secondary metabolite synthesis (Oksman–Caldenty and Hiltunen, 1996). However, through the use of genetic engineering, these roots can be transformed to become a valuable tool to study secondary metabolism and they can be used for the production of specific secondary metabolites.

2.3.3.2 Hairy root cultures – a transgenic system

Hairy roots are differentiated tissues, which are produced during the infection of plants with *Agrobacterium rhizogenes*, a Gram-negative soil bacterium (Veena and Taylor, 2007) of which several different strains occur in nature. *Agrobacterium rhizogenes* strains can be classified

depending on the genes which are transferred to the plant genome. These genes code for a specific type of opine (agropine, mannopine and mikimopine) which are produced after the incorporation of their genes into the plant genome (Veena and Taylor, 2007). Alternatively the bacterial strains can also be classified depending on the polarity of infection of the plant tissue (Veela and Taylor, 2007). Agropine strains for example can only induce hairy root formation on the apical surface of the tissue (polar), possibly due to the basipetal transport of auxin in plants. All the other strains can induce hairy roots on both the apical and basal surfaces (non-polar) (Veena and Taylor, 2007).

In nature, *A. rhizogenes* have been found to only infect a limited number of plant species such as the apple, cucumber, tomato and melon (Veena and Taylor, 2007). However, when tested under laboratory conditions, *A. rhizogenes* transformation has been applied to more than 450 different plant species (Veena and Taylor, 2007). This included a diverse range of dicotyledonous and cotyledonous plant species and some gymnosperms (Georgiev *et al*., 2007) (refer to Christey, 2001 for examples of transformed plants). The mechanism involved in *A. rhizogenes* transformation has been elucidated.

2.3.3.3 *A. rhizogenes* transformation biology and effects of *rol* genes

Agrobacterium rhizogenes contains a virulence plasmid, known as the root-inducing (Ri) plasmid (Sinkar *et al*., 1987). The Ri plasmid contains three segments of DNA: 1) the segment known as the transfer DNA (T-DNA) which is transferred to the plant; 2) border sequences of the T-DNA and 3) a segment known as the virulence region (*vir*) which is responsible for the transfer of the T-DNA (Oksman-Caldenty and Hiltunen, 1996).

1) T-DNA

The genes contained in the T-DNA are of a bacterial origin but they are regulated by sequences of eukaryotic origin which allow them to be expressed in the plant genome (Giri and Narasu, 2000). The T-DNA segment of the agropine strains contain two segments known as left T-DNA (T_L-DNA) and right T-DNA (T_R-DNA), which are independently transferred to the plant cell (Figure 2.3.1) (Oksman-Caldenty and Hiltunen, 1996). The transfer of the T_L-DNA is essential for the production of hairy roots, whereas the transfer of only the T_R-DNA does not stimulate the production of hairy roots. The T_R-DNA contains genes which are homologous to the tumour-inducing (Ti) T-DNA of *Agrobacterium tumefaciens*, and these genes are

responsible for auxin synthesis. The T_R-DNA also contains genes responsible for opine (mannopine and agropine synthase (*ags*)) synthesis. The products of these genes provide the bacterium with a source of carbon and nitrogen (Sinkar *et al*., 1987). The T_L-DNA contains 18 open reading frames (ORF) of which four; ORF10, ORF11, ORF12 and ORF15 corresponding to *rol* (root locus) A, *rol* B, *rol* C and *rol* D respectively, are important for hairy root formation. *Rol* B is the gene which is responsible for hairy root induction (Georgiev *et al*., 2007) whilst the other *rol* genes synergistically promote the root proliferation (Aoki and Syong, 1999).

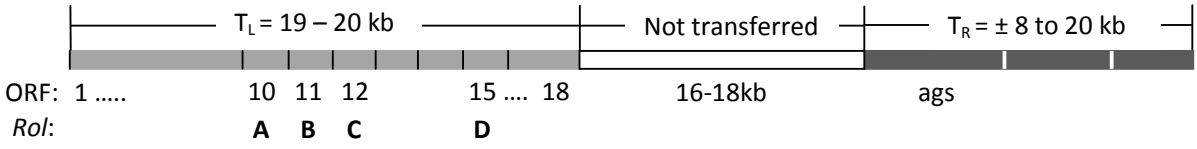


Figure 2.3.1: A segment of the Ri plasmid of *A. rhizogenes* (constituted using information from Tiwari *et al*., 2007)

2) **Virulence (*vir*) region**

The *vir* region is 35 000 bp long and codes for six transcriptional loci: *vir* A, B, C, D, E and G. These genes are located on the bacterial plasmid, and are not transferred to the plant genome (Oksman-Caldenty and Hiltunen, 1996). When activated (through the action of Vir A and Vir G proteins) the gene product of Vir D attaches to the border sequence of T-DNA on the plasmid and causes the production of single stranded T-DNA (T – strand). The T – strand is a linear piece of DNA packaged in protein coded for by *vir* E (Oksman-Caldenty and Hiltunen, 1996) and this T-DNA package is transferred to the plant cell.

2.3.3.3.1 *A. rhizogenes*-mediated transformation

In nature, when a plant cell is wounded it releases phenolic compounds (such as acetosyringone) into the surrounding area. The *A*. *rhizogenes* bacteria can sense and recognize these compounds and they are attracted towards the wounded area by chemotaxis[3] (Oksman-Caldenty and Hiltunen, 1996; Veena and Taylor, 2007). In the bacterial cell the phenolic compounds stimulate the expression of the virulence genes which are responsible for excising; transferring and (possibly) incorporating the T-DNA segment of the Ri plasmid into

[3]Chemotaxis – the ability of a bacterium to sense and migrate towards increasing levels of an attractant chemical or away from a repellent one (http://www.britannica.com/EBchecked/topic/109049/chemotaxis)

the plant genome (Georgiev *et al*., 2007). Single or multiple copies of single strand T-DNA is randomly inserted into the plant genome and the homologous strand is replicated by the plant genome structures. The genes on the T-DNA segment are responsible for the synthesis of hormones (cytokinins and auxins) and opines (Guillon *et al.,* 2006). It is thought that the increased production of auxins upon transformation stimulates the production of hairy roots (Guillon *et al.,* 2006; Georgiev *et al*., 2007).

2.3.3.3.2 Characteristics of hairy root cultures

Hairy roots possess certain characteristics which make their use for research highly beneficial and interesting. Unlike normal roots, hairy roots have plagiotropic (loss of gravity response) growth, a high degree of lateral branching resulting in several meristems (Srivastava and Srivastava, 2007), a profusion of root hairs and an enhanced growth rate similar to that of suspension cultures (Oksman-Caldenty and Hiltunen, 1996; Veena and Taylor, 2007). Due to the stable integration of the *rol* genes, roots are also biochemically and genetically stable (Shanks and Morgan, 1999; Srivastava and Srivastava, 2007). These roots can also be cultivated in a plant growth regulator (PGR) free medium (Veena and Taylor, 2007), which is advantageous as some PGRs (such as 2,4-D) are toxic at high levels and residual contamination in the end product may be highly undesirable (Georgiev *et al*., 2007).

The roots are also able to synthesize high levels of several natural products that are similar to those in intact plants. These are usually specific to that plant species and they can accumulate to higher levels than in the undifferentiated cultures (Srivastava and Srivastava, 2007). Secondary metabolites, which are normally synthesized in the roots and are transported to the aerial organs, can also accumulate in the transgenic hairy roots. Some hairy roots have been found to secrete secondary metabolites into the growth medium, which is highly advantageous for product recovery. Often novel compounds, which do not occur in the untransformed roots or plants, can be produced in hairy roots and these compounds may be highly desirable to the phytochemical and pharmaceutical industries (Shanks and Morgan, 1999; Georgiev *et al*., 2007). However, although secondary metabolite production is controlled by specific genes, their synthesis is also influenced by certain nutritional and environmental factors which mean that their synthesis can be manipulated in the culture system (Giri and Narasu, 2000). The *rol* genes in the Ri plasmid can also be replaced by genes coding for biosynthetic enzymes or regulatory factors for key metabolic pathways. Since whole plants can be regenerated from these roots, this system can be used to produce

transgenic plants which can be used to study the synthesis of important metabolites (Oksman-Caldenty and Hiltunen, 1996).

2.3.3.3.3 Hairy roots as a scientific tool

Hairy roots are scientifically valuable as they can be used to study secondary metabolism, gene function and its regulation; and to investigate the root biology of plants (Veena and Taylor, 2007). Hairy roots can also be used to alter secondary metabolism and develop *de novo* metabolic pathways by genetic engineering through incorporating the necessary genes for the enzymes for the particular pathway (Guillon *et al*., 2006). Attempts are also being made to use this system for the production of pharmaceutically important proteins, enzymes, foreign proteins and ribosome-inactivating proteins.

During this study co-workers and I were interested in using *in vitro* cultivated shoots and hairy root cultures to study the effect of stress on the physiology and metabolism of *S. frutescens* plants. However, since no information was available on the secondary metabolites, the synthesis of the four primary metabolites (GABA, asparagine, canavanine and arginine) was used as a marker to investigate how stress affects their metabolism. Three stresses were investigated and they were selected by looking at the natural environment in which these plants grow in the Western Cape. As these plants are found near the coastal regions it was predicted that they experience limited nutrient (nitrogen and phosphate) availability, a low water supply during certain times of the year as well as high salt deposits in the soil. These three stresses, which represent nutrient deficiency, drought and salinity (respectively), were induced *in vitro* by the manipulation of the cultivation medium. For the first stress (mineral deficiency) the effect of nitrogen availability was investigated as this macronutrient is not only important for the synthesis of the four metabolites, but it is also critical for the growth of plants.

2.4 NITROGEN STRESS

Due to the importance of nitrogen the impact of nitrogen fertilization has been investigated on several medicinal and aromatic plants (MAPs) such as geranium (Araya *et al*., 2006) and palmorosa (Rajeswara Rao, 2001). Nitrogen as a macronutrient is used for the synthesis of several cell components such as amino acids, proteins, enzymes and nucleic acids (Taiz and Zeiger, 2002). Nitrogen is important for plant growth and a nitrogen deficiency results in chlorosis (leaf yellowing) in the older leaves. The older leaves are usually affected first since the nitrogen is exported to the younger leaves during stress. However, the type of response to nitrogen fertilization can vary depending on the environmental conditions, genotype, cultivar, type of fertilizer (ammonium nitrate, ammonium sulphate and urea) applied and the time during

the plant's growth cycle during which it is applied (Carrubba, 2008). Nitrogen metabolism is one of the primary metabolic pathways in plants and it is connected to other primary metabolic pathways as they often share common intermediates.

2.4.1 A brief overview of some of the primary metabolic pathways in plants

The chloroplast is the location where the light reactions of photosynthesis occur. Inside the chloroplast is an internal membrane system known as thylakoids which contain the chlorophyll and this is the site where the light reactions occur. When these thylakoid membranes are stacked on top of each other they form a granum. Those membranes which are not stacked but are exposed to the stroma are known as stroma lamellae. The chloroplast is surrounded by a double lipid layer known as the envelope (Figure 2.4.1). The carbon reduction reactions which are catalyzed by water-soluble enzymes, occur in the stroma which is the region outside the thylakoids (Taiz and Zeiger, 2002).

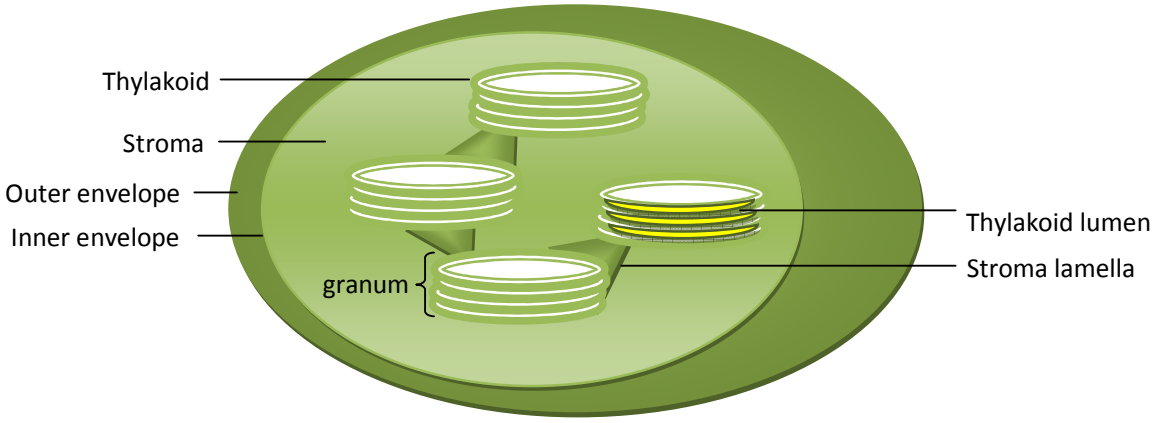


Figure 2.4.1: The chloroplast structure (modified from Taiz and Zeiger, 2002)

2.4.1.1 Photosynthesis

Photosynthesis is the process during which plants use water and CO_2 to convert light energy into the chemical energy of organic molecules (carbohydrates) (Taiz and Zeiger, 2002). In most plants, light photons are received by photosystem I (PSI) and photosystem II (PSII) and this results in an electron transport cascade which generates ATP and NADPH. During this process PSII oxidizes water to O_2 and H^+ in the thylakoid lumen (Figure 2.4.2). The electrons from water are then transferred via plastoquinone to cytochrome b_6f which transfers the electrons to PSI using plastocyanin (Pc). At the same time cytochrome b_6f also transports extra protons (H^+) from the stroma to the lumen of the thylakoid lamella. Photosystem I reduces $NADP^+$ to NADPH in the stroma via ferredoxin (Fd) and the flavoprotein-NADP

reductase (FNR). The transfer of protons to the lumen generates a proton gradient and this is used by ATP synthase to synthesize ATP in the stroma (Taiz and Zeiger, 2002). ATP, CO_2 and NADPH are then required for the light independent reactions which follows.

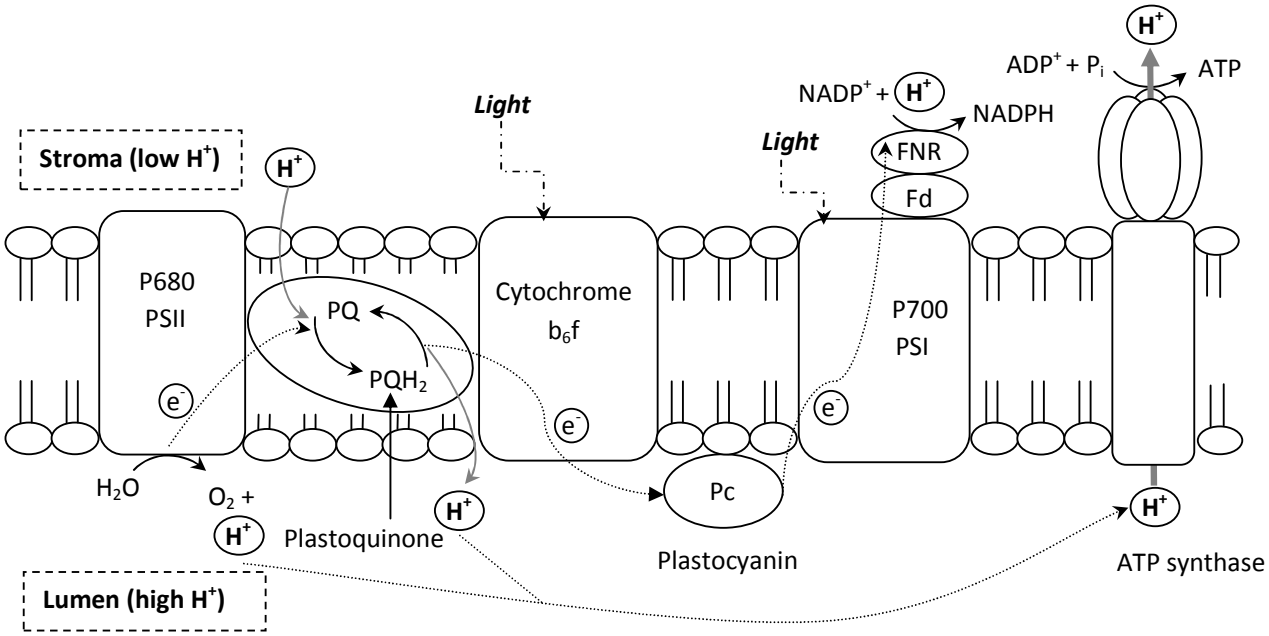


Figure 2.4.2: The light reactions of photosynthesis (Taiz and Zeiger, 2002)

2.4.1.2 The Calvin cycle in the chloroplast

The Calvin cycle involves three steps, firstly the enzyme Ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) uses CO_2 to carboxylate ribulose-1,5-bisphosphate resulting in the production of 3-phosphoglycerate (Figure 2.4.3). The second step involves the reduction of 3-phosphoglycerate to triose phosphate and finally the ribulose-1,5-bisphosphate is regenerated (Taiz and Zeiger, 2002). The triose phosphates which are produced can be used to synthesize phosphoenolpyruvate (PEP) in the cytosol. This reaction product, PEP, can be decarboxylated to produce oxaloacetate via PEP carboxylase. Oxaloacetate in turn can be converted to malate by the enzyme malate dehydrogenase using the reducing equivalents of NADH (Taiz and Zeiger, 2002). This malate is then transported to the mitochondrion where it can be used to replace the tricarboxylic acid cycle (TCA) intermediates (Taiz and Zeiger, 2002). Alternatively the triose phosphates can be used for the synthesis of starch in the chloroplast or it can be exported to the cytosol where it is converted to sucrose (Taiz and Zeiger, 2002).

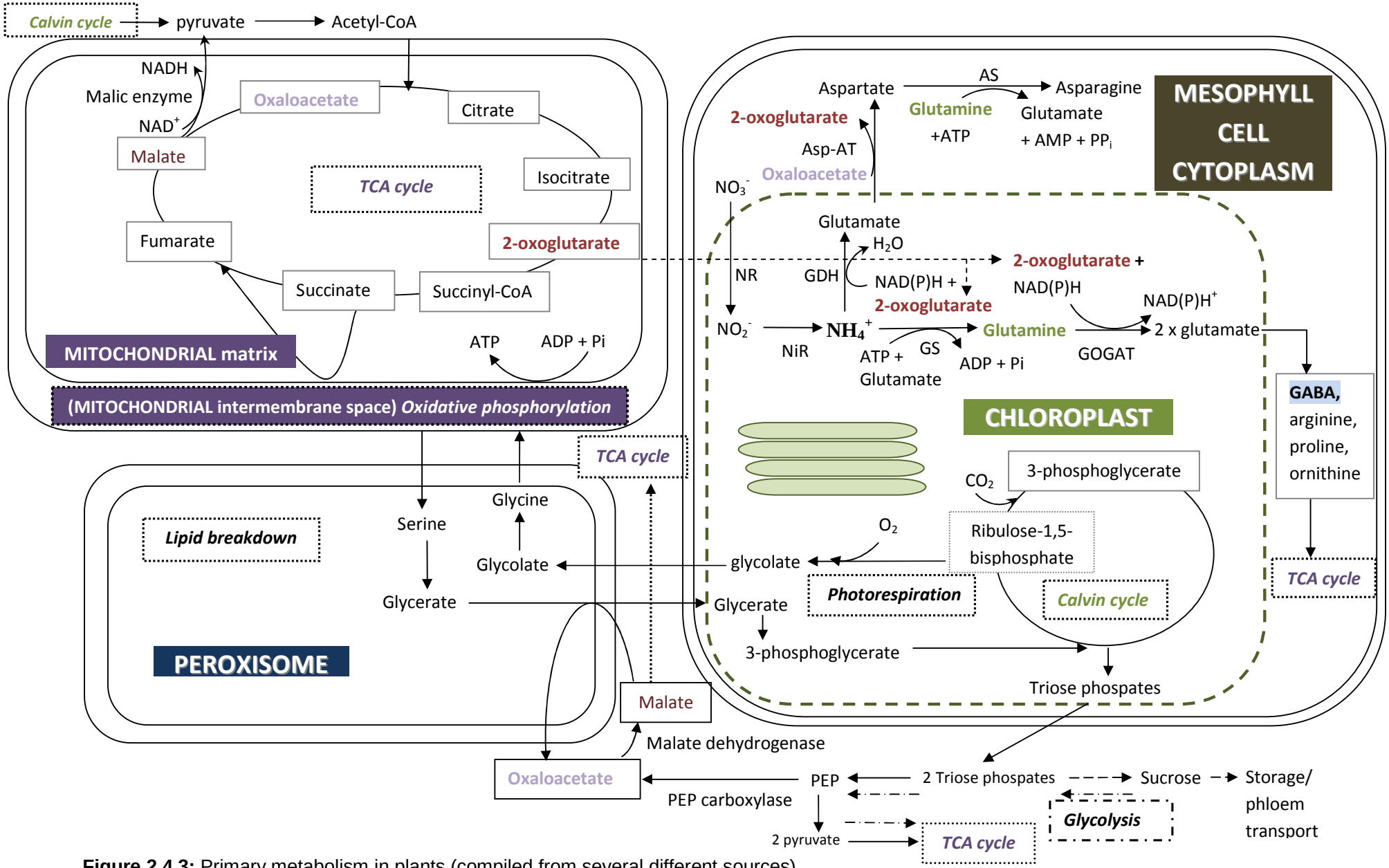


Figure 2.4.3: Primary metabolism in plants (compiled from several different sources)

2.4.1.3 Glycolysis in the cytoplasm

Glycolysis involves the conversion of carbohydrates to hexose phosphates which are split into two molecules of triose phosphate (Figure 2.4.3). The triose phosphates are then oxidized and rearranged to produce two pyruvate molecules which can be used for the TCA cycle (Taiz and Zeiger, 2002). During this process a few ATP and NADH molecules are produced.

2.4.1.4 Tricarboxylic acid (TCA) cycle in the mitochondrial matrix

The pyruvate generated in the cytosol during glycolysis or from triose phosphates derived from the Calvin cycle (Figure 2.4.3), is transported to the mitochondrial matrix where it is decarboxylated by the enzyme pyruvate dehydrogenase resulting in the production of acetyl-CoA (Figure 2.4.3). Acetyl-CoA enters the TCA cycle by reacting with oxaloacetate (OAA) catalyzed by the enzyme citrate synthase resulting in the production of citrate. The oxidation of one molecule of pyruvate results in the release of three CO_2 molecules and the free energy is used to synthesize a total of four NADH, one $FADH_2$ and one ATP molecule (Taiz and Zeiger, 2002). The pyruvate can be replenished by using malate (one of the intermediate products of TCA) as a substrate. Malate can be converted to pyruvate via the malic enzyme which also results in the production of NADH and the release of CO_2 (Taiz and Zeiger, 2002).

2.4.1.5 Electron transport / oxidative phosphorylation in the mitochondrial membrane

An energy carrying compound (ATP) is used by cells to drive metabolic processes. The chemical energy stored in the reducing equivalents (NADH and $FADH_2$) during the reactions in the TCA cycle has to be converted to ATP to be useful (Taiz and Zeiger, 2002). This is achieved by reactions of the oxidative phosphorylation pathway which occurs in the inner mitochondrial membrane space (Figure 2.4.4). The reducing equivalents (NADH and $FADH_2$) produced during sucrose oxidation (glycolysis) and the TCA cycle are reoxidized by complex I (NADH dehydrogenase enzymes) which is attached to the inner membrane of the mitochondrion. Secondly, succinate from the TCA cycle is oxidized by the enzyme (succinate dehydrogenase) of complex II which also forms a part of this chain and results in the production of fumarate. The reducing equivalents from succinate oxidation are transferred via $FADH_2$ to the electron pool in the inner membrane space. Complex III is involved with the pumping of protons (H^+) out of the mitochondrion as well as the transfer of electrons. Complex IV transfers the electrons to O_2 to produce water and it also pumps H^+ out of the mitochondrion. Another complex known as the alternative oxidase (AOX) can also use the electrons to reduce oxygen and produce water when complex IV is inactivated. Thus, during

oxidative phosporylation the electrons are transported from the reducing equivalents (NADH, $FADH_2$) to oxygen (O_2) resulting in the production of water (H_2O) and NAD^+. Some of the energy is used to create a proton gradient across the inner mitochondrial membrane (by pumping H^+ across the membrane) which can be used for the synthesis of ATP via complex V in the mitochondrial matrix.

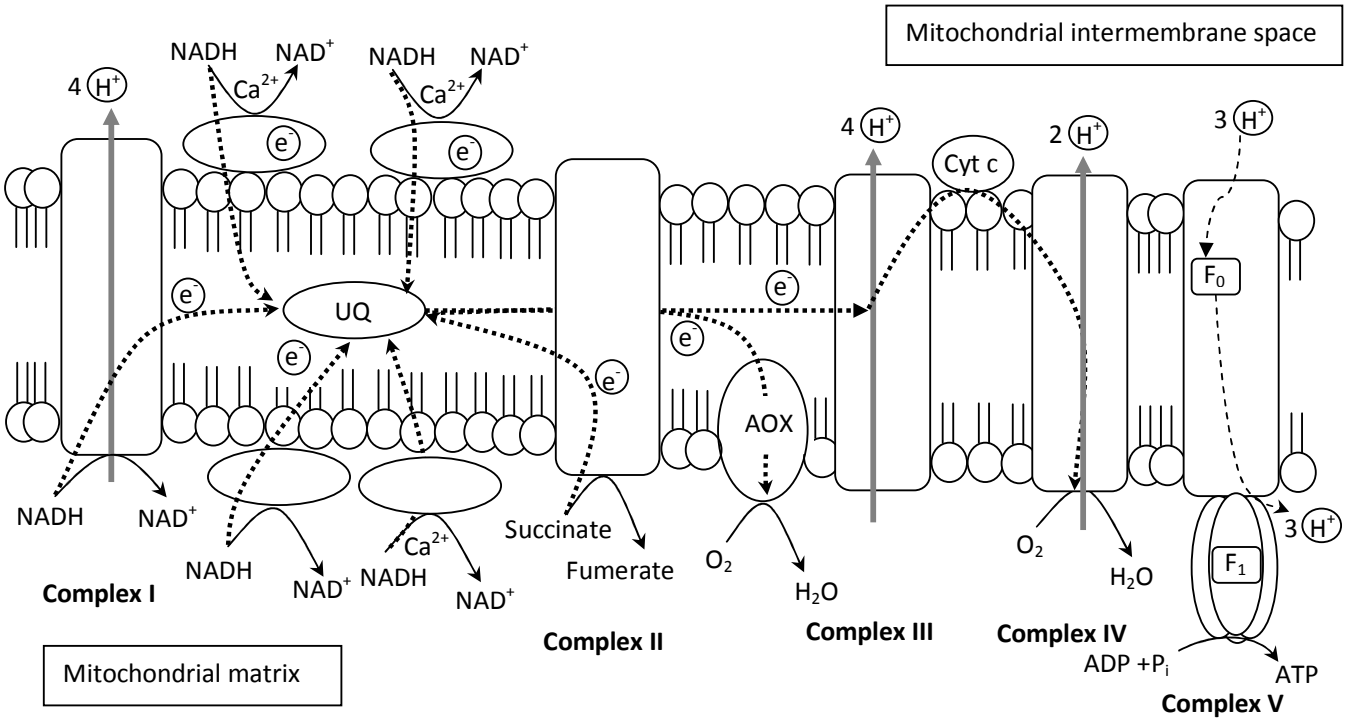


Figure 2.4.4: Electron transport chain in the mitochondrial membrane (Taiz and Zeiger, 2002)

2.4.1.6 Photorespiration links the chloroplast, peroxisome and mitochondrion

In plants the enzyme, Rubisco, which catalyzes the binding of CO_2 with Ribulose-1,5-bisphosphate (carboxylation), can also oxygenate this substrate with O_2 but this reaction results in a loss of carbon (Taiz and Zeiger, 2002). However, this carbon can be recovered by photorespiration which cycles the products through the metabolic pathways in the peroxisome and mitochondrion and finally back to the chloroplast where it is used by the Calvin cycle (Figure 2.4.3) (Taiz and Zeiger, 2002). During this process CO_2, NADH and NH_4^+ are produced as products (Taiz and Zeiger, 2002).

2.4.2 Nitrogen assimilation

Nitrogen assimilation, as indicated in Figure 2.4.3, is connected to the other metabolic pathways such as the TCA cycle as the products of the TCA pathways (such as 2-oxoglutarate) are used for amino acids synthesis. This is the reason why during a nitrogen deficiency, excess carbohydrates accumulate as they are normally used for the synthesis of amino acids and other nitrogen compounds (Taiz and Zeiger, 2002). During a nitrogen deficiency the excess carbohydrates can result in enhanced synthesis of anthocyanins which is observed as a purple colouration of the leaves (Taiz and Zeiger, 2002).

During nitrogen assimilation, nitrate (NO_3^-) in the soil is absorbed by the roots (also transported to the leaves) and converted to nitrite (NO_2^-) by the enzyme nitrate reductase (NR) (Figure 2.4.3). Nitrate reductase appears to be a rate limiting enzyme during the acquisition of nitrogen (Flores *et al*., 2004). The activity and expression of the genes for this enzyme are controlled by NO_3^- availability as well as by light and/or sugar levels (Flores *et al*., 2004).

The enzyme nitrite reductase (NiR) converts the NO_2^- to ammonium (NH_4^+) (Figure 2.4.3). Glutamine synthetase (GS) combines NH_4^+ with the amino acid glutamate using energy from ATP to form glutamine. Glutamate synthase (GOGAT) can convert glutamine to two glutamate molecules using 2-oxoglutarate (TCA intermediate) and energy from NADH. Alternatively glutamate dehydrogenase (GDH) can reversibly convert NH_4^+ and 2-oxoglutarate using energy from NADPH to form glutamate and water.

Therefore it is to be expected that the availability of nitrogen will influence the synthesis of four key metabolites (asparagine, arginine, canavanine, GABA) studied in this thesis as they are all synthesized in the nitrogen metabolic pathway in plants. The discussion of these metabolites of interest follows below (Sections 2.4.2.1 to 2.4.2.4).

2.4.2.1 Asparagine synthesis

During asparagine synthesis (Figure 2.4.3), glutamate and oxaloacetate (TCA intermediate) are converted to aspartate and 2-oxoglutarate by the enzyme aspartate aminotransferase (Asp-AT) (Figure 2.4.3). Asparagine synthetase (AS) combines the aspartate with glutamine using the energy from ATP to produce asparagine and glutamate as products (Taiz and Zeiger, 2002).

Asparagine synthetase is located in the cytosol of leave and root cells as well as in nitrogen-fixing nodules. High light and carbohydrate levels inhibit the expression of the genes coding

for this enzyme as well as the activity of AS (Taiz and Zeiger, 2002). In plants asparagine is one of the forms in which nitrogen is transported throughout the plant (Taiz and Zeiger, 2002).

2.4.2.2 Arginine synthesis

Arginine is an amino acid with a positively charged polar side chain (Voet and Voet, 2004). Arginine has a high nitrogen: carbon ratio (4: 6) and along with asparagine it acts as a major nitrogen storage compound in plants (Forde and Lea, 2007). Few studies have focused on elucidating arginine synthesis in plants. Therefore, the enzymes, their metabolic control and subcellular compartmentation are still poorly understood (Slocum, 2005). During arginine synthesis (Figure 2.4.5), glutamate is converted to ornithine via a series of acetylated intermediates (Slocum, 2005). Ornithine combines with carbamoyl phosphate and is converted to citrulline by ornithine transcarbamoylase (OCT) (Slocum, 2005). Citrulline and aspartate are reversibly converted to argininosuccinate (a very unstable intermediate) by the enzyme argininosuccinate synthase (ASS) and this is the rate-limiting enzyme for the synthesis of arginine (Hwang *et al*., 1996a).

Argininosuccinate synthase is inhibited by high levels of aspartate and ATP. The direction of this metabolic pathway (forward or reverse) depends on the cellular pH (Hwang *et al*., 1996a). The forward reaction (synthesis of argininosuccinate) functions when the pH is between 8 to 9, whilst the reverse reaction (synthesis of citrulline) functions optimally at pH 6 (Hwang *et al*., 1996a). Argininosuccinate is converted to arginine by the enzyme argininosuccinate lyase (ASL). The cycle is completed when arginine is degraded by arginase regenerating ornithine and releasing urea (Nabais *et al*., 2005; Slocum, 2005). The urea can undergo further degradation by urease to produce NH_4^+ and CO_2. Following this, the NH_4^+ can be reassimilated during asparagine or glutamine synthesis (Nabais *et al*., 2005).

Arginine and ornithine are two important substrates for the synthesis of stress related solutes (osmolytes) such as the polyamines (putrescine, spermine and spermidine) (See Section 2.5 for details on water and salt stress) (Forde and Lea, 2007). In humans, arginine is active against viral infections and it assists with nitric oxide synthesis (Sia, 2004).

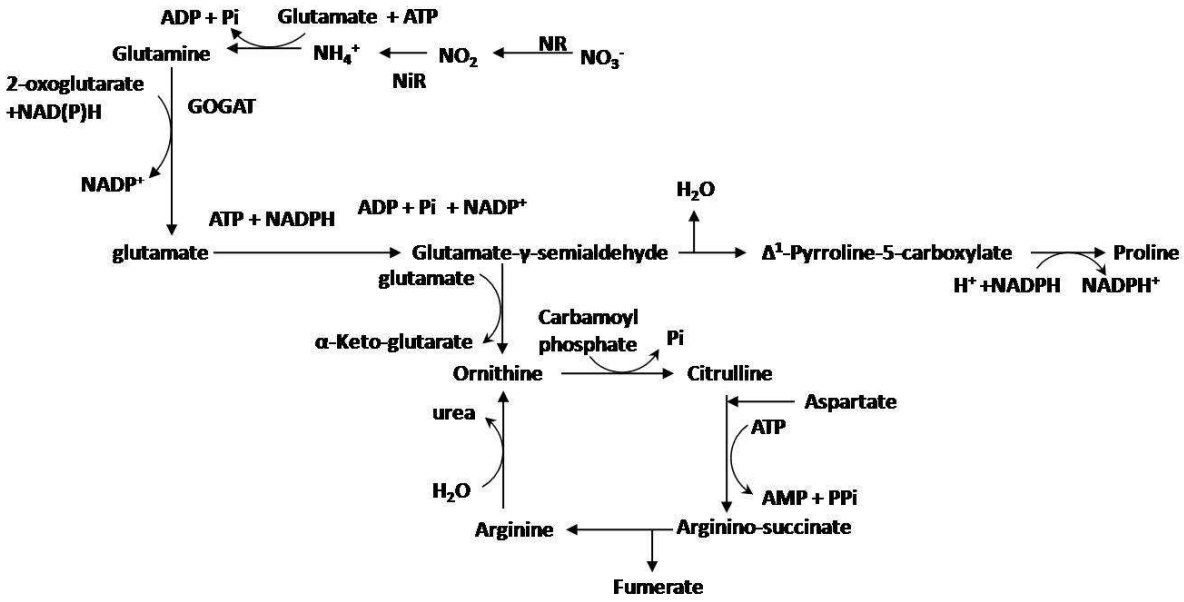


Figure 2.4.5: Metabolic pathway for arginine synthesis (modified from Simon-Sarkadia *et al*., 2007)

2.4.2.3 Canavanine synthesis

Canavanine (Figure 2.4.3) is a non-protein amino acid analogue of arginine (Hwang *et al*., 1996a). Canavanine accumulates in the seeds of many leguminous plants (Bence *et al*., 2003; Van Wyk and Albrecht, 2008). Taxonomists have also used the presence or absence of this compound to distinguish between plants from subfamilies of the family Fabaceae as canavanine is restricted to species of the subfamily *Papilinoideae* (Bence *et al*., 2003; Onyilagha *et al*., 2009). Approximately 1 500 species of 246 genera of legumes contain canavanine (Ekanayake *et al*., 2007). Different plants such as *Canavalia ensiformis*, *Canavalia gladiata*, *Canavalia cathartica* (Sridhar and Seena, 2006), *Colutea arborescens*, *Caragana arborescens*, *Dioclea megacarpe*, *Vicia gigantean*, *Robinia pseudoacacia* and *Wisteria floribunda* (Rosenthal, 2001) store varying levels of canavanine in their seeds, where this compound probably functions as a nitrogen storage molecule (Hwang *et al*., 1996b; Rosenthal and Nkomo, 2000). Canavanine, with its reported anti-tumourigenic properties has also been extracted from *Sutherlandia* seeds (Bell *et al*., 1978; Mosche *et al*., 1998; Tai *et al*., 2004).

Canavanine appears to be synthesized in a similar pathway to the Krebs and Henseleit cycle (also known as the urea cycle), which excretes excess nitrogen in the human body. The biosynthetic pathway of canavanine is indicated in Figure 2.4.6 (Rosenthal, 1982; Hwang *et al*., 1996b).

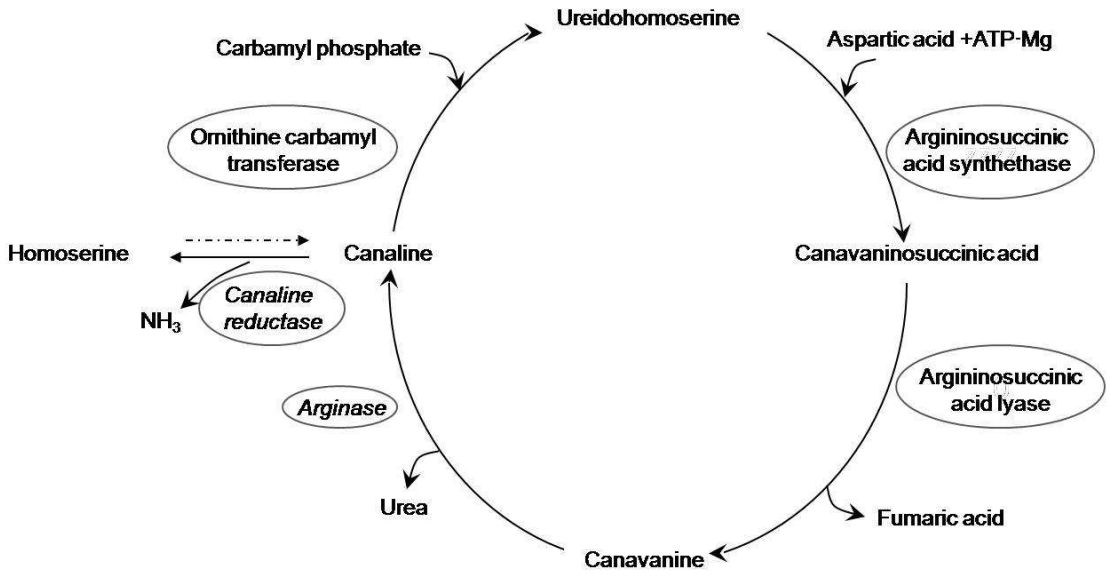


Figure 2.4.6: Metabolic pathway for canavanine synthesis (Rosenthal, 1982; Hwang *et al*., 1996b)

Canavanine synthesis in plants appears to be dependent on the presence of chlorophyll as this metabolite was only detected in the green cells in suspension cultures of *Canavalia lineate* (Hwang *et al*., 1996b), whilst some of the enzymes (argininosuccinic acid synthethase (ASS), argininosuccinic acid lyase (ASL), ornithine carbamyl transferase) involved in canavanine synthesis were also detected at higher levels in green callus than in white callus (Hwang *et al*., 1996a). During canavanine synthesis, ureidohomoserine and aspartic acid are converted to canavaninosuccinic acid by the enzyme ASS. Similarly to arginine synthesis, ASS is reported to be the rate-limiting enzyme in the cycle (Hwang *et al*., 1996a). Canavaninosuccinic acid is converted to canavanine by ASL, resulting in the release of fumaric acid. In *C. lineate,* canavanine was found to accumulate in the vacuoles of the leaves (Hwang *et al*., 1996b). Canavanine is degraded by arginase resulting in the production of canaline and urea. Canaline can be converted to homoserine by canaline reductase with NH_3 as a byproduct or it can be converted back to ureidohomoserine using carbamyl phosphate as a substrate for the enzyme ornithine carbamyl transferase (Hwang *et al*., 1996b). Hwang *et al*. (1996a) concluded that canavanine synthesis is regulated by the coordination of the three enzymes ornithine carbamyltransferase, ASS and ASL.

Canavanine is often reported to be responsible for the reported pharmacological activity in *S. frutescens* (Tai *et al*., 2004; Ojewole, 2008; Stander *et al*., 2009). The properties of canavanine have been studied since the early 1980's and research on canavanine is therefore abundant (Rosenthal, 1982; Hwang *et al*., 1996a; Hwang *et al*., 1996b; Nakajima *et al*., 2001). In humans, canavanine displays anti-bacterial, anti-fungal, anti-viral (Ekanayake *et al*., 2007) and anti-cancer activity. It also appears to inhibit the levels of influenza and retroviruses (Van Wyk and Albrecht, 2008; Van Wyk, 2008). This makes canavanine a valuable pharmaceutical product.

Reports on the anti-cancer properties of canavanine include work by Green and co workers (1983) who indicated that canavanine could enhance the anti-proliferative effects of radiation treatment on human colorectal cancer cells (Bence *et al*., 2003). Bence *et al*. (2003) also concluded that canavanine could potentially act as a radio-sensitization agent against human pancreatic cancer cells. In cancer cells, the canavanyl proteins can disrupt the critical processes required for tumour growth (Bence *et al*., 2003) hence arresting the spread of the cancer. However, the precise mechanism behind the anti-proliferating activity of canavanine remains to be elucidated. As a result, this compound has potential as a novel chemotherapeutic agent. The compound canaline (which is produced when canavanine is degraded) has also been suggested as an anti-cancer drug for pancreatic cancer (Reid *et al*., 2006). Canaline is a vitamin B6 antagonist, inhibiting pyridoxal-phosphate-dependent enzymes and inhibits cell growth in plants (Ekanayake *et al*., 2007). Canaline is also found to be toxic to human peripheral blood mononuclear cells in culture due to its ability to disrupt polyamine biosynthesis (Sia, 2004).

Animal studies have suggested that canavanine could play a role in diabetic retinopathy (one of the main causes of blindness in middle-aged diabetics) by the induction of molecular chaperone proteins[4] (heat shock, glucose-regulated or stress proteins) which ensure that proteins which are misfolded during stress are quickly degraded (Mihály *et al*., 1998; Ekanayake *et al*., 2007). Furthermore, canavanine also inhibits nitric oxide (NO) synthesis in macrophages and polymorphonuclear leukocytes (Ekanayake *et al*., 2007) by inhibiting inducible nitric oxide synthase (iNOS) enzyme and can potentially be used for the treatment of septic shock syndrome (Van Wyk and Albrecht, 2008).

[4] **Molecular chaperones** – proteins which guide other proteins to their final destination in the cell and assist them with reaching and maintaining their correct conformation (Mihály *et al*., 1998)

The mechanism behind canavanine's pharmacological activity appears to be correlated with its structural similarity to arginine. Due to this similarity and a lack in the ability of enzymes (tRNA synthetase enzymes) to distinguish between the two substrates, canavanine can be incorrectly incorporated into proteins in nearly all the enzymes which use arginine as a substrate (Rosenthal, 2001). When canavanine is incorrectly incorporated into proteins, it changes the protein's conformation due to a lack in the capacity to form the crucial ionic interactions; thus disrupting the enzyme/protein's activity (Mattei *et al*., 1992; Bence *et al*., 2003). Canavanine also has an effect at the transcriptional and translational levels due to its anti-metabolic activities (Rosenthal *et al*., 1998; Ekanayake *et al*., 2007).

Since canavanine occurs in several legume species consumed by humans and animals, toxicity testing on canavanine indicated that this compound causes a reduction in feed intake, growth, as well as a significant decrease in the plasma concentration of the basic amino acids (arginine, lysine and histidine) in animals which consumed canavanine-containing legumes (Ekanayake *et al*., 2007). One study involved feeding monkeys with alfalfa seeds containing canavanine and these monkeys proceeded to develop systemic lupus erythematosus (a chronic inflammation caused by an autoimmune disease). Upon re-challenge with 1% canavanine in their diet, the monkeys displayed a relapse of the disease (Akaogi *et al*., 2006). The reason for this autoimmune response is linked to the apoptosis of cells which are phagocytosed in proteosomes, resulting in the production of antigens. However, when canavanine is inserted in the proteins of proteosome enzymes it disrupts the production of antigens causing the immune system to turn on itself (auto-immune disease) (Akaogi *et al*., 2006). However the antagonistic effect of canavanine in the body is only observed when the arginine levels are low (Ekanayake *et al*., 2007).

In plants canavanine, functions to deter insects (Hwang *et al*., 1996b; Rosenthal, 2001), mammals and micro-organisms (bacteria, fungi and viruses; Bass *et al*., 1995) which makes it useful as an herbicide or insecticide. Canavanine also acts as an allelochemical compound suppressing the growth of plant competitors. In 2001, Nakajima and co-workers investigated this allelochemical property of canavanine on rice seedlings. The growth of rice seedlings was inhibited by canavanine, but this effect could be reduced by the concomitant application of arginine, which either indicates that canavanine and arginine compete as substrates for the same enzymes or that the inhibitory effect is due to interference of canavanine with arginine metabolism. Such allelochemicals are often translocated from the site of synthesis to the soil as root exudates.

2.4.2.4 Gamma-aminobutyric acid synthesis

Gamma-aminobutyric acid (GABA) is a four carbon non-protein amino acid (Figure 2.1.9D), which is highly soluble in water (Shelp *et al*., 1999). Gamma-aminobutyric acid is a zwitterion (carries both positive and negative charge) at physiological pH values and it can assume several conformations including a cyclic structure which is similar to proline (Shelp *et al*., 1999). It is synthesized and degraded by a pathway known as the GABA shunt which consists of three enzymes (Figure 2.4.7) (Bartyzel *et al*., 2003). The first step in GABA synthesis involves the irreversible decarboxylation of L-glutamate by cytosolic glutamate decarboxylase (GAD) which releases a CO_2 molecule (Bartyzel *et al*., 2003). Glutamate decarboxylase (GAD), is a cytosolic Ca^{2+}-dependent calmodulin-binding enzyme which functions optimally in an acidic pH and is activated by high levels of L-glutamate and increasing cytosolic H^+ and calcium (Ca^{2+}) levels (Shelp *et al*., 1999; Bartyzel *et al*., 2003; Allan *et al*., 2008). Gamma-aminobutyric acid synthesis from glutamate is enhanced when glutamine synthesis is inhibited, protein synthesis is reduced or protein degradation is enhanced (Shelp *et al*., 1999).

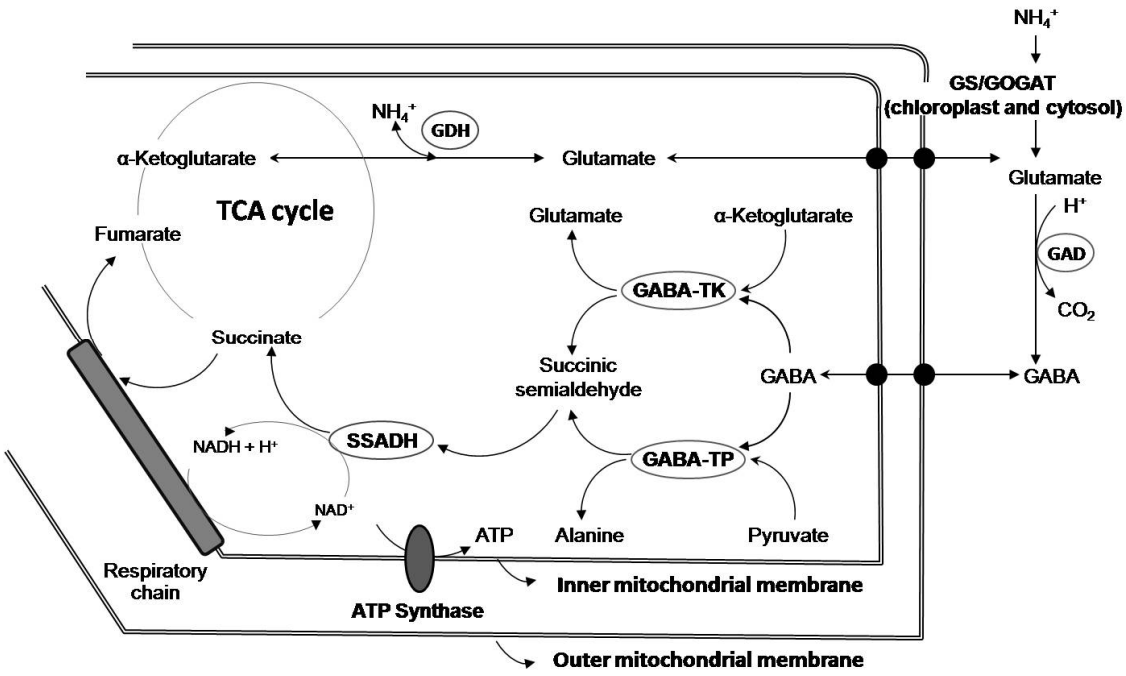


Figure 2.4.7: Metabolic pathway for GABA synthesis (modified from Bouché and Fromm, 2004)

Gamma-aminobutyric acid is transported into the mitochondrion where it is reversibly transaminated by GABA amino-transferase (GABA-T) to succinic semialdehyde using either

pyruvate or ά-ketoglutarate as substrate (Shelp *et al*., 1999). This reaction produces alanine or glutamate respectively depending on the substrate. Succinic semialdehyde dehydrogenase (SSADH) located in the mitochondrion, irreversibly converts succinic semialdehyde to succinate, also producing NADH. The succinate feeds into the TCA cycle and NADH can be used during oxidative phosphorylation for the synthesis of ATP (Bartyzel *et al*., 2003). The regulation of SSADH by ATP indicates that there is tight control of the rate at which products (NADH and succinate) of GABA degradation are shunted to the electron transport chain (Bouché and Fromm, 2004). Succinic semialdehyde dehydrogenase (SSADH) is strongly inhibited by NADH and ATP (Allan *et al*., 2008). However, a balance in the NAD(P)H: $NAD(P)^+$ ratio is important for the efficient functioning of the electron transport chain in the mitochondrion and chloroplast membranes (Allan *et al*., 2008). The SSADH pathway produces less energy than the TCA cycle. Environmental stress decreases the TCA cycle and can cause a reduction in the NAD^+: NADH ratio which limits SSADH activity and the accumulation of succinic semialdehyde can inhibit GABA degradation via the GABA-T shunt (Shelp *et al*., 1999). In plants, glutamate and GABA are also produced during protein storage and mobilization to help recycle arginine-derived nitrogen and carbon (Allan and Shelp, 2006).

In the human body, GABA functions in the mammalian central nervous system by blocking neuro-transmission (Bartyzel *et al*., 2003). Gamma-aminobutyric acid induces a calming effect which could explain its use for relieving anxiety and stress. Furthermore, GABA also appears to inhibit tumour cell migration *in vitro* (Tai *et al*., 2004; Chadwick *et al*., 2007; Xaba, 2007; Van Wyk, 2008; Van Wyk and Albrecht, 2008).

Gamma-aminobutyric acid plays several roles in plants. GABA can act as a nitrogen storage metabolite, it can also manage the carbon flux into the TCA cycle to control the carbon: nitrogen balance in the plant (Bouché and Fromm, 2004; Allen and Shelp, 2006). The compound can also buffer glutamate production and supplies most of the carbon chains of this amino acid as succinate to the TCA cycle (Bartyzel *et al*., 2003; Bouche and Fromm, 2004). Furthermore, GABA can also assist with regulation of the pH as GABA synthesis consumes H^+ ions (Shelp *et al*., 1999; Bartyzel *et al*., 2003; Bouché and Fromm, 2004). Other roles include a potential role in regulation of plant growth, development and defense by acting as a signaling molecule and binding to the appropriate receptors (Bartyzel *et al*., 2003) to elicit signal transduction cascades.

Gamma-aminobutyric acid is also involved in the adaptation of plants to biotic and abiotic stress, however, it does not always accumulate (Bartyzel *et al*., 2003; Bouché and Fromm, 2004). Studies have indicated that Ca^{2+}-signaling is involved during the production of reactive oxygen species (ROS) during stress. Since Ca^{2+} may activate the GABA enzymes, it is suggested that the GABA shunt may play an important role during an oxidative stress condition linked to the production of ROS (Bouché and Fromm, 2004).

2.5 WATER AND SALT STRESS

The other two abiotic stresses investigated during this study involve water (drought) and salt (salinity) stress. Plants exposed to salinity and drought stress both experience problems with the uptake of water. Additionally plants subjected to high levels of salt also suffer from the effects of ion toxicity.

2.5.1 Water limitation

When water becomes limiting or the salt content in the soil increases it causes a reduction in the soil water potential. Water uptake in plants is a passive process and plants can take up water if the water potential of the plant is below the soil water potential (Taiz and Zeiger, 2002). Thus one of the key responses observed in plants during both water and salt stress is the reduction in water potential in the cells (Shibli *et al*., 2003; Ahmad *et al*., 2007). Failure to absorb water can result in a loss of turgor pressure in the plant (Taiz and Zeiger, 2002). Plants have to maintain turgor pressure in the cells as this mechanism is essential for optimal functioning of metabolism, cell expansion, biochemical, physiological and developmental processes (Liu and Van Staden, 2001). However, plants can only decrease the water potential until it reaches the permanent wilting point. At this point the water potential of the cells becomes so low that the plant fails to regain turgor pressure even when water loss through the stomata has stopped. Beyond this point the plant will likely die due to dehydration (Taiz and Zeiger, 2002). The permanent wilting point for plants is usually about -1.5 MPa but it varies with different species (Taiz and Zeiger, 2002).

Plants under salt and water stress exhibit some similarities in the mechanism to lower their water potential during stress. One mechanism involves a reduction in the osmotic potential by increasing the total solute concentration in the cells in a process known as osmotic adjustment (Shibli *et al*., 2003). These solutes can come from newly-synthesized compounds or from the

break down products of proteins (amino acids such as proline) and other compounds (carbohydrates, sugars; polyols), polyamines (putrescine, spermine, spermidine) and quaternary amines (glycine betaine) (Ahmad *et al*., 2007). These solutes prevent dehydration, cellular damage and can function as nitrogen storage compounds, which can be used when the stress has been relieved (Di Martino *et al*., 2003; Ahmad *et al*., 2007).

2.5.2 Salt toxicity

Initially plants subjected to salt stress can take up ions (Na^+ and Cl^-) which assist with the reduction of their osmotic potential. However, these ions are accumulated in the vacuole to prevent damage to the cell. Although plants can tolerate mild salt concentrations in the soil, high levels of salt can result in ion toxicity. Ultimately, these stresses (water deficit and drought) can affect the key metabolic pathways in the cell such as photosynthesis, respiration and nitrogen assimilation.

2.5.3 The effect of water and salt stress on metabolism and photosynthesis

Water plays a key role during photosynthesis as it provides the electrons which are used to transfer the absorbed light energy through the thylakoid membrane of the chloroplast (Taiz and Zeiger, 2002). A reduction in water content decreases the number of electrons available for photosynthesis. This results in a reduction in the production of NADPH and ATP in the chloroplasts (Taiz and Zeiger, 2002). More intense water stress also leads to the inhibition of cell division and cell wall synthesis and protein translation (Taiz and Zeiger, 2002). One of the first responses in plants during drought is closing of the stomata to reduce water loss. However, this response further reduces photosynthesis as less CO_2 enters the leaves (Taiz and Zeiger, 2002).

Plants under salt stress can experience a reduction in the uptake of minerals (nitrogen, phosphorus, potassium, calcium and magnesium), which can change the mineral composition *in vivo*. This can result in specific mineral deficiency symptoms observed (Shibli *et al*., 2003). An example is the increase in chlorosis observed in some stressed plants which is due to the reduction in the uptake of iron with increase in salinity in the medium which reduces iron levels *in situ* (Shibli *et al*., 2003).

Salinity also decreases the NO_3^- uptake by roots, NO_3^- loading in the xylem as well as NO_3^- flux from the roots to the shoots (Flores *et al*., 2004). All species and cultivars are not equally affected by similar stress levels and this is due to a difference in their tolerance or sensitivity to specific types and intensity of stresses (Shibli *et al*., 2003).

2.5.4 Identification of tolerant and sensitive species

Some of the parameters which have been used to investigate the sensitivity of plants to stress include investigation of the rooting response. The root development of tolerant plants is not as susceptible to salt as the sensitive plants. Nutrient uptake is also one of the parameters which can be used to examine tolerance to salinity (Shibli *et al*., 2003). For example tolerant plants have a smaller depletion of potassium ions (K^+) during salt stress than sensitive plants (Shibli *et al*., 2003). Tolerant plants are also able to maintain a higher membrane stability (Shibli *et al*., 2007). No previous information on the tolerance or sensitivity of *S. frutescens* can be found in the literature. Therefore, this study is a pioneer report on the impact of nutritional, water and salinity stress on this particular species.

2.6 PROBLEM STATEMENT

Pharmacological assays on *S. frutescens* conducted by Chinkwo (2005) investigated the ability of *S. frutescens* extracts prepared from three regions in South Africa (Western Cape, Bloemfontein and the Free State) to induce apoptosis in neoplastic cells (cervical carcinoma) and Chinese Hamster Ovary cells (CHO). The extracts prepared from the Western Cape populations exhibited more apoptotic activity than the extracts from the other two regions. This was attributed to certain environmental challenges which induce the synthesis of elevated, different or novel compounds in the plants. Plants from various regions can grow in soil with a different soil composition. They also experience a diversity of environmental growth factors and these factors could contribute to the synthesis of the various secondary metabolites (Chinkwo, 2005). Over the years, plants could have adapted to these adverse conditions on a metabolic and/or physiological manner which would enable them to survive better. It is, however, not clear how these unfavourable conditions affected the metabolism of the plant, which environmental factors would have the largest impact on the phytochemistry or whether some other unknown variables were responsible in the variation in the pharmacological activity of the wild populations.

However no attempt was made by Chinkwo (2005) to identify the secondary metabolite(s) which may have been responsible for the difference in activity. One of the likely reasons for this is a lack of knowledge on all the secondary metabolites which are synthesized by *S. frutescens*. The other reason is that several of the research conducted on the biological activity of *Sutherlandia* identified metabolites (which are synthesized in the primary metabolic pathways) such as the amino acids (asparagine and arginine) and the non-amino acids (GABA and canavanine) as being important for the noted pharmacological actions of *S. frutescens*. It therefore remains unclear whether the health-promoting effect of *Sutherlandia* is due to the popularly reported metabolites; or an unidentified compound(s) or whether it is due to the synergistic effect of several metabolites (primary and secondary). Currently only the quantities of five compounds (GABA, asparagine, canavanine, arginine and pinitol) are routinely monitored during the production of *Sutherlandia* herbal products (personal communication with Dr M Stander of the Central Analytical Facility, Stellenbosch University). For this reason, these metabolites (canavanine, arginine, asparagine and GABA) were monitored in this study and reported on later in this thesis.

2.7 AIMS AND OBJECTIVES

The commercial demand for *Sutherlandia* is on the increase. This is precipated by current work on this species to validate pharmacological claims. Recent reports on the immunomodulatory effects of *Sutherlandia* extracts and this effect on regulating glucose levels in the blood have led to a greater number of phytopharmaceutical products. It is difficult to envisage the ongoing demand for *Sutherlandia* and its popularity is thus predicted to increase. This was one of the motivating factors for using a biotechnological approach to study this species and this would benefit future aims of conservation should these plants face threats. *Sutherlandia* often grows along coastal areas and in the Western Cape many of these are now becoming urban developments which may lead to a loss of wild populations and habitat. A better understanding of the propagation schemes and agricultural requirements for medicinal plants is needed to assist with successful domestication. Currently, little is known about *Sutherlandia* as a cultivated crop. This novel study was initiated in order to address some of these concerns.

The aims and objectives of this study were to establish *in vitro Sutherlandia* shoot and hairy root cultures to meet the potential commercial demand for these plants and its metabolites. These cultures also provided a biotechnological tool to study the effects of three abiotic

stresses (nitrogen availability, water and salt stress) on the synthesis of arginine, GABA, canavanine and asparagine. Additionally, the impact of stress on the growth and development of *S. frutescens* was monitored. Finally, *in vitro* organ cultures were compared to wild plants of *Sutherlandia frutescens* in an attempt to understand the differences in secondary metabolism using various chromatographic techniques available. This work also included anti-bacterial analysis.

Currently, as far as can be ascertained, no reports in the literature on the micropropagation of *S. frutescens* or the induction of hairy roots with *A. rhizogenes* are available. I report here for the first time on the generation of plants in tissue culture using a phytohormone-free system for multiplication and successful *Agrobacterium*-mediated transformation. This work will form an important basis for future research on the phytochemistry of *S. frutescens* and will establish a system for investigation of the impact of various stresses (biotic and abiotic) on this particular species.

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CHAPTER 3:

Micropropagation of *S. frutescens*

3.1 INTRODUCTION

3.1.1 Commercial cultivation of medicinal plants

Commercial cultivation of medicinal plants can be problematic due to a lack of seed stock for the medicinal plants and a lack of knowledge on their germination (Jäger and Van Staden, 2000). Mass propagation of selected medicinal plants can be achieved by micropropagation. However, information is still lacking on the proper *ex vitro* cultivation (soil, fertilizer, shading, planting to harvest time, yield, shelf life) of these plants (Jäger and Van Staden, 2000). In the future it will be an important requirement to chemically fingerprint the cultivated plants and to conduct pharmacological assays to prove that cultivated plants or lines remain active (Jäger and Van Staden, 2000).

3.1.2 Advantages of the micropropagation of medicinal plants

Micropropagation provides several advantages such as a) *in vitro*-derived 'seedlings' may be utilized to initiate medicinal gardens, sold to pharmaceutical companies and/or ethnobotanical markets; b) this technique has a low production costs; c) there is convenience in handling, storage, shipping and d) limited quarantine restrictions (Mithila *et al*., 2001). These micropropagated plants often produce similar compound profiles to those found in the native plants and can therefore be used as an alternative source of these compounds (Kolewe *et al*., 2008). Whole plants can be regenerated from protoplasts[5], pollen, cryopreserved and cell suspension cultures. The use of micropropagation can therefore contribute to conserving valuable plant diversity and somatic embryogenesis can help to produce breeding material from wild populations (Canter *et al*., 2005). The result is the mass production of plant material, which can be used for selection or genetic engineering (Canter *et al*., 2005). Somaclonal variation, the result of genetic disturbances in plants cultivated in tissue culture, can be very beneficial for the plant breeder as it contributes to the available variation in the plants (Canter *et al*., 2005).

[5] Protoplasts – plant, bacterial or fungal cells of which the cell wall was removed either through mechanical or enzymatic methods (http://www.biology-online.org/dictionary/Protoplast)

3.1.3 Aims of the chapter

The aim of this chapter was to investigate the factors that limit seed germination and to establish a protocol for *in vitro* germination of sterile seeds of *S. frutescens*. The germinated seeds were used to multiply *Sutherlandia* shoots *in vitro,* which was important to develop a stock culture for further experiments. *In vitro* rooting was not intensively investigated as shoots were found to root spontaneously. A few rooted plants were transferred to the glasshouse in a preliminary attempt to acclimatize plants. Although different plant growth regulators (BA, kinetin, NAA and IAA) for shoot multiplication and elongation were investigated for purposes of this thesis, this part of the study has not been included in this chapter as plantlet production was prolific without inclusion of PGRs using nodal explants. This chapter thus reports on a continuous culture system which was found to be optimal for plantlet regeneration as it was easy to establish and resulted in expedient proliferation of healthy *Sutherlandia frutescens* shoots in a PGR-free system. Plant stock cultures facilitated the manipulation of environmental propagation conditions *in vitro*.

3.2 MATERIALS AND METHODS

3.2.1 *In vitro* seed germination

Seeds were purchased from Silverhill seeds (Kenilworth, South Africa) and kept in a dark, dry place until use. *Sutherlandia frutescens* seeds are collected from populations within the Western Cape region by Rod and Rachel Saunders (http://www.silverhillseeds.co.za/collecting.asp). Seeds were not kept for periods longer than three months prior to experimentation. The seeds were firstly incubated in the presence of light or in the dark (control) to determine the requirement of light for germination. This was followed by other treatments which include soaking seeds overnight in water and using chemical or physical scarification or fire treatment to remove the seed coat, prior to light incubation. Untreated seeds were used as the control for this experiment.

3.2.1.1 Decontamination of seeds and investigation of the requirement of light for germination

Fifty seeds were decontaminated by a 1 min immersion in absolute ethanol, followed by a 20 min wash in a commercial bleach solution (3.5% (v/v) NaOHCl) prior to three wash steps for 10 min each in sterile distilled water (dH_2O). After the wash steps the seeds were blotted dry on sterile filter paper (Whatman® no.10) and transferred to medium with a quarter strength of

the macro- and micronutrients described by Murashige and Skoog (1962) (Table A1; Appendix), referred to here as ¼-strength MS. The medium was solidified using 1% (w/v) Agar (Biolab, Gauteng, South Africa) and the pH adjusted to 5.8 using 1 M NaOH or 1 M HCl prior to autoclaving. All the media used in this study were autoclaved at 103 kPa (1.1 kg cm^{-2}) and 121˚C for 20 minutes and transfer of all plant material was conducted under laminar flow conditions. The cooled germination medium was poured into Petri dishes (100 mm x 20 mm, Corning®, USA). Five seeds were placed on a Petri dish which was sealed with a 1.5 cm strip of Parafilm 'M'® (American National Can™, USA). Five Petri dishes were placed in the light growth chamber under an illumination of 50 µmol m^{-1} s^{-2} photosynthetic photon flux density (PPFD) and a 16 hour light and 8 hour dark cycle. The light was provided by 'cool-white' fluorescent tubes (L75W/20XOsram, USA, code number F96712). The temperature was at 24˚C ± 2˚C for all seed lots. The remaining five Petri dishes were placed in the dark growth chamber. The number of seeds that germinated daily per Petri dish was scored for 21 days. A seed was considered to be germinated when the radicle (1 mm) protruded from the seed capsule.

3.2.1.2 Pre-germination treatments

Several treatments were applied to seeds prior to incubation in the light growth chamber: a) seeds were chemically scarified by incubating 25 seeds in 10 ml concentrated sulphuric acid (H_2SO_4) for 15 minutes. This acid scarification was repeated twice. The acid was removed and the seeds were washed three times for 10 min each in dH_2O; b) seeds were physically scarified by gently rubbing the seeds on fine sandpaper for 5 seconds; c) the third treatment involved soaking seeds overnight in cold dH_2O; or d) the seeds were briefly dipped in absolute ethanol (EtOH) prior to setting them on fire with the flame of the Bunsen burner. After treatment the seeds were decontaminated as described above. Five seeds were placed on a Petri dish and these were incubated in the light growth chamber and germination was monitored as described previously.

3.2.2 Continuous culture

The hypocotyls and cotyledons (explants) of germinated seeds were used test the effect of different plant growth regulators (PGR) on the induction of callus, roots or shoots. This involved cultivation of the explants on several different plant PGR combinations using BA, NAA, Kinetin and IAA (results not included in this manuscript). However those seedlings (with the primary root removed) which were transferred to PGR-free medium, elongated best over

the 28 days. Therefore the most suitable medium for plantlet regeneration was free of PGRs. All other combinations were thus disregarded for use in further experiments. Continuous culture was thus performed by growing explants in full strength Murashige and Skoog medium (1962); solidified with 0.8% (w/v) agar (pH 5.8) containing 3% (w/v) sucrose and 0.1 g L^{-1} myo-inositol. During subculture, the shoot was sectioned into the apex and (1-2 cm) nodal segments (containing an axillary bud) and these were routinely transferred onto new medium every 28 days. Shoots were left to multiply and elongate in glass vessels (90 x 50 mm) prior to subculture. All shoot cultures were kept in the 18/6h light-dark conditions. These cultures provided a stock of microplants for subsequent experimentation.

3.2.3 *In vitro* rooting

The *in vitro* shoots spontaneously produced roots in the PGR-free medium. The shoots (2 cm) were transferred to a glass vessel (90 x 50 mm) containing 25 ml full strength MS medium supplemented with 3% (w/v) sucrose, 0.1 g L^{-1} myo-inositol, pH 6.0, solidified with 0.8% (w/v) agar. In total, 45 *in vitro* shoots rooted successfully with this protocol. The physiological parameters (length of the shoot, length of the longest root and the number of roots) were determined after 28 days.

3.2.4 Acclimatization in the glasshouse

During *in vitro* rooting, the glass vessels containing plants for acclimatization were closed with a vented lid. Vented lids with a 10 mm hole covered with a 0.22 µm polypropylene membrane (Magenta® B-cap, Sigma), were used. After a month the rooted shoot was carefully removed from the agar medium and the roots were gently washed with water to remove any excess agar medium. Residual agar-medium can result in microbial infections *post vitrum* and this hinders acclimatization. The rooted shoots were transferred to glass vessels containing an autoclaved vermiculite: compost (1: 1; v/v) mixture. The vented lids were essential for this experiment as it allows for more effective gaseous exchange between the *in vitro* environment and the out of culture atmosphere. Vented lids therefore aid with acclimatization as plants are less hyperhydric and this is related to the lowered relative humidity (Pierek, 1987) and allows for transpiration (Cassells and Walsh, 1994; Offord and Tyler, 2009). These plants were placed in a thermostatically-controlled glasshouse (Stellenbosch University, South Africa) with natural sunlight in March 2009 (Southern Hemisphere). The culture vessels containing the plantlets were exposed to a PPFD range of 540 – 800 µmol m^{-2} s^{-1} (midday irradiance). The glasshouse thermostat was set to regulate the minimum temperature at 15˚C and the

maximum temperature at 25˚C. During the acclimatization period the glasshouse conditions were maintained. The plantlets were watered every third day with $^{1}/_{10}$th strength MS medium (lacking sucrose and myo-inositol). After one week the lids of the glass vessels were gradually loosened over the course of two weeks to allow plants to acclimatize and towards the end of the acclimatization period they were completely removed.

3.2.5 Data collection and statistical analysis

Fifteen seeds (five per Petri dish) were used per treatment and this experiment was repeated three times. The number of germinated seeds per Petri dish for each treatment was noted daily for 21 days. The percentage data for the seeds germinating for each treatment were arcsine transformed prior to analysis using the Kruskal-Wallis test. The normality of the data was determined using the Shapiro-Wilk's W test prior to further analysis to assess the normal distribution of the data. All data were analyzed with the use of Statistica Release 8 (Statsoft Inc. 2007). The differences between means reaching a minimal confidence level of 95% were considered as being statistically significant.

3.3 RESULTS AND DISCUSSION

3.3.1 Germination of seeds

Previously Shaik *et al*. (2008) studied the *in vitro* germination of *Sutherlandia frutescens* seeds. Different pre-sowing treatments were investigated which included 1) mechanical scarification using sand paper; 2) physical scarification by soaking seeds in water at ambient temperature (25˚C) for 24 h or in hot water (80˚C) for 15 and 30 min; 3) chemical scarification was investigated by soaking seeds in 97% H_2SO_4 or 4) soaking mechanically scarified seeds in potassium nitrate (KNO_3). The final treatment 5) involved soaking seeds in Gibberillic acid (GA_3). Of all the treatments mechanical scarification was best for inducing germination (Shaik *et al*., 2008).

During my study, I first investigated the requirements of light for germination (Table 3.4.1). The seeds that were placed in the dark did not germinate effectively (29.2%) whereas germination was slightly improved in the light (35.1%; Table 3.4.1). However this difference was not statistically significant. Even so, germination rates were still considered as low as they were below 50%. This result further indicated that dormancy in *S. frutescens* is not solely under a physiological control. The dormancy-breaking mechanism of light is linked to phytochrome function, which in turn activates biochemical and genetic changes which

eventually lead to increased GA_3 inside the seed. This GA_3 is essential for activating α-amylases and other hydrolytic enzymes in the seed resulting in the degradation of stored resources (starch) as well as the endosperm (Taiz and Zeiger, 2002). The starch degradation products are used as a carbon source and the softer endosperm allows for the radicle to push through it until the seed has germinated (Taiz and Zeiger, 2002).

Table 3.3.1: The germination of *S. frutescens* seeds in the light

Treatment	Percentage germination (%)
Control (dark)	29.2a
Light	35.1a

Different letters indicate statistical significance at the 95% confidence level.

The first seeds germinated (Figure 3.4.1A) after one day for all treatments investigated. Presoaking seeds in cold water was not an effective treatment as it only resulted in 25.7% of the seeds germinating (Table 3.4.2). Increased temperature may be a requirement for this treatment (seed soaking) to work more effectively as evidenced by Shaik *et al*. (2009). The higher temperature is more effective to soften the hard seed coat which may prohibit imbibition and subsequent germination. Chemical scarification of seeds with sulphuric acid resulted in 86.3% of the seeds germinating. This result suggests that the seeds might have been sensitive to the time and concentration of acid exposure as Shaik *et al*. (2009) achieved a higher germination frequency (97.5%) using chemical scarification. The reduced germination frequency can possibly be attributed to the repeated exposure to concentrated sulphuric acid which could have damaged the embryos. Previously Cruz *et al*. (2007) reported that the duration of exposure to an acid is important and this treatment should be optimized for each new species.

The treatment, flaming of the seeds, also resulted in a high germination frequency (91.9%). However, this result was not significantly different from the chemically and physically scarified seed germination. Sugii (2003) suggested the use of this technique to germinate Fabaceae seeds. Flaming is effective as it can weaken the hilum which allows the uptake of water. The heat of the flame is also effective to decontaminate the seeds for *in vitro* cultivation (Sugii, 2003). The best treatment for germination was physical scarification of seeds which resulted in 98.6% of the seeds germinating. Therefore, the overall results suggest that those treatments which remove the seed coat, significantly improved germination.

Successful germination after removal of the seed coat is often an indicator of coat-imposed dormancy (Taiz and Zeiger, 2002). In such instances, the seed coat is made up of several palisade layers containing lignified cells which are packed tightly together and contain water repelling compounds (Baskin, 2003). These layers act as a mechanical (physical) barrier to water absorption and gaseous exchange. Water imbibition is essential for enzymatic function in a seed and exchange of gases is crucial for respiration.

Table 3.3.2: The effect of various treatments on the germination of *S. frutescens* seeds

Treatment	Percentage germination (%)
Presoaked	25.7a
Chemical scarification	86.3b
Physical scarification	98.6b
Flamed	91.9b

Different letters indicate statistical significance at the 95% confidence level.

These results further confirms that of Shaik *et al*. (2009) that *Sutherlandia* seed dormancy is seed-coat imposed as those treatments which removed the hard testa were best for seed germination. Although physical scarification was most effective for germination, a great number of seedlings died post germination due to an internal fungal contamination which coincided with seedling emergence. Chemical scarification may have resulted in lowered germination, but this is likely to have decontaminated seeds of this type of infection.

The advantage of using *in vitro* treatments to induce germination is that it will increase the likelihood that all seeds will germinate at the same time, which is essential for further experiments. On day four in culture, 91.5% of the physically-scarified seeds had already germinated (results not shown). Xaba (2007) noted that 90% of the seeds sown in soil only germinated after 10–15 days. Interestingly, a brown-coloured substance was released into the cultivation medium surrounding the seeds. This may be an indication of the allelopathic properties of *Sutherlandia* seeds, as reported by Rosenthal, (1982); Nakajima *et al*. (2001) and Rosenthal, (2001).

Figure 3.3.1: *Sutherlandia frutescens* A) seedling and B) *in vitro* cultivated shoot

The seedlings which germinated during the germination experiments were used as explants for micropropagation studies (Figure 3.4.1A). Although PGRs are usually used for the multiplication of shoots *in vitro*, I was able to multiply *Sutherlandia* shoots very effectively without the addition of PGRs, generating a large number of plantlets *in vitro* which could be used for the other experiments. This technique worked well as each axillary bud developed into a new shoot which could be used as explants for the next subculture. As each 2 cm microshoot segment elongated to approximately 5.6 ± 0.3 cm after one month, it generated one meristematic shoot and approximately five nodes (Figure 3.4.1B). During subculture, a 2 cm meristematic shoot (which usually included one node) and the four nodal segments (1 cm) were transferred to new media and cultured for a month, after which this procedure was

repeated. This system therefore has the potential (if every axillary node gives rise to a shoot) to develop five viable explants after a month, 25 viable explants after 2 months and 125 explants after three months. Other leguminous plants are generally multiplied through the use of PGRs. For example proliferation of the axillary shoots of the cotyledonary nodes of *Clitoria ternatea* (L.), a perennial leguminous twiner, required the addition of a cytokinin, with 1.0 mg L^{-1} BA being most efficient for shoot regeneration (Barik *et al*., 2007). In *Canavalia lineate*, the highest percentage bud break from single nodal explants was observed when BA (10 µM) and NAA (1 µM) was used in combination (Hwang *et al*., 1996). The current protocol reported here, is thus simpler, more cost-effective and a high regeneration capacity is maintained.

The plantlets generated *in vitro* were healthy with little signs of somaclonal variation (Figure 3.4.1B). Somaclonal variation during tissue culture is often associated with hyperhydricity and difficulties in the *ex vitro* acclimatization of plants (Kevers *et al*., 2004). Hyperhydricity can result in the irreversible loss of the regenerative ability of cells (Ivanova and Van Staden, 2008). The regime developed for *S. frutescens* yielded plants that were normal and none of the regenerated plantlets became hyperhydric. Absence of hyperhydricity is likely associated with the lack of added PGR in this system. It must be emphasized that PGRs are one of the factors which induce or evoke hyperhydricity of cultures which are stressed due to suboptimal cultivation conditions. This includes the use of a suboptimal medium, container or explants choice or unfavourable environmental conditions (Deberg *et al*., 1992). Phytohormone supplementation in tissue culture often leads to genetic aberrations, more especially if the PGR combinations are suboptimal for growth (Kevers *et al*., 2004). It can be concluded that the *S. frutescens* cultures established in this study had sufficient endogenous hormones to continue proliferating in the absence of additional (exogenous) PGRs.

3.3.2 Rooting and acclimatization

Spontaneous rooting (Figure 3.4.2A and B) was observed in 85% of the shoots with an average of 13 (13.2 ± 1.4) roots being produced per shoot. The length of the longest root was approximately 7.7 ± 1.1 mm. Spontaneous rhizogenesis in PGR-free medium was also observed in the legume, *Canavalia lineate* shoot cultures (Hwang *et al*., 1996). The spontaneous rooting of plants in tissue culture is very advantageous as it reduces the cost and time to maintain plants and this is highly desirable for commercial production. Furthermore, none of the roots were associated with callus, another beneficial aspect of this particular system. Rooting *in vitro* is generally induced by treating the plantlet base with auxins such as

NAA, IAA or IBA (Sharp *et al*., 1995). The legume, *Clitoria ternatea* (L.) required the addition of the auxin (IBA) to induce root development. However, high auxin levels in the medium (more than 1.0 mg L^{-1}) resulted in the production of callus. The type and concentration of auxin required for rooting may vary with the type of explants as Rout (2005) found that NAA was more effective than IBA for rhizogenesis of shoots derived from nodal explants of *C. ternatea* (Barik *et al*., 2007). Rooting in *C. ternatea* could be further improved by combining the auxin (IBA) with reduced MS strength as full strength MS increased the callugenic response of the shoots (Shahzad *et al*., 2007). At times, in some species callugenesis occurs concomitantly with rhizogenesis. Callus production at the bases of plants lowers the frequency of acclimation post culture (Shahzad *et al*., 2007) as the vascular system of the root and the stem is disrupted (George and Sherrington, 1984).

Six of these rooted shoots were transferred to the glasshouse to investigate their *ex vitro* acclimatization (Figure 3.4.2C and D). Plantlets acclimated easily to *ex vitro* growth without apparent morphological or growth disturbances as a result of tissue culture propagation. All six plants survived. Rooted shoots of *C. ternatea* were transferred to sterile soilrite (75% Irish peat moss and 25% perlite) and the pots were covered with polythene membranes to maintain a high humidity. Plants were successfully acclimated after four weeks and could be transferred to normal soil and placed in the glasshouse (Shahzad *et al*., 2007). This method resulted in a 70% survival rate. Problems with *ex vitro* survival may arise due to *in vitro* developed roots which may not be functional *ex vitro* due to a lack of root hairs which restricts water uptake (Preece and Sutter, 1991). An alternatively strategy to improve *ex vitro* survival would be to simultaneously acclimatize and root plants from *in vitro* derived shoots, however this will require the optimization of the growth conditions (humidity, temperature and light) and other factors (eg. the size of the microshoot) for successful *ex vitro* establishment of plants (Preece and Sutter, 1991).

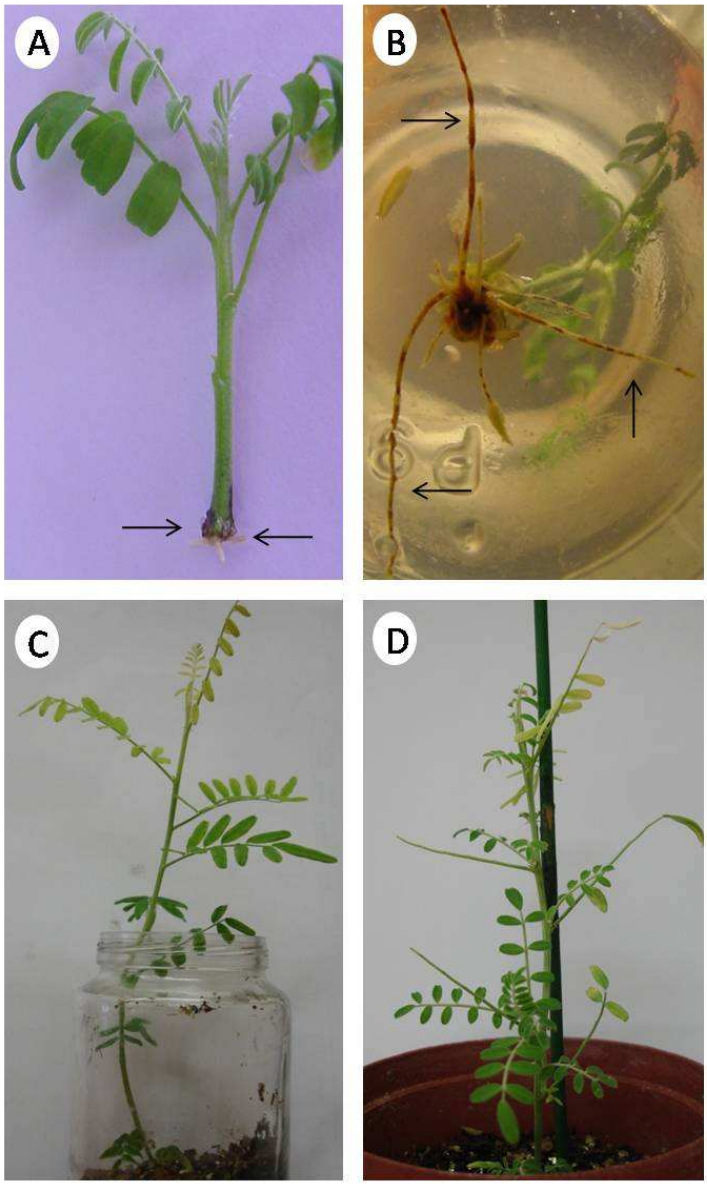

Figure 3.3.2: Micropropagation of *Sutherlandia frutescens*. A) spontaneous development of roots (indicated by the arrows) of shoots cultivated on PGR-free medium; B) induction of multiple roots which elongated in the medium; C) elongated shoot transferred to soil during acclimatization, and, D) fully acclimatized shoot after three months in the glasshouse

3.4 CONCLUSION

This study confirmed that seed germination of *S. frutescens* species is seed-coat imposed and dormancy is easily broken by scarification. The protocol for producing multiple clones over a

short period of time was optimal, providing a continuous supply of healthy propagules. One of the advantages of this regime is that it uses renewable shoot material and it has the potential to generate approximately 625 explants over a 4 month cycle. This is highly desirable for commercial micropropagation of this sought-after medicinal plant as it is not labour intensive. Secondly, this simple protocol serves aims of using tissue culture as a conservation mechanism. As the protocol was highly regenerative, sufficient material was available for routine genetic engineering (transformation) in studies that followed this chapter. The *in vitro* shoots rooted spontaneously which was beneficial as this reduced time and costs to establish such a protocol. Preliminary acclimation served to prove that micropropagated plants can be used successfully to supply microplants for commercial purposes.

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CHAPTER 4:

Genetic transformation for hairy root establishment

4.1 INTRODUCTION

4.1.1 Hairy root induction by *Agrobacterium rhizogenes*-mediated transformation

The process of transformation involves inoculating the wounded explants with *Agrobacterium rhizogenes* bacterial suspensions (Toivonen, 1993). The inoculation of plant parts may be direct (the plants are inoculated with the bacterial suspension and then cultivated on solid media) or indirectly (explants and bacteria can be co-cultivated in liquid media). After two to three days of co-cultivation, during which the plant tissue and bacteria divide, the T-DNA is transferred from the bacteria to the plant cells (Kim *et al*., 2007). After co-cultivation the explants are transferred to solid media containing antibiotics to remove the bacteria. The first roots can start to appear after between one to four weeks (depending on the plant species under investigation) and these can be excised and cultivated on Murashige and Skoog or Gamborg B5 hormone-free medium (Toivonen, 1993; Hu and Du, 2006; Georgiev *et al*., 2007). For some species, hairy root induction may require finding the optimal conditions for transformation to work.

4.1.2 Improving the transformation efficiency

Successful transformation of plants in the laboratory is dependent on the optimization of a few critical factors (Georgiev *et al*., 2007) which include: 1) determining the most efficient bacterial strain for transforming a particular species; 2) the density of the bacterial suspension should be optimal (bacterial suspensions that are in high concentrations lead to competitive inhibition and those that are too low, causes the production of bacterial cells which are ineffective for transformation); 3) the tissue type (hypocotyl, leaf, stem, stalk, petiole, shoot tip, cotyledon, protoplast, storage root or tuber) and age of the explants (juvenile tissue works optimally) should be experimented with (Hu and Du, 2006); 4) the optimization of the co-cultivation conditions is essential; 5) the proper antibiotic (cefotaxime sodium, carbecillin disodium, vancomycin, ampicillin sodium, streptomycin sulphate or tetracycline) or the combination and 6) the concentration of antibiotics (ranging from 100 to 500 µg ml^{-1}) needs to be determined to remove residual *Agrobacterium* contamination (Hu and Du, 2006); and, finally 7) the culture medium (carbon source and concentration, ionic concentration, pH and phytohormones) and

conditions (light and temperature) needs to be optimized for hairy root cultivation post-transformation (Hu and Du, 2006).

4.1.3 Whole plant regeneration of hairy roots

Whole plants can be regenerated from these transgenic roots and they often display typical traits such as wrinkled leaves, shortened internodes, an abundant plagiotropic root system and reduced apical dominance (Srivastava and Srivastava, 2007). The abnormal phenotypes are assigned to the insertion of *rol* genes. Insertion of *rol* A results in shortened internodes, leaf wrinkling, changes in the root architecture, reduced flower bud length, floral hyperstyly, reduced flowering and a loss of pollen viability (Chaudhuri *et al*., 2006). Insertion of *rol* B results in shortened internodes, reduced apical dominance, protruding stigma and reduced length of the stamens (Chaudhuri *et al*., 2006). *Rol* C insertion resulted in dwarfism due to internode shortening, reduced apical dominance which causes an enhanced lateral shoot development, loss of pollen viability (causes male sterility), enhanced shoot and root regeneration in explants and the appearance of annual flowering in biennial plants (Chaudhuri *et al*., 2006). Unfortunately, these transgenic plants often show higher mortality rates than normal non-transformed plants (Hu and Du, 2006). However, these transgenic plants are ideal as they can be used as a source of secondary metabolites, especially when the compounds of interest are not being expressed in the hairy root cultures.

4.1.4 Aims of the chapter

The induction of hairy roots in *Sutherlandia frutescens* was an important first step towards generating stable cultures which can be used to study the phytochemistry of the transgenic roots of this species and to determine if these transgenic roots can successfully be used as an alternative source to harvest metabolites (instead of the shoots). The aim for this chapter was the development of a successful protocol for the induction of hairy root cultures. This involved testing the efficiency of three *Agrobacterium* strains A4T, LBA9402 and C58C1 to induce hairy roots on different explants; the hypocotyls and cotyledons. The transformation of two model legumes (*Medicago truncatula* and *Lotus japonicus*) using the three *Agrobacterium* strains was also investigated. The transformation of *S. frutescens* has never been studied before. For this reason an efficient, simple but reproducible transformation system was thus investigated. A preliminary study was conducted to investigate shoot regeneration from *S. frutescens* hairy roots and in future this will be followed up by analysis of the phytochemistry and the pharmacological activity of extracts from these transgenic plants.

4.2 MATERIALS AND METHODS

As this type of study has never previously been conducted on this species, several of the techniques used have to be optimized and the experimental approach which was used is summarized in Figure 4.2.1. This is followed by the complete description of the techniques which were found to be optimal for transformation. Optimization steps have thus not been reported in this chapter.

4.2.1 Plant material and media

The MS salts and vitamins were purchased from Highveld Biological (Pretoria, South Africa) and prepared according to the manufacturer's instructions. All full-strength MS and half strength 0.5xMS media were supplemented with 0.1 g L^{-1} myo-inositol and 3% (w/v) sucrose (the term 0.5xMS denotes the use of half the concentration of MS salts in the growth medium). The pH was adjusted to 6.0 with 1 M NaOH or 1 M HCl, unless stated otherwise. Solid medium was generated by including 0.8% (w/v) agar (Biolab, South Africa). All media were autoclaved at 103 kPa and 120˚C for 20 min and the transfer of all tissue was conducted under laminar flow conditions. Cultures were either incubated in the light (16 h light/8 h dark; light conditions as previously stated in Chapter 3, Section 3.2.1.1) or a dark growth chamber at a constant temperature of 25±2˚C. Seedlings of *S. frutescens*, *M. truncatula* and *L. japonicus* were used for transformation experiments. The seedlings were obtained from germinated seeds which were decontaminated and germinated as follows:

a) *Sutherlandia frutescens* seeds were chemically scarified by two incubation steps in 10 ml concentrated H_2SO_4 for 15 min each. After treatment, the seeds were washed three times for 10 min each with sterile dH_2O;

b) *Medicago truncatula* seeds (Paraggio, Australia) were decontamination by incubating seeds for one minute in 70% (v/v) ethanol, followed by a 10 min wash in commercial bleach solution (3.5% (w/v) NaOHCl). Finally, seeds were rinsed five times for 5 min each in dH_2O, and,

c) *Lotus japonicus* seeds (Gifu, Japan) were decontaminated by incubating seeds in 5 ml concentrated H_2SO_4 for 15 min and the decontamination step was repeated twice. Next, seeds were washed four times for 5 min in dH_2O. All seeds were blotted dry on sterile filter paper prior to transfer to glass tubes (25 x 90 mm) containing 10 ml solid ¼-strength MS medium (solidified with 0.8% (w/v) agar, pH adjusted to 5.7). Tubes were sealed with plastic lids, thereafter the tubes were placed in the light growth chamber and germination was monitored. Germinating seeds were cultivated for a further two weeks until the hypocotyls

were approximately 2 cm long. Tobacco (*Nicotiana tabacum*) plants in continuous culture were cultivated in glass culture vessels (90 x 50 mm) containing autoclaved 25 ml medium 0.5xMS supplemented with 3% (w/v) sucrose; 1 µM BA, pH adjusted to 5.8 and solidified with 2.2 g L^{-1} Gelrite (Sigma, USA).

4.2.2 *Agrobacterium* cultivation

Agrobacterium rhizogenes strains LBA9402 and A4T and the *Agrobacterium tumefaciens* strain C58C1 were used in this part of the study. All three agrobacterial strains were cultivated on YMB medium (5 g L^{-1} yeast extract; 10 g L^{-1} mannitol; 0.1 g L^{-1} magnesium sulphate; 0.5 g L^{-1} potassium dihydrogen phosphate; (pH 7.0) solidified with 15 g L^{-1} agar) (Hooykaas *et al*., 1977). Strains C58C1 and A4T were also cultivated on YMA medium (5 g L^{-1} Yeast extract; 0.5 g L^{-1} casein hydrolysate; 8 g L^{-1} mannitol; 2 g L^{-1} ammonium sulphate; 5 g L^{-1} sodium chloride (pH 6.6) solidified with 15 g L^{-1} agar). The antibiotic Rifampicin (Rimactane®, Sandoz Ltd, South Africa) was added to the cooled autoclaved medium at a concentration of 100 mg L^{-1}. After streaking out bacteria on the media, each plate was wrapped with a 1.5 cm parafilm strip and incubated in a dark incubator at 28˚C for three days.

Liquid cultures of the agrobacterial strains were established by transferring a single colony of each strain to 50 ml YMA or YMB media containing Rifampicin (100 mg L^{-1}) and 250 µM filter-sterilized acetosyringone (3′, 5′-dimethoxy-4′-hydroxyacetophenone, Sigma, Germany). The liquid cultures were placed on a rotary shaker (60 rpm) in the dark incubator (28˚C). The bacteria were left to grow till the mid logarithmic phase (OD_{600} = 1 – 2). Freezer stocks of bacterial strains were prepared by mixing 800 µl bacterial solution and 800 µl cryopreservation solution [65% (v/v) glycerol, 0.1 M $MgSO_4$, 0.025 M Tris-HCl (pH 8.0)] in cryo-vials and these were stored at -80˚C.

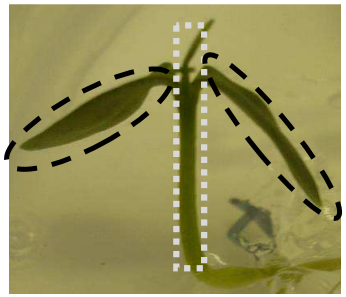
Seed germination (section 4.2.1)

↓

Different explants (hypocotyls, cotyledons and seedlings)

Four different species:

A) *S. frutescens*: Hypocotyls (indicated in white) and cotyledons (indicated in black)
B) *Medicago* and *Lotus* seedlings
C) Tobacco leaves in a continuous culture system on $^{1}/_{2}$ MS agar medium containing 1 µM BA, 3% (w/v) sucrose; pH 5.8, solidified with 0.8% agar)



↓

Incubate in *Agrobacterium* suspensions for 45 min

Three strains: *A. rhizogenes* A4T and LBA9402 and an *A. tumefaciens* strain C58C1 with *rol* T-DNA

↓

Co-cultivate for two days on 250 µM acetosyringone-containing MS medium

↓ Note the percentage of explants on which roots is initiated for each bacterial strain

Cultivate on medium containing 250 µM cefotaxime

↓ Transfer roots with residual *agrobacterial* growth to 500 µM cefotaxime-containing medium for a month or until roots are free bacterial

Initially select five root clones from each A4T, LBA9402 and C58C1-treated explants

↓ Select only four *Sutherlandia* clones and one tobacco clone for further studies

A ←

Establish hairy root cultures on 0.5xMS medium (0.8% (w/v) agar, pH 5.8)

↓

Extract DNA from hairy roots with CTAB RNA extraction method

Extract DNA from liquid *A. rhizogenes* strain LBA9402 suspension culture

↓

B **C**

← Study the growth kinetics of hairy roots in liquid media

Transfer 0.1 g hairy root tips to MS; 0.5xMS, Gamborg B5 and White's media

↓

Liquid suspension cultures

Note the fresh and dry mass after 28 days

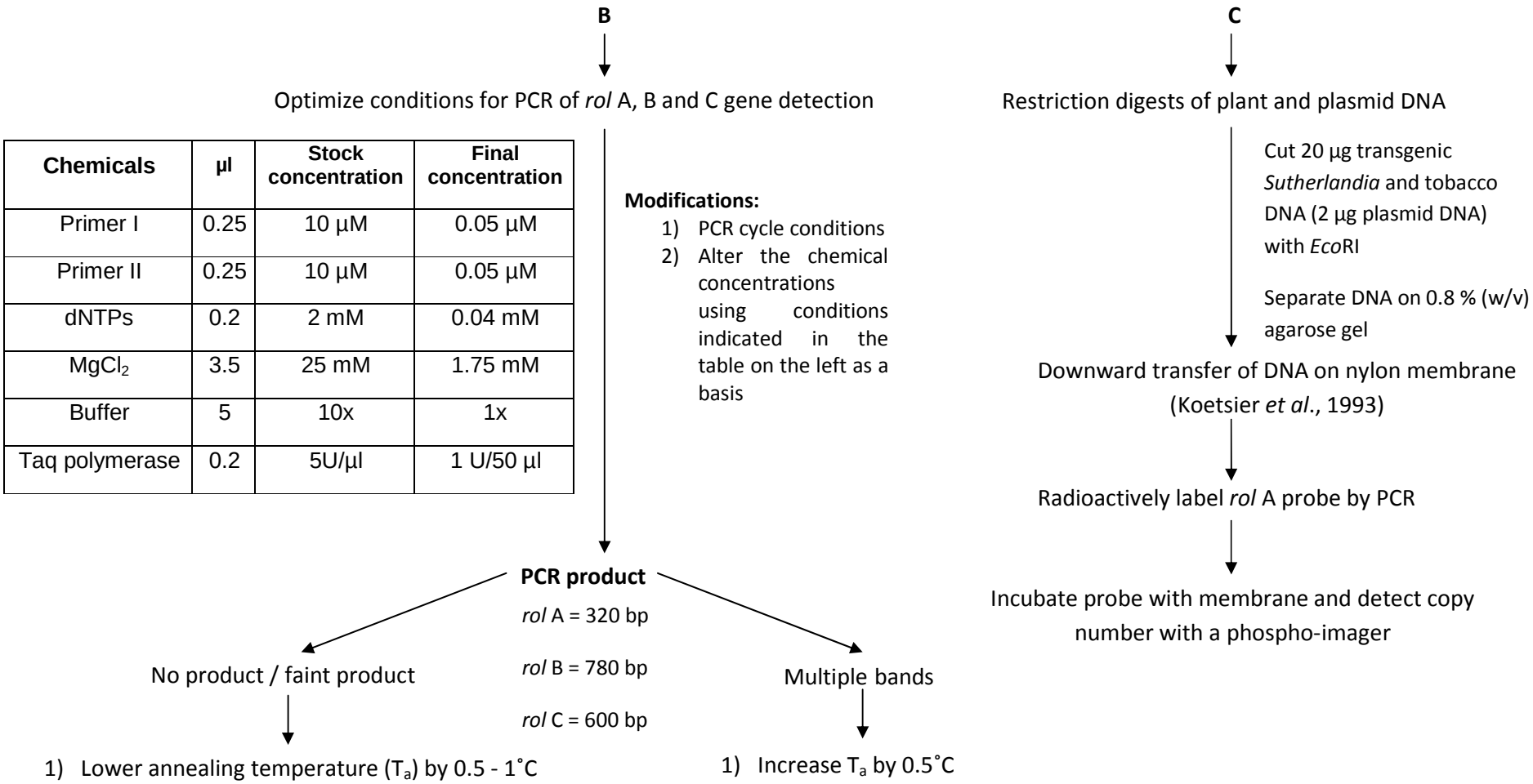


Figure 4.2.1: A summary of the methods used to investigate hairy root induction in *S. frutescens* and a molecular analysis of putative transgenic roots

4.2.3 *Agrobacterium* genetic transformation of explants

Prior to transformation, 13 ml of the saturated bacterial solution was transferred to a 15 ml centrifuge tube (Sterilin, U.K.) and centrifuged in a desktop centrifuge (Sigma 302) at 4 000 rpm (20˚C) for 15 min. The supernatant was discarded, the pellet washed and resuspended in 15 ml liquid MS medium to remove the antibiotics. The bacterial solution was centrifuged once more and the pellet was resuspended in 90 ml liquid MS medium. Five hypocotyls (1 cm) and 10 cotyledons of *S. frutescens*, 10 *Medicago* (1 cm) seedlings and 10 *Lotus* (1 cm) seedlings, of which the primary root had been removed, were used as explants. Ten primary leaves (cut in 10 x 10 mm squares) of tobacco plants (positive control) were also incubated separately in the different bacterial solutions for 45 min. Explants incubated in MS medium lacking the bacterial solution were used as the negative control. After incubation, the explants were blotted dry on sterile filter paper (Whatman® no.1) and placed horizontally (leaves placed with the abaxial side in contact with the medium) on PGR-free solid MS medium containing 250 µM acetosyringone. Plates were incubated in the dark growth chamber for two days.

After co-cultivation, the explants were washed three times for 15 min per wash in sterile dH_2O containing 250 mg L^{-1} cefotaxime (Claforan from Aventis, South Africa). The explants were dried on sterile filter paper and transferred to Petri dishes (5 explants per plate) containing solid 0.5xMS medium supplemented with 250 mg L^{-1} cefotaxime. These plates were placed back in the dark and monitored daily for root development using a light microscope. The number of explants showing hairy root induction for each bacterial strain and type of explant was noted for 21 days. This experiment was repeated three times.

4.2.4 Induction of liquid and solid cultures

After 30 days, five root tips from *S. frutescens* and tobacco explants were randomly selected for each bacterial strain and used to establish hairy root cultures. Excised roots were initially cultivated on 0.5xMS medium (250 mg L^{-1} cefotaxime) for a month, after which those explants with residual agrobacterial growth were transferred to medium with a higher cefotaxime concentration (500 mg L^{-1}). This treatment with the antibiotic was repeated until all the explants were free of residual agrobacterium infection. Out of the 15 hairy root clones established, only four [one A4T, one LBA and two C58C1 (g and e)] *Sutherlandia* and one tobacco (A4T) clone were selected for further analysis. The root tips of these clones were cultivated on solid 0.5xMS medium in the dark and subcultured on a monthly basis. Root cultures of untransformed roots were established by excising the roots from germinated seeds

and culturing them on solid 0.5xMS medium. These roots were subcultured on a monthly basis.

4.2.5 Growth kinetic studies of *S. frutescens* hairy roots

Liquid shake cultures of the hairy roots were initiated by incubating 100 mg putative transgenic roots in 50 ml medium in a 250 ml Erlenmeyer flask closed with a cotton wool plug and a tinfoil cap. The four types of media investigated for hairy root biomass accumulation included: MS (full strength); 0.5xMS (half strength); full strength Gamborg B5 (Gamborg *et al*., 1968) and full strength White's medium (White, 1934 and White, 1954). All media were supplemented with 3% (w/v) sucrose, 0.1 g L^{-1} myo-inositol, the pH was adjusted to 6.0 with 1 M NaOH or 1 M HCl. Media for this experiment was purchased from Duchefa (Duchefa Biochemie, Netherlands) and used as indicated by the manufacturer. The flasks were placed on an orbital shaker (120 rpm) in the dark. The fresh weight of the roots was determined after 28 days by blotting the roots dry on tissue paper prior to weighing. The roots were then placed in brown paper bags and dried in the oven for two days at 50˚C after which they were weighed once more to determine the dry mass (g). Five flasks were used per clone for each medium and this experiment was repeated three times.

4.2.6 Plantlet regeneration from hairy roots

Five root tips (2 cm) were transferred to a Petri dish with MS medium modified to contain 0.5 or 1 mg L^{-1} BA or Kinetin (pH 6.0), hairy roots placed on a medium containing no PGRs were used as the control for this experiment. Four Petri dishes (one Petri dish represents one replicate) were used per treatment for each clone and this experiment has only been repeated once. The plates were wrapped with a 1.5 cm parafilm strip and placed in the light (16h) growth room for eight weeks. The light intensity was the same as described previously in Section 3.2.1.1. After cultivation, the response (induction of callus, somatic embryos or shoots) was recorded (however this data was not included in this thesis as these experiments are still ongoing). Upon transfer to the light, the hairy roots of tobacco spontaneously regenerated shoots (without PGR in the medium) and these were excised and cultivated on 0.5xMS medium in the light growth chamber.

4.2.7 DNA isolation from *S. frutescens* hairy roots and regenerated tobacco shoots

The total genomic DNA was extracted using a modified cetyl trimethyl ammonium bromide (CTAB) RNA isolation method (White *et al*., 2008). This RNA method was modified to isolate

only DNA. For DNA isolation, (4 g) young *Sutherlandia* hairy root tissue or (2 g) untransformed *in vitro* shoot material and (2 g) tobacco leaf material (from regenerated transgenic plants) were used. The tissue was rapidly frozen in liquid nitrogen and homogenized with a mortar and pestle. The powdered tissue was transferred to 25 ml preheated (65˚C) autoclaved CTAB buffer (2% (w/v) CTAB, 2% (w/v) polyvinyl pyrrolidone (PVP), 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 0.5 g L^{-1} spermidine) containing 3% (v/v) β-mercaptoethanol (added immediately prior to extraction to the heated buffer). The centrifuge tubes were briefly vortexed for 10 s and incubated in the waterbath for 30 min. The samples were then centrifuged for 10 min at 5 000 revolutions per minute (rpm) in an ultracentrifuge (Sorval RC26 Plus). An equal volume of chloroform: isoamyl-alcohol (24: 1; v/v) solution was added to the supernatant followed by a vortex step for 30 s. After centrifugation (5 000 rpm for 15 min) the clear layer was transferred to a new tube and the chloroform: isoamyl alcohol extraction was repeated. The aqueous phase was transferred to a clean tube and the DNA was precipitated by adding ($^{1}/_{10}$ th of the volume of the supernatant) 4.4 M ammonium acetate (105 ml H_2O, 50.5 ml glacial acetic acid, 45 ml NH_4OH; pH 5.2) and an equal volume ice-cold isopropanol. The tubes were briefly inverted to precipitate the DNA and kept at -20˚C overnight. The DNA was collected by centrifuging at 5 000 rpm for 25 min followed by a wash with 1 ml 70% (v/v) EtOH, prior to a second wash with 1 ml 100% (v/v) EtOH. After the wash steps, the pelleted DNA was left to air-dry and then resuspended in 300 µl de-ionized water (ddH_2O). The residual polysaccharide and protein contamination of the DNA was removed with a Promega DNA Wizard cleanup kit (Promega, USA). The protocol outlined by the manufacturer was followed. The DNA was finally resuspended in 100 µl TE (10 mM EDTA, 25 mM Tris pH 8.0) buffer and stored at -20˚C until required for other experiments.

4.2.8 Plasmid DNA isolated from *A. rhizogenes* strain LBA9402

The method of Li *et al*. (1995) was followed for isolation of plasmid DNA. A single colony of *A. rhizogenes* strain LBA9402 was inoculated in 15 ml YMB media containing rifampicin (100 mg L^{-1}) and acetosyringone (250 µM) and placed on a rotary shaker (60 rpm) at 28˚C and cultivated for three days. Four hours prior to extraction, the cultures were incubated at 37˚C until the OD_{600nm} = 1.0 – 1.5 (Li *et al*., 1995). The tubes were centrifuged in a table top centrifuge at 4 000 rpm for 15 min to pellet cells. The pellet was resuspended in 1 ml buffer (50 mM glucose, 25 mM tris-HCl pH 8.0, 10 mM EDTA). Next, the bacterial cells were lysed in two volumes of a 0.2 M NaOH-1% (w/v) sodium dodecyl sulphate (SDS) solution. The tube was mixed and kept on ice for 15 min. The solution was neutralized by adding 1.5 volumes of pre-cooled (-20˚C) 5 M potassium acetate. The tubes were mixed gently and kept on ice for

30 min followed by centrifuging at 4 000 rpm for 5 min. The supernatant was transferred to a clean tube and centrifuged once more at 4 000 rpm for 5 min. The supernatant was transferred to a new tube and an equal volume of chloroform: isoamyl solution (1: 24; v/v) was added to the tube and mixed for 2 min on a table top shaker. The tubes were centrifuged for 5 min (4 000 rpm) and the aqueous phase was collected. The DNA was precipitated with an equal volume of ice cold isopropanol and samples were kept at -20˚C for one hour. The pelleted DNA was washed with 1 ml 70% ethanol, dried on the fume cabinet and resuspended in 100 µl dH_2O.

The genomic and plasmid DNA concentration was determined at A_{260nm} with a nanodrop (ND-1000 spectrometer; Thermo-scientific, USA) and the DNA quality was assessed by ethidium bromide (EtBr)-UV (A_{366nm}) visualization (Alpha Innotech^{TM} 2000; Alpha Innotech Corporation, San Leandro, CA) after separation on a 0.8% (w/v) agarose gel (Top vision^{TM}, Fermentas) in 0.5x TBE (5.4 g L^{-1} tris, 2.75 g L^{-1} boric acid, 2 ml 0.5M EDTA, pH 8.0, and made up to 1 L with dH_2O). A Savant HG 360 Electrophoretic gel system was used for gel electrophoresis. To track the migration of samples, 5 µl loading buffer was added to the DNA samples prior to electrophoresis at 100 V for 1 h.

4.2.9 PCR of *rol* A, B and C genes

The PCR reactions were carried out in a 50 µl volume containing 100 ng template DNA and 80 ng plasmid DNA (positive control). During the optimization of the PCR reaction different concentrations of DNA, $MgCl_2$ and *Taq* (Fermentas) as well as the addition or exclusion of dimethyl sulphoxide (DMSO) was investigated (refer to Figure 4.2.1). The final PCR master mix (indicated in Table 4.2.1) contained 5 µl of 10xPCR buffer (200 mM $(NH_4)_2SO_4$ (Fermentas)), 0.2 µl of 2 mM dNTPs (Fermentas) and 0.25 µl of 10 µM of the forward and reverse primers [Primer pairs A_1 and A_2 (for *rol* A); B_1 and B_2 (*rol* B) and C_1 and C_2 (*rol* C) (Table 4.2.2)]. For the amplification of *rol* A and *rol* C, 2.0 mM $MgCl_2$ was used whereas 1.75 mM $MgCl_2$ was used for *rol* B amplification. Additionally, 5% (v/v) DMSO was added during *rol* C amplification. During this optimization different cycle regimes were also investigated and the final cycle regime conditions for each *rol* gene is indicated in Table 4.2.3. To verify the absence of contamination, a negative control reaction, a) either replacing DNA by de-ionized water (dH_2O) or b) using DNA isolated from untransformed *Sutherlandia* shoot tissue was carried out with each PCR reaction.

The PCR reactions were conducted in a GeneAmp PCR system 9700 and the PCR products (17 µl of the reaction) were separated by electrophoresis in a 1.5% (w/v) agarose gel (prepared in 0.5x TBE) containing 10 mg ml^{-1} ethidium bromide (EtBr) and electrophoresed at 100 V for 30 min. The size of the bands which were amplified was compared to a Roche III or VIII (Roche) or Pst λ molecular marker. This Pst λ DNA ladder was generated by digesting lambda DNA with a Pst I enzyme. This digestion generates 17 fragments which range in size from 11 501 bp to 247 bp. This ladder was used routinely for size analysis of size fractionated DNA. Bands were visualized with EtBr-UV (wavelength 366 nm) light.

Table 4.2.1: The final concentrations of reagents used for PCR of *rol* genes of *S. frutescens* hairy roots and *A. rhizogenes* strain LBA plasmid DNA

Reagents	***rol* A**	***rol* B**	***rol* C**
$MgCl_2$	2.0 mM	1.75 mM	2.0 mM
DMSO	0	0	5 %
Primer I (forward)	0.05 µM		
Primer II (reverse)	0.05 µM		
dNTPs	0.04 mM		
10x Buffer $(NH_4)_2SO_4$	1x		
Taq polymerase (Fermentas) (U/50 µl reaction)	2.5 U		
Deionized water (ddH_2O)	To make up to 50 µl		

Table 4.2.2: Primer sequences for amplification of *rol* genes

Primer	**Sequence**	**PCR product size**
A_1	5'-CAG AAT GGA ATT AGC CGC ACT A-3'	320 bp
A_2	5'- CGT ATT AAT CCC GTA GGT TTG TTT-3'	
B_1	5'-ATG GAT CCC AAA TTG CTA TTC CTT CCA CGA-3'	780 bp
B_2	5'-TTA GGC TTC TTT CTT CAG GTT TAC TGC AGC-3'	
C_1	5'-CAT TAG CCG ATT GCA AAC TTG -3'	600 bp
C_2	5'-ATG GCT GAA GAC GAC CTG -3'	

Rol B primers were manufactured by MWG Biotech (Germany) and *rol* A and C primers were purchased from IDT (USA).

Table 4.2.3: PCR cycle regime for amplification of *rol* genes

PCR step	*rol* A	*rol* B	*rol* C
Initial denature	94˚C – 2 min	94˚C – 3 min	94˚C – 2 min
Denature	94˚C – 5 min	94˚C – 50 s	94˚C – 5 min
Anneal (Ta)	54˚C – 45 s	53˚C – 1 min	52˚C – 5 min
Elongate	72˚C – 2 min 30 s	72˚C – 1 min	72˚C – 5 min
Number of cycles	**2x**	**30x**	**2x**
Denature	94˚C – 1 min		94˚C – 1 min
Anneal	54˚C – 45 s		52˚C – 1 min
Elongate	72˚C – 2 min 30 s		72˚C – 1 min 50 s
Number of cycles	**35x**		**35x**
Final elongation	72˚C – 10 min	72˚C – 10 min	72˚C – 10 min

4.2.10 Southern hybridization analysis with downward transfer

DNA from plant cultures was extracted as described above using the CTAB extraction protocol. During restriction digest, 20 μg genomic DNA from *S. frutescens* hairy roots and tobacco shoots and 2 μg of the control LBA9402 plasmid DNA were digested. Each reaction contained eight units (U) of the restriction enzyme *Eco*R1 (Fermentas); 0.1 M spermidine, 1x restriction buffer and ddH_2O. The DNA was incubated in a 37˚C water bath for 16 h to ensure complete digestion by the enzyme. One sample without restriction enzyme was used as a negative control. After the digest, the DNA was separated on a 0.8% (w/v) agarose gel (50 V) for three hours. Thereafter, the gel was transferred to 250 ml 0.25 M HCl solution and swirled on a table top shaker for 10 min, until the bromophenol indicator turned yellow, so as to depurinate the DNA. This was followed by a brief rinse in ddH_2O and soaking in 250 ml rinsing solution (0.3 M NaOH/3 M NaCl) for 15 min. Finally the gel was placed in a 250 ml transfer solution (0.5 M tris-HCl (pH 7.0)/3 M NaCl) and swirled for a further 20 min.

To transfer the DNA from the gel to a Hybond-N+ membrane (Amersham Biosciences; UK) the method of Koetsier *et al*. (1993) was utilized. The blotting membrane and one layer of filter paper were cut to the size of the gel and the top left corner of the membrane was clipped to indicate the first lane. One filter paper square was cut slightly (1 cm) larger than the size of the gel. During gel depurination, the membrane and filter papers were submerged in transfer

neutralization solution prior to transfer. The depurinated gel was placed on the base of a Petri dish with the DNA side facing upwards. The blotting membrane was carefully applied on top of the gel and all air bubbles were smoothed out with a clean glass rod. The larger filter paper which was pre-wetted in transfer solution was smoothed over the membrane using the glass rod. A four cm stack of folded paper towels was then placed on top of the gel-membrane stack and the entire pile was inverted and the gel was slid off the Petri dish. The smaller filter paper square was soaked in transfer solution and placed on top of the gel. Wet handy wipes (soaked in the transfer solution) were folded to the same size as the gel and placed on top of the gel to prevent the gel from drying out. The transfer time was approximately 3 h (depending on the thickness of the gel) after which the dehydrated gel was checked for complete DNA transfer under the UV light. After the transfer, the membrane containing the DNA was rinsed in a solution [0.5 M tris-HCl (pH 7), 1 M NaCl] for 10 min and blot dried with a paper towel. The DNA was fixed to the membrane by baking the membrane between filter papers at 80˚C for two hours. The membranes were stored between filter papers in a dark dry area until further use.

4.2.10.1 Radioactive PCR labeling of *rol* A probe

The membrane was pre-hybridized and hybridized using the procedures detailed in the ULTRhyb™ Ultrasensitive Hybridization buffer system (Ambion, USA). Redivue (α-^{32}P) dCTP (3 µl; Amersham, 50 µCi, 3000 Ci/mmol) was used to radioactively label a *rol* A probe during PCR using 45 ng plasmid DNA. The reagents for PCR was added as indicated in Table 4.2.1 and the PCR cycle included an initial denaturation at 94˚C for 4 min followed by 35 cycles of 94˚C for 1 min; annealing at 54˚C for 45 s and elongation at 72˚C for 2 min 30 s and a final elongation step at 72˚C for 10 min. After PCR, the probe was cleaned using a Promega kit, according to the manufacturer's instructions. After the cleanup the tube containing the probe was placed in boiling water for 5 min to separate the double strands. This was followed by a snap cooling (to prevent re-annealing) on ice for 2 min. Successful amplification of the *rol* A gene during PCR was checked by running one PCR reaction on a gel and visualizing the band under UV light.

4.2.10.2 Southern hybridization and signal detection

The membranes were placed inside a hybridization tube and pre-hybridized in Rapid Hyb buffer (GE Healthcare, Amersham, UK) according to the manufacturer's instructions at 56˚C in a hybridization oven with continuous constant rotation for two hours. The probe (10 µl) was carefully added to the hybridization buffer of each tube as direct contact with the membrane

causes smears. The tubes were placed back in the hybridization chamber and hybridization of the probe to the membrane was conducted overnight at 56˚C with continuous constant rotation for 16 h. The unhybridized probe was removed by post-hybridization washes.

The first wash with 100 ml 2x SSC / 0.5% (w/v) SDS, was conducted at room temperature for 15 min and this was repeated twice. This was followed by two more post hybridization washes in 1x SSC / 0.5% (w/v) SDS at 65˚C for 15 min. Finally the membrane was placed in a Saran Wrap and the excess post-hybridization buffer on the membrane was removed prior to sealing the wrap. The blot dried membrane was exposed to autoradiography film (Packard, USA) and developed for 72 hours using a HyperScreen™ (Amersham Biosciences; UK). Positive hybridization of the probe was recorded using phospho-imaging (Packard Instrument Co. Meriden, CT) and an AlphaImager™ 2000 documentation and analysis system (Alpha Innotech Corporation, San Leandro, CA).

4.2.11 Data collection and statistical analysis

A random block design was used for all experiments and all experiments were repeated three times. The experimental layout and data collection set up, was as follows:

1) Induction of hairy root cultures

Five explants (hypocotyls, cotyledons or whole seedlings) were placed per Petri dish. Each explant represented a replicate. For *Sutherlandia*, there were three Petri dishes for each strain and for *Lotus*, *Medicago* and tobacco there were two Petri dishes per strain. The explants were monitored daily for root development for a period of 21 days to determine the percentage of explants producing roots per Petri dish for each strain/explant and species. This experiment was repeated three times.

The data for the frequency of explants producing roots were arcsine transformed prior to analysis using the Kruskal-Wallis test. The normality of the data was determined using a Shapiro-Wilk's W test prior to further analysis using a Tukey test or Kruskal-Wallis test depending on the normal distribution of the data. A Kruskal-Wallis (for comparing multiple independent samples) or a Mann-Whitney U test (for comparing two independent samples) was used to compare transformation efficiency between the different bacterial strains of the various species. The differences between means reaching a minimal confidence level of 95% were considered as being statistically significant.

2) Accumulation of mass of hairy root cultures

Five flasks were prepared for each medium and each flask represented a replicate. The fresh mass and dry mass of the roots were determined after 28 days. This experiment was

repeated three times. The Kruskal-Wallis test was used to analyze the results for the fresh and dry mass accumulation data.

All statistical testing was performed with the aid of the Statistica Release 8 (Statsoft Inc. 2007) program.

4.3 RESULTS AND DISCUSSION

Transformation in plants using *Agrobacterium* is at times difficult, particularly as *Agrobacterium* naturally only transforms Solanaceous plants. Recalcitrance of plants to *Agrobacterium* transformation may be overcome by optimizing several different factors (strain used, target tissue, co-cultivation parameters amongst others). This can prove to be laborious. Even though transformation is now routinely used in many laboratories in the world, transformation protocols exist mainly for model species such as *Arabidopsis*, tobacco and tomato.

4.3.1 The induction and establishment of hairy roots

Agrobacterium rhizogenes strains LBA9402 (a hypervirulent strain) and A4T, represent two agropine synthesizing strains that naturally occur in the wild. This type of agrobacterium is most often used for transformation as they possess the strongest induction ability (Hu and Du, 2006). Strain C58C1 is a genetically-engineered *Agrobacterium tumefaciens* strain which contains a GUS and kanamycin (*npt* II) gene on a separate vector from the virulence genes on the Ri plasmid (Tepfer personal communication). Inclusion of acetosyringone was important as it is responsible for activation of the *vir* genes in the virulence region, however these genes do not enter the plant cell, but their gene products are essential for the transfer of T-DNA (Oksman-Caldentey, 1996). Here, all three strains were used to infect the hypocotyls and cotyledons of *S. frutescens* for hairy root induction. The first roots appeared after four days on the hypocotyl explants infected with strains A4T and C58C1, whilst the hairy roots on the cotyledons only appeared after 12 and 14 days for the two strains respectively (Table 4.3.1). In contrast, the first roots appeared earlier (eight days) on the cotyledons but later (12 days) on the hypocotyls during transformation using the strain LBA9402 (Table 4.3.1). The hairy roots generally appeared directly (not associated with an initial callus phase) at the base of the hypocotyl explants (Figure 4.3.1A), whilst the hairy roots on the cotyledons appeared both directly or they appeared from the callus which was induced on the cotyledonary tissue (Figure 4.3.1B). Formation of wound callus was also observed during *A. rhizogenes*-mediated transformation of *Tylophora indica*. The production of hairy roots from this callus was

attributed to the requirement of cell division of the host target tissue for transformation (Chaudhuri *et al*., 2005).

The highest transformation response for *S. frutescens* was obtained during the transformation of hypocotyl tissue using strain A4T (93%). This strain was also more efficient at transforming the cotyledons with a frequency of 47%. The transformation efficiency of the other two strains with hypocotyls (ranging between 83% and 87%) and cotyledons (between 41% to 42%) was very similar. Statistical analysis indicated that transformation varied upon the type of tissue used ($p = 0.000$) with the hypocotyls being more responsive than the cotyledons. There was, however, no significant difference between the transformation efficiency of the bacterial strains ($p = 0.7335$). The hypocotyl and cotyledonary tissue were selected as explants as it was suggested that juvenile tissue is more amenable to transformation than older explants (Hu and Du, 2006). Previously, Nilsson and Olsson (1997) reported that the *rol* B and *rol* C promoter activity is activated by auxins and high sucrose levels, respectively. Both of these compounds are therefore required for hairy root induction. The IAA (auxin) levels are usually highest in the vascular cambium tissue and the phloem is a rich source of both sucrose and auxin as it transports various compounds between the leaves and the roots in the intact plant (Nilsson and Olsson, 1997). The high levels of these compounds in the hypocotyls might explain their higher hairy root induction capacity as the cotyledons were detached from the hypocotyls which probably resulted in reduced auxin and sucrose levels in this tissue.

However, *A. rhizogenes* transformation in *Alhagi pseudoalhagi* (a forage legume) was more successful in the cotyledons than in the hypocotyls. The improved transformation efficiency of the cotyledons was attributed to an enlarged cut area which allowed a greater number of bacteria to attach to the explants (Wang *et al*., 2001). Alternatively, the difference in the physiology of the different tissue influences the susceptibility of the explants and this causes a diverse response in the different tissue of the same plant (Wang *et al*., 2001). Furthermore, the inclusion of acetosyringone in the bacterial cultivation medium and the co-cultivation medium during transformation may have improved the transformation frequency. Acetosyringone is a phenolic compound which is released by wounded cells of some dicotyledonous species and this compound may stimulate the bacteria to transfer the T-DNA to the host cells more efficiently (Wang *et al*., 2001). Pretreatment of agrobacteria with acetosyringone prior to exposure to explants also greatly enhanced the number of explants of *A. psuedoalhagi* producing hairy roots (Wang *et al*., 2001).

Table 4.3.1: Frequency of hairy root induction on different explants by three strains of *Agrobacterium* in *S. frutescens*. (The values are the means ± SE, n = 30)

Plant species	Type of explant	Strain	Hairy root (%) ± SE	Root emergence (days)
Sutherlandia frutescens	Hypocotyl	A4T	93.3 ± 6.7a	4
		C58C1	86.7 ± 6.7a	4
		LBA	83.3 ± 8.3a	12
	Cotyledon	A4T	46.7 ± 16.9b	12
		C58C1	40.0 ± 12.6b	14
		LBA	41.7 ± 9.7b	8

Data were analyzed using the Kruskal-Wallis test to study the effect of the type of strain on % hairy root formation (p = 0.7224) and the effect of explant type on % hairy root induction (p = 0.000). The interaction between the type of bacterial strain used and the type of explant was investigated using a factorial ANOVA (p = 0.96335). Different letters indicate statistical significance at the 95% confidence level.

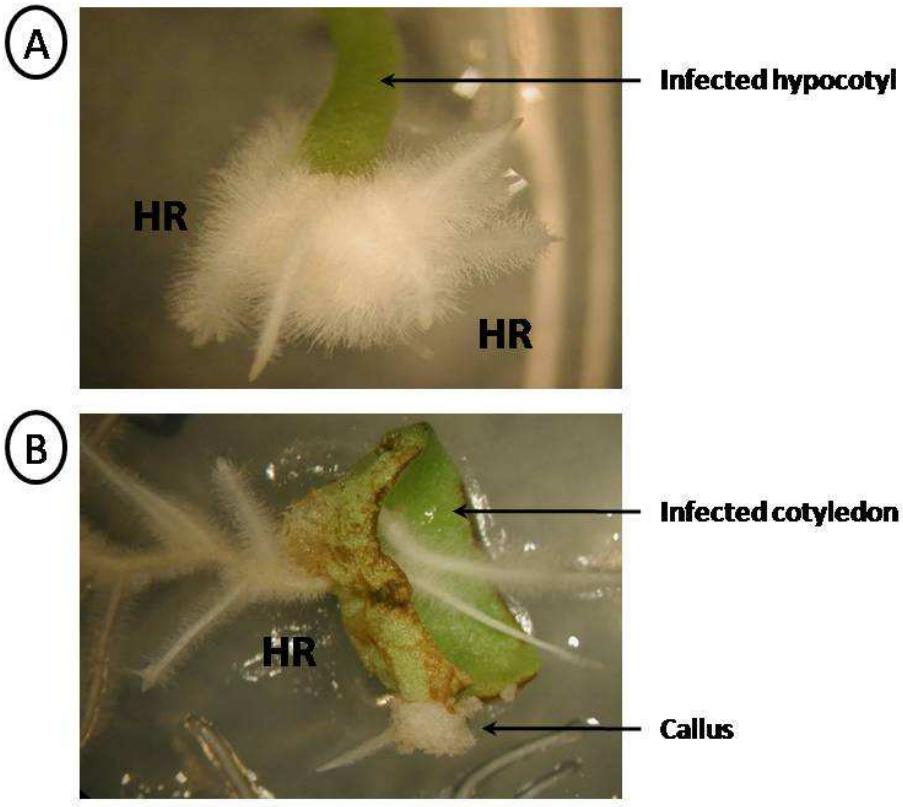


Figure 4.3.1: Induction of hairy roots on *S. frutescens* A) hypocotyl and B) cotyledons (HR-hairy roots).

In the control plants (tobacco) the first roots appeared on day six with strain A4T, a day later for strain LBA9402 whilst explants infected with strain C58C1 only responding after nine days (Table 4.3.2, Figure 4.3.2).

Table 4.3.2: Frequency of hairy root induction on tobacco leaves by three *Agrobacterium* strains. (The values are the means ± SE, n = 30)

Plant species	Type of explant	Strain	Hairy root induction (%) ± SE	Root emergence (days)
Tobacco	Leaf	A4T	59.4 ± 16.8a	6
		C58C1	20.5 ± 14.2a	9
		LBA	68.8 ± 11.0a	7

Data were analyzed using the Post HocTukey HSD test to study the effect of the type of strain on % hairy root formation (p = 0.0782). Different letters indicate statistical significance at the 95% confidence level.

In the tobacco explants, transformation was highest with strain LBA9402 (69%) and lowest when using strain C58C1 (21%) whilst strain A4T resulted in 60% transformation of explants (Table 4.3.2). The statistical analysis indicated no significant difference at the 95% level between the transformation efficiency of the three strains (p = 0.0782). The leaves used for transformation are derived from cultures which had been cultivated *in vitro* for more than a year. The reduction in the total percentage of transformed explants emphasizes the previous observation that transformation is more efficient in younger tissue. However, the transformation efficiency of the bacterial strains can also vary with the type of species and the type of tissue. For example Jain *et al*. (2008) investigated transformation of *Maytenus senegalensis in vitro* root segments using strains A4T and LBA9402 and found that transformation was most successful when using the strain LBA9402.

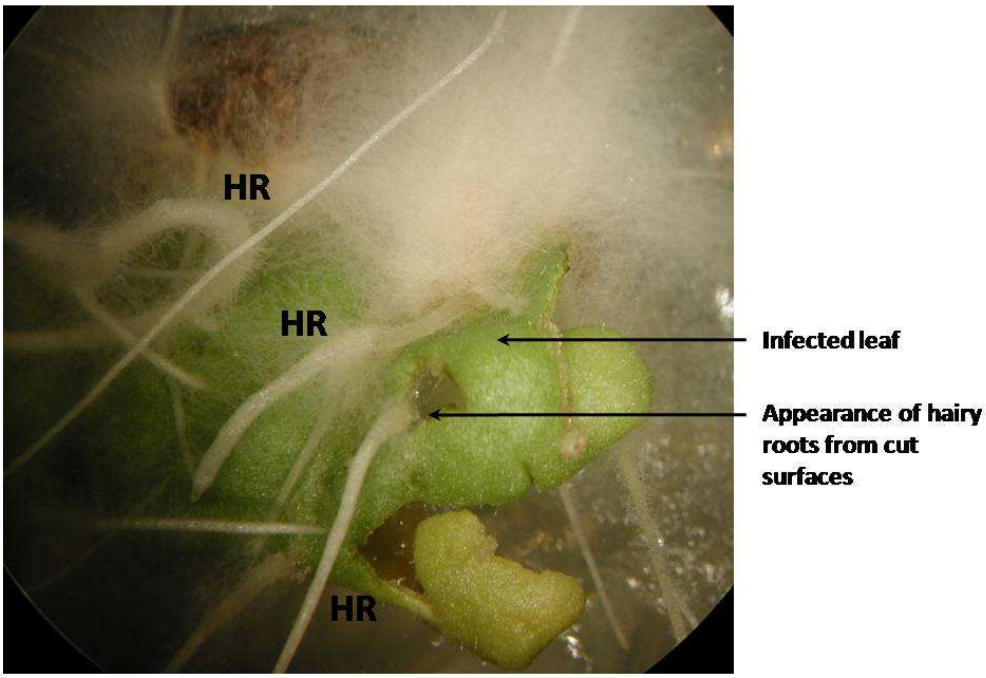


Figure 4.3.2: Induction of hairy roots on tobacco leaves (HR – hairy roots)

During this study the transformation of two model legume species *L. japonicus* and *M. truncatula* was also investigated to determine their response to the three bacterial strains investigated. In *Lotus*, the appearance of hairy roots was more rapid than the response in *Sutherlandia* and tobacco. The first roots were evident after two (LBA9402) to three (A4T and C58C1) days (Table 4.3.3; Figure 4.3.3A and B). In contrast to *S. frutescens* the transformation of *Lotus* seedlings was very successful using strains C58C1 and LBA9402 with all the explants producing roots (100% transformation efficiency). Strain A4T was, however, less effective but still resulted in a high transformation efficiency (89% of explants producing hairy roots). During the transformation of *Medicago*, the first roots appeared after three days for the strain LBA9402; four days for the strain C58C1 and five days for the strain A4T. Table 4.3.4 shows that the three strains were equally effective at inducing hairy roots on the intact seedlings. Hairy roots for both species appeared from both the base of the hypocotyl as well as the cotyledonary tissue of the intact seedlings (Figure 4.3.3). Statistical analysis indicated that there was no significant difference between the transformation efficiency of the three strains in *Medicago* seedlings ($p = 0.47$).

Agrobacterium rhizogenes virulence varies with the plant host and transformation efficiency of the host species varies between the different bacterial strains (Chaudhuri *et al*., 2005). Although in this study the roots were excised and cultivated independently *in vitro*, other researchers have used *A. rhizogenes*-mediated transformation to generate composite plants (Kereszt *et al*., 2007). These plants have a wild type shoot and transgenic roots. Composite plants have been generated by inserting the apical stem of young vegetative phase plants in an *A. rhizogenes*-containing rockwool medium (Kereszt *et al*., 2007). The hairy roots on these plants develop from a teratoma. Alternatively the bacteria can be injected in the cotyledonary node or the proximal part of the hypocotyl. The advantage of this latter method is that the hairy roots develop without the associated callus or teratoma phase (Kereszt *et al*., 2007). Hairy roots have been induced on many leguminous plants such as *Phaseolus vulgaris*, *Trifolium pretense*, *M. sativa*, *L. japonicus* (to name a few) using the latter method (Kereszt *et al*., 2007). These composite plants are ideal as they are capable of nodulation and can therefore be used to study the gene function of relevant genes (Kereszt *et al*., 2007).

Table 4.3.3: Frequency of hairy root induction using different *Agrobacterium* strains in *Lotus japonicus.* (The values are the means ± SE, n = 30)

Plant species	Explant	Strain	Hairy root induction (%) ± SE	Days to root emergence
Lotus japonicus	Seedling	A4T	89.98 ± 5.4a	3
		C58C1	100a	3
		LBA9402	100a	2

A Mann-Whitney U test was performed to compare transformation efficiency with the different bacterial strains between the two species (*Lotus* and *Medicago*) and the Kruskal-Wallis test was used to investigate the transformation efficiency of each strain in each species. Different letters indicate statistical significance at the 95% confidence level.

Table 4.3.4: Frequency of hairy root induction using different *Agrobacterium* strains in *Medicago truncatula*. (The values are the means ± SE, n = 30)

Plant species	Explant	Strain	Hairy root induction (%) ± SE	Days to root emergence
Medicago truncatula	Seedling	A4T	88 ± 8b	5
		C58C1	88 ± 12b	4
		LBA9402	84 ± 4b	3

A Mann-Whitney U test was performed to compare transformation efficiency with the different bacterial strains between the two species (*Lotus* and *Medicago*) and the Kruskal-Wallis test was used to investigate the transformation efficiency of each strain in each species. Different letters indicate statistically significant difference at the 95% confidence level.

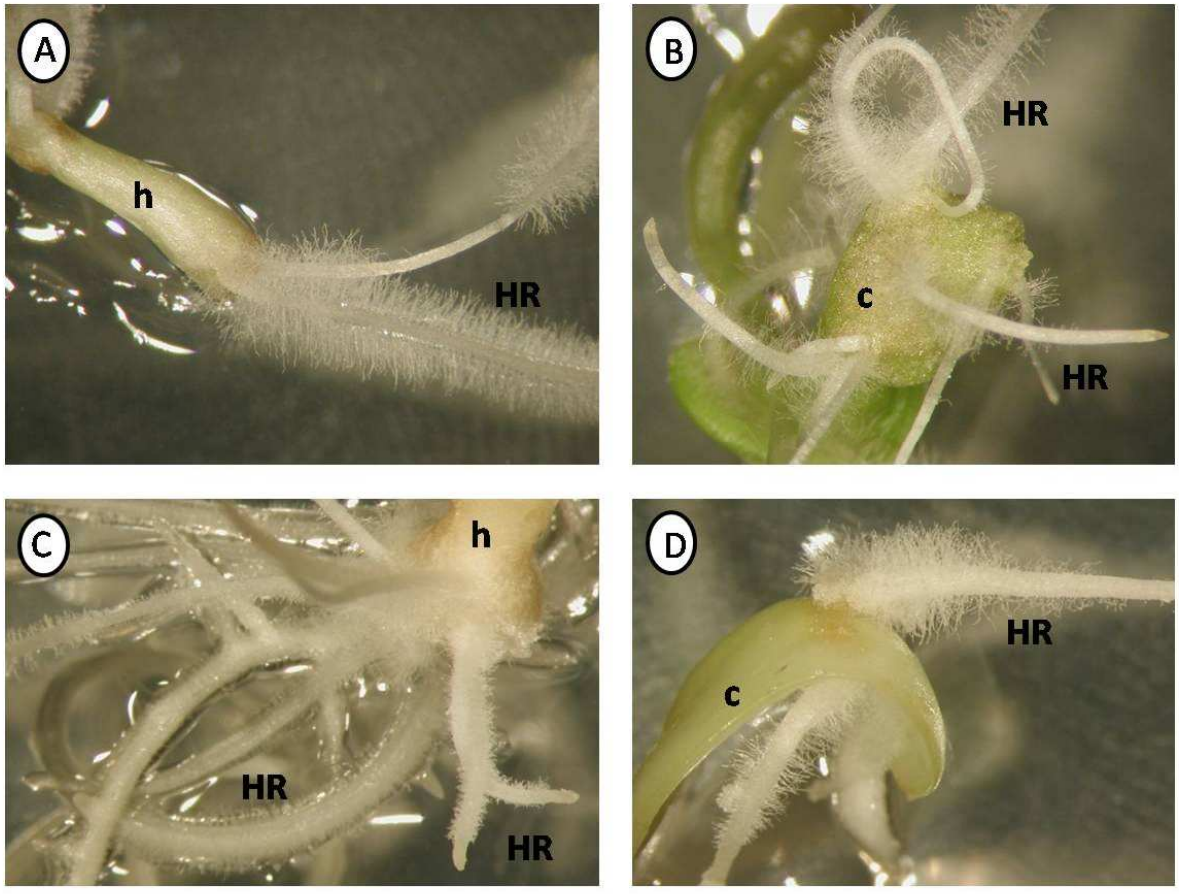


Figure 4.3.3: Hairy root induction on *Lotus* and *Medicago* seedlings using different *Agrobacterium* strains. Induction of hairy roots (HR) in *Lotus* A) hypocotyl (h) and B) cotyledon (c), and in *Medicago* C) hypocotyl (h), and, D) cotyledonary (c) explants

4.3.2 Solid and liquid hairy root cultures

Initially 15 root tips (five for each bacterial strain) of *S. frutescens* were excised and used to establish hairy root cultures on solid media. These clones were cultivated on cefotaxime-containing medium to kill the remaining bacteria. The clones derived from C58C1 transformation in particular contained residual agrobacterial growth and they were cultivated on medium with a higher cefotaxime concentration (500 mg L^{-1}). These roots were subcultured at least three times on this medium or when needed, until the roots were free of residual agrobacterial growth. The strategy of Wang *et al*. (2001) was to initiate the hairy root cultures on the highest concentration of cefotaxime (500 mg L^{-1}) and to gradually reduce the concentration during each subculture until the cultures were free of bacteria. Four clones (A4T, LBA, C58C1-g and C58C1-e) which demonstrate expedient growth were selected for further analysis.

During cultivation the hairy roots cultivated on the solid medium displayed typical hairy root characteristics such as the ability to grow prolifically in PGR-free media as well a typical anti-gravitropic growth (Figure 4.3.4A and B) (Srivastava and Srivastava, 2007; Veena and Taylor, 2007). According to Giri and Narasu (2000) this response is very advantageous as it improves the aeration of roots, resulting in an enhanced biomass accumulation in the liquid medium. An interesting response, the appearance of nodule-like structures (Figure 4.3.4C and D) was observed during cultivation of roots of clone C58C1-g in the liquid suspension medium. Nodule development is usually a response associated with the symbiotic interaction with rhizobacteria which provide the host plant with nitrogen (Taiz and Zeiger, 2002). However, since the roots were cultivated in aseptic conditions, it would be interesting to determine what these structures are and what their function may be. This anatomical observation was associated with cultures that were close to stationary phase and it was usually noted closer to the time for subculture. Nodular-like structures may possibly be induced in *S. frutescens* transgenic roots when nutrients become limiting and the medium poorly aerated. The advantage of this hairy root system is that the roots are established as independent organs in the medium and they can be clonally propagated by subculture of root tips (Boisson-Dernier *et al*., 2001).

Interestingly, initially during cultivation of roots in the liquid medium a difference in the morphology of the roots of clone C58C1-g and e was observed (Figure 4.3.4E). The differences in these morphologies may be due to the type of tissue (root tips versus intact tissue) selected for the initiation of cultures or alternatively the time during the growth cycle during which the roots were harvested may have influenced this growth response. Alternatively, this may have been due to the insertion of the T-DNA which is associated with

random positioning on the plant genome. Trangenes inserted in heterochromatic regions may be poorly expressed whereas those in closer proximity to enhancer elements may be strongly upregulated in expression. Problems associated with morphological characteristics (intra-clonal variation) may also arise due to somaclonal effects of tissue cultures.

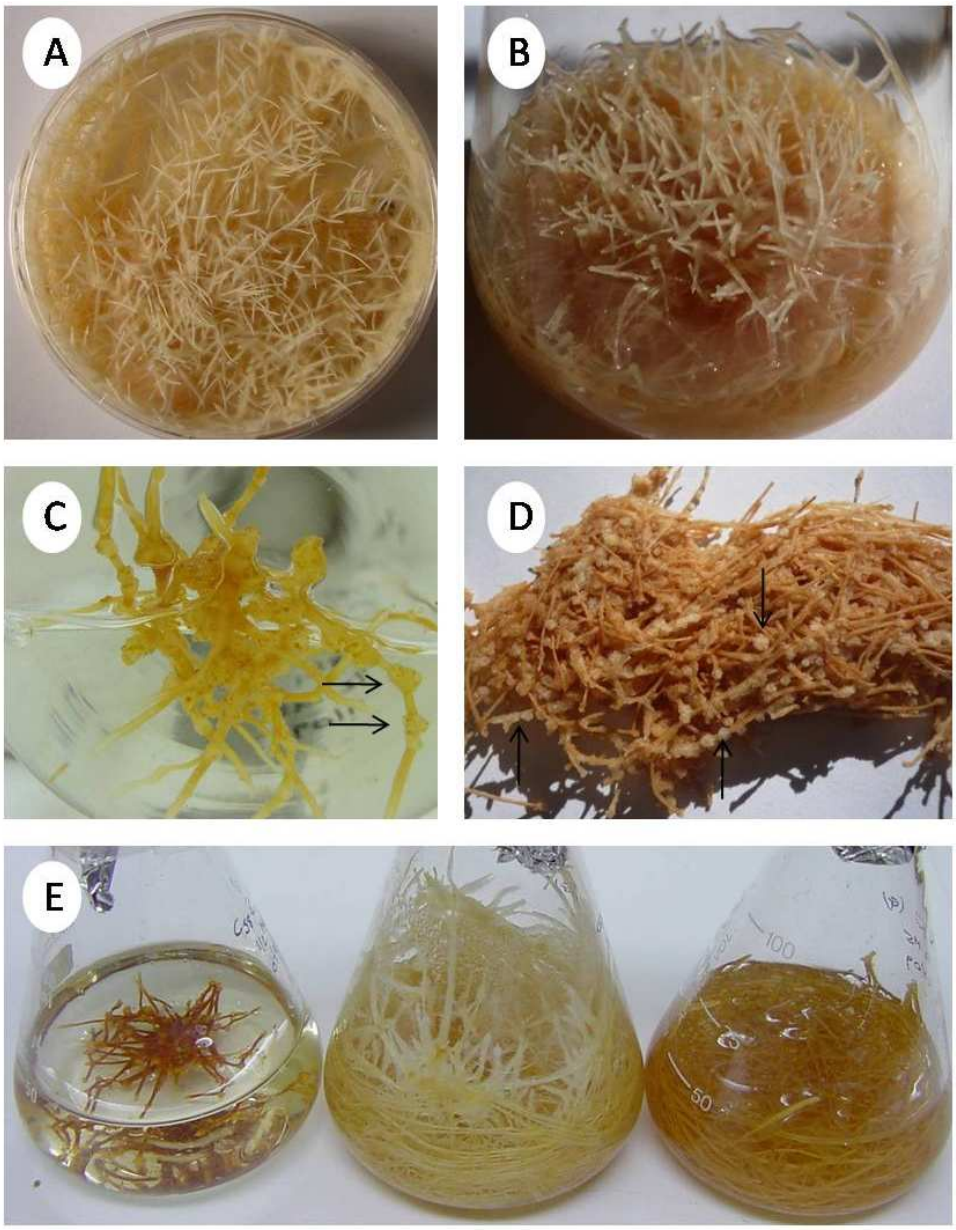


Figure 4.3.4: Hairy roots cultured in solid and liquid cultures displaying a typical hairy root response. A) Anti-gravitropic root mass growing on solid medium after 28 days of culture; B) Liquid-shake culture of clone C58C1-g showing abundant proliferation of hairy roots. Cultures were grown for 28 days; C) Initial formation of nodular structures on hairy roots (clone C58C1-g); D) Intense nodulation of hairy root clone C58C1-g (arrows indicate nodule-like structures on hairy root clones) was observed at the end of culture, and, E) Intraclonal morphological variation within one clone (C58C1-g) cultivated on 0.5xMS medium

4.3.3 Growth kinetic analysis

The optimal cultivation conditions (composition of nutrients, carbon source and concentration, PGRs, ionic concentration, pH, light and temperature) are important as they can affect both biomass accumulation and secondary metabolism (Hu and Du, 2006). During this study the effect of the medium composition on the accumulation of biomass was investigated to determine the optimal growth medium for the four hairy root clones. Hairy roots can accumulate between 0.1 to 2.0 g dry weight per liter per day due to a high rate of linear extension and development of several meristems produced by the lateral branches or secondary roots (Oksman–Caldenty and Hiltunen, 1996). However the individual clones may require different culture conditions due to the random insertion and integration of T-DNA. This can also cause differences in their secondary metabolite accumulation in the different media (Hu and Du, 2006).

Table 4.3.5 and Table 4.3.6 represent the effect of different growth media on the four hairy root clones studied. White's medium did not favour hairy root growth compared to the other media tested. For instance, at the end of the growth phase clone C58C1-g grew up to 1.25 g fresh mass (FW) or 0.18 g dry mass (DW) on White's medium. The same clone (which had the lowest growth rate on White's medium) accumulated 5.38 g FW and 0.60 g DW biomass on B5 medium. Halving the MS salts decreased the biomass (DW) accumulated by the roots of this clone by 20% (0.47 g DW), therefore growth on MS medium was better for the C58C1-g clone (0.59 g DW). The difference in the growth of this hairy root clone (C58C1-g) can be observed in Figure 4.3.5. During this experiment both the dry mass and fresh mass was reported on. To obtain an accurate view of the growth characteristics for hairy root cultures, it is better to report on dry weight as an indicator of growth as this is a measurement of the total mass of the desiccated tissue (Taiz and Zeiger, 2002). This measurement is therefore useful as it excludes the fluctuations that can occur in the fresh mass of the tissue (Taiz and Zeiger, 2002).

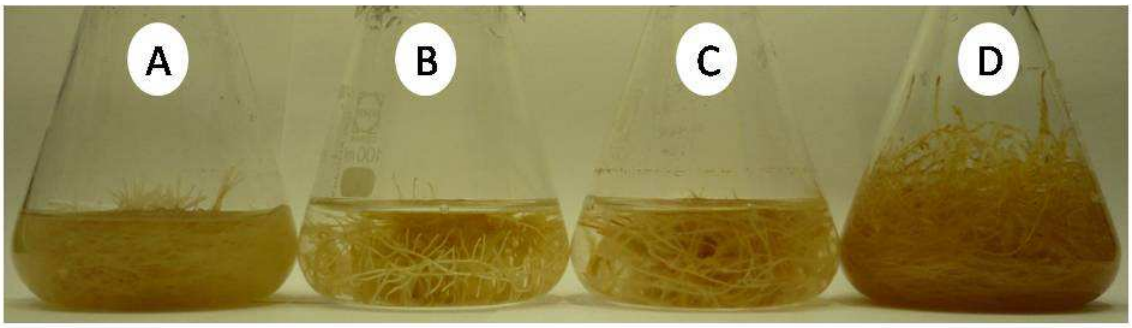


Figure 4.3.5: Liquid cultures of the hairy roots of clone C58C1-g cultivated on four media; A) MS; B) White's, C) 0.5xMS, and, D) B5 medium; displayed differences in their growth and morphology patterns

Even though, B5 medium promoted prolific growth for most of the clones, this result was in some instances statistically similar to those hairy roots growing on 0.5xMS or MS medium. For example the growth of clone C58C1-g was recorded at 0.59 g (DW) in MS medium and this is compared to growing this clone on B5 medium (0.62 g DW). The difference between these two media is that MS medium has a high salt content (potassium and nitrogen) and a low inorganic nitrogen source, whereas B5 medium has a low salt content and a high organic nitrogen source (Giri and Narasu, 2000; Tiwari *et al*., 2008). The higher level of thiamine (10.0 mg L^{-1}) in B5 medium than in the MS medium (0.1 mg L^{-1}) may have an influence, as Beyl (2000) commented that root cultures require thiamine for growth. These differences in the salt concentration and nitrogen source and organic nutrients may therefore account for the difference in growth observed. From these results, it can be concluded that halving MS salts resulted in a reduced growth, whilst cultivation of roots on White's medium was very unfavourable for growth of all four hairy root clones. As there was little statistical variation on B5 and MS medium for growth mass accumulation, MS medium was thus used for continuous culture of hairy roots.

Table 4.3.5: Fresh mass (g) accumulated by hairy root clones in different cultivation medium. (The values are the means ± SE, n = 30)

Clone	Fresh mass (g)			
	0.5xMS	**MS**	**B5**	**White's**
A4T	0.92 ± 0.21a	3.27 ± 0.70b	3.56 ± 1.10b	0.81 ± 0.12a
C58C1-g	4.21 ± 0.45b	7.12 ±0.69b	5.38 ± 0.81b	1.25 ± 0.11a
C58C1-e	5.96 ± 0.46b	8.54 ± 0.65b	8.12 ± 0.40b	1.60 ± 0.10a
LBA	1.37 ± 0.26ab	2.41 ± 0.39bc	4.52 ± 0.52c	0.75 ± 0.05a

Data were analyzed using the Kruskal-Wallis test to study the effect of the type of medium on the accumulation of fresh mass. Different letters indicate statistically significant difference at the 95% confidence level.

Table 4.3.6: Dry mass (g) accumulated by hairy root clones in different cultivation medium. (The values are the means ± SE, n = 30)

Clone	Dry mass (g)			
	0.5xMS	**MS**	**B5**	**White's**
A4T	0.10 ± 0.03a	0.21 ± 0.05ab	0.34 ± 0.09b	0.10 ± 0.01 ab
C58C1-g	0.47 ± 0.03ab	0.59 ± 0.03b	0.60 ± 0.03b	0.18 ± 0.01 a
C58C1-e	0.44 ± 0.06ab	0.46 ± 0.05ab	0.62 ± 0.02b	0.16 ± 0.01a
LBA	0.19 ± 0.03a	0.26 ± 0.05ab	0.46 ± 0.04b	0.10 ± 0.00 4a

Data were analyzed using the Kruskal-Wallis test to study the effect of the type of medium on the accumulation of dry mass. Different letters indicate statistically significant difference at the 95% confidence level.

Inter-clonal differences in biomass accumulation can be linked to the fact that each hairy root clone came from a separate transformation event. The T-DNA may therefore have inserted into different places in the genome (areas which are more or less highly expressed) and/or a different number of gene copies may have been inserted which may contribute to these differences (Hu and Du, 2006). A certain amount of heterogeneity has been identified in

different hairy root clones and repeated selection is therefore important for obtaining a stable, highly-productive hairy root line (Yukimune *et al*., 1994). These results also indicate the importance of determining the best medium for root growth.

Untransformed *S. frutescens* roots were cultured on medium without PGR, but these roots grew very slowly. This further concludes the requirement for supplementing the medium of non-transgenic cultures with auxin. However upon transfer to medium containing 0.1 mg L^{-1} NAA, callus production in these roots was observed. The ability for hairy root cultures to exhibit a prolific growth rate in the absence of growth regulators is one of the key characteristics which differentiate this type of culture system from normal roots (Veena and Taylor, 2007). The capacity of the hairy root clones to grow prolifically in a month period on auxin-free medium was regarded as evidence for their transgenic status. Further proof was provided by molecular evidence (Section 4.3.5, this Chapter). This tendency of hairy root cultures to maintain a high growth rate that is stable from one culture cycle to another is crucial for purposes of industrialization. In contrast *in vitro* roots of untransformed or wild-type roots are generally found to grow slowly and have a short life span (Giri and Narasu, 2000).

4.3.4 DNA isolation from hairy root cultures

Several problems are associated with the isolation of DNA from hairy roots. These include a low yield and a high phenolic and polysaccharide content. The presence of phenolic compounds (released from the vacuoles during cell lysis) is problematic as they bind to proteins and nucleic acids and form high molecular weight complexes. Oxidization of phenolics by cellular oxidases also results in the browning of the DNA pellet (Moyo *et al*., 2008). The polysaccharides are also problematic as they co-precipitate with nucleic acids in the presence of alcohols (Gambino *et al*., 2008) and they can interfere with DNA quantification by spectrophotometric methods (Zidani *et al*., 2005). Both of these compounds are unfavourable as they also obstruct enzymatic reactions (Weishing *et al*., 1995) and can therefore interfere with further downstream reactions (PCR and Southern hybridization).

Over the years several methods have been developed to extract nucleic acids which are free from polysaccharides and polyphenolics, however they do not always work effectively as they can be time consuming, complex and can result in a low nucleic acid yield (Gambino *et al*., 2008). The RNA extraction protocol developed by White *et al*. (2008) was efficient for the isolation of DNA from hairy root tissue. The extraction buffer contained the following chemicals

1) CTAB, a detergent which lyses plant cells (Liao *et al*., 2004) and effectively breaks them open; 2) EDTA which chelates magnesium ions (a important co-factor of nucleases) therefore protects DNA from endogenous nuclease enzymes (Zidani *et al*., 2005), 3) β-mercaptoethanol, a reducing agent which prevents oxidization of phenolic compounds and binding to the nucleic acids (Liao *et al*., 2004), and, 4) PVP, (polyvinyl-pyrrolidone polymers) which efficiently removes the phenolics (Khanuja *et al*., 1999). The only problem with this method was the high RNA levels which co-precipitated with the DNA (Figure 4.3.6A). Use of the Promega kit was efficient to purify DNA which is essential for further downstream experiments (Figure 4.3.6B). However, the only disadvantage of this kit was that the DNA quantity was reduced and several extractions were therefore required to obtain sufficient DNA for further downstream molecular examination.

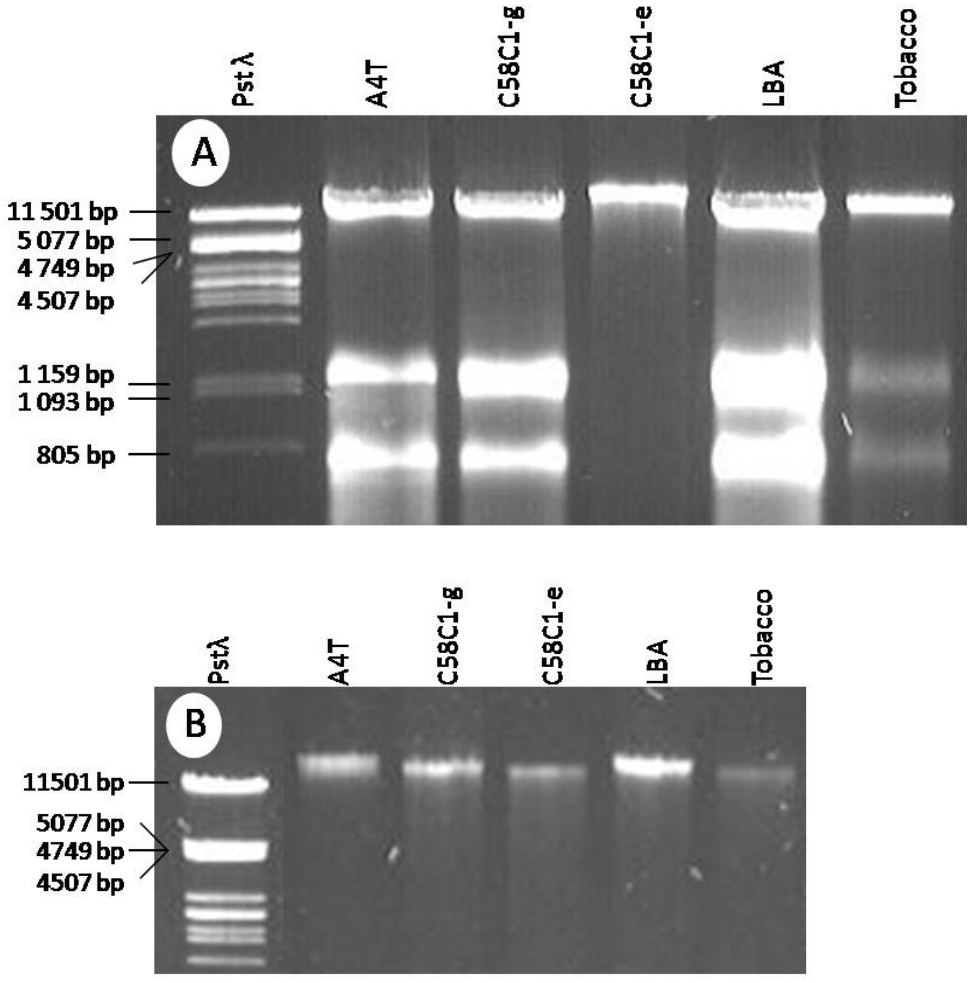


Figure 4.3.6: DNA isolated from *Sutherlandia* and tobacco hairy roots. A) Isolation using the method by White *et al*. (2008) resulted in high levels of DNA and RNA; and, B) Purification of samples using the Promega kit successfully removed RNA

4.3.5 PCR of *rol* genes and Southern hybridization

The integration of the T_L-DNA from *Agrobacterium* was confirmed by PCR analysis of *rol* A, B and C fragments respectively (Figure 4.3.7B-D). PCR analysis resulted in successful amplification of a 320 bp (*rol* A) and a 780 bp (*rol* B) products in all the hairy root clones analyzed. During the analysis of *rol* C, a 600 bp product was detected in all hairy root clones except clone C58C1-g which contained a larger product with a size between 900 and 1114 bp, which can indicate the presence of a truncated gene. *Agrobacterium* pRiC58 type strains often result in inverted repeats in transformed roots (Karimi *et al*., 1999).

The DNA of the hairy root clones and plasmid DNA (positive control) was digested with *Eco*RI and separated on a 1.0% (w/v) agarose gel (Figure 4.3.8A). Southern hybridization analysis of the DNA was conducted using the radioactively labeled *rol* A probe (Figure 4.3.8B). The preliminary results confirm that *rol* A was successfully integrated in clones C58C1-g; C58C1-e; LBA and tobacco DNA. However, no binding of the probe to the plasmid DNA was observed. The reason for this may be that too little plasmid DNA was digested and therefore very little to no detection was observed. Although PCR analysis indicated the presence of *rol* A in clone A4T no binding of the radioactive probe to the DNA was observed from Figure 4.3.8B either. It is possible, that the reason for this observation is that the DNA sample was loaded into two wells on the gel (Figure 4.3.8A) which may have resulted in the DNA not being concentration enough to allow for detection during the Southern analysis. This problem can be resolved by concentrating the DNA prior to restriction digestion and loading onto the gel. More comprehensive efforts to optimize the Southern hybridization technique are therefore required for future analysis.

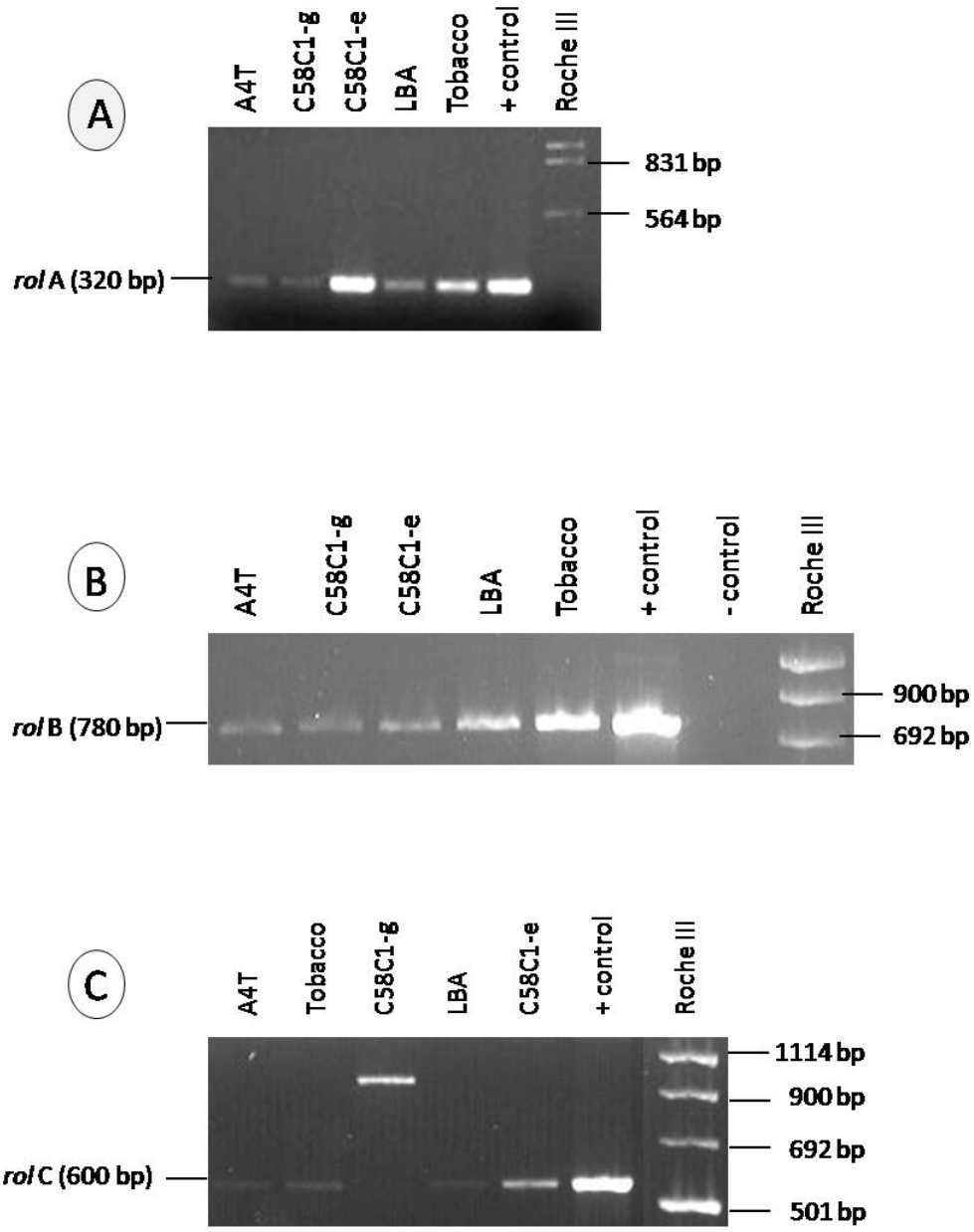


Figure 4.3.7: PCR amplification of *rol* genes. A) Amplification of *rol* A revealed a 320 bp fragment; B) A 780 bp band was detected for the *rol* B primer pair; C) Positive amplification of *rol* C resolved a 600 bp fragment after PCR for all the clones except for the C58C1-g clone where a larger fragment (between 900 and 1114 bp) was present. In all cases '+ control' refers to tobacco DNA which was used as a positive control and '– control' denotes a PCR reaction where no DNA was added to test for artifactual amplification (negative control)

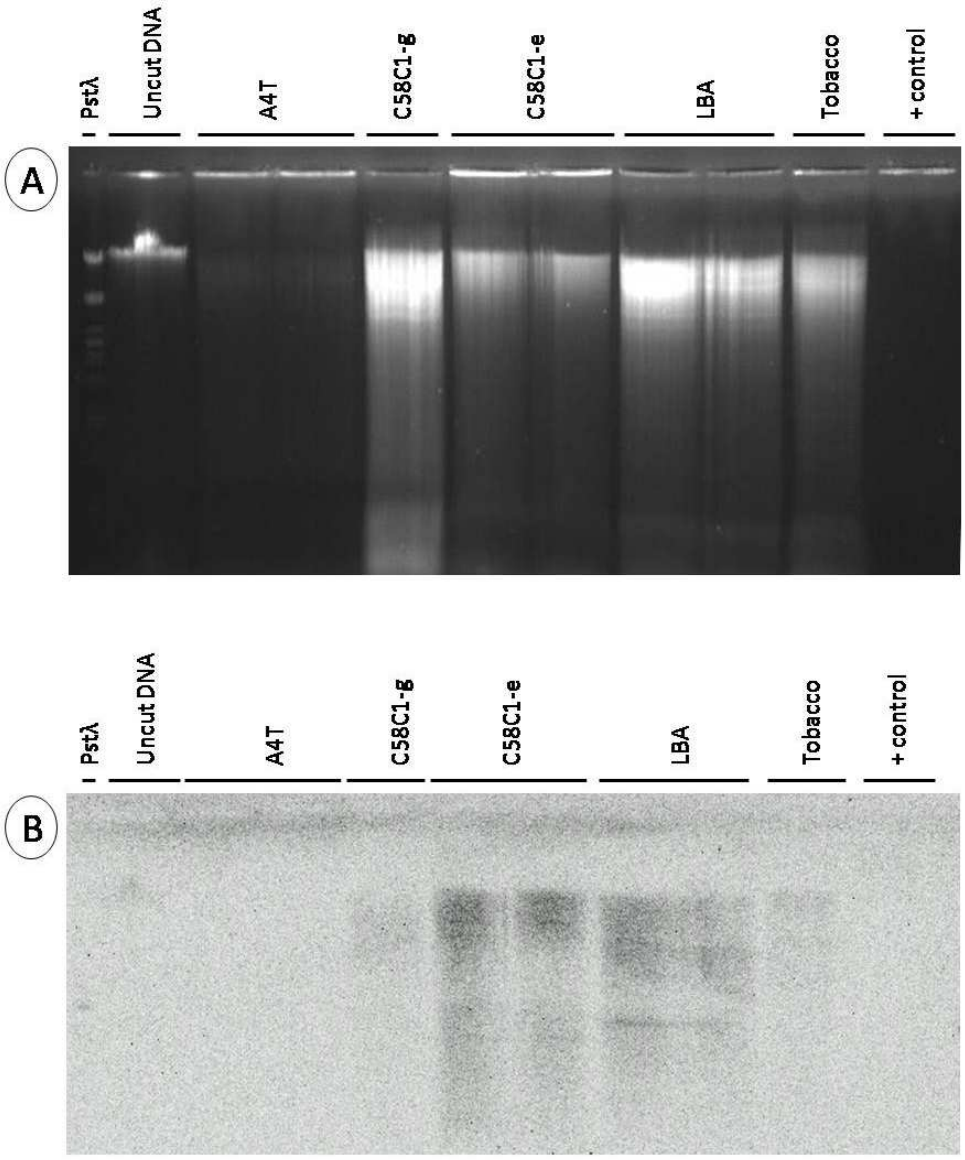


Figure 4.3.8: Restriction digest and Southern hybridization. A) Twenty microgram hairy root DNA and two microgram LBA plasmid DNA (+ control) was digested using the enzyme *Eco*RI, and, B) Southern hybridization was performed using a radioactively labeled *rol* A probe

4.3.6 Shoot regeneration from hairy roots

Regeneration of shoots from transformed roots can be spontaneous or it may require the use of plant growth regulators (Chaudhuri *et al*., 2006). In certain species the spontaneous regeneration of the shoots may be light dependent (eg. *Convolvulus arvensis*) or light independent (eg. *Nicotiana hesperis*) (Chaudhuri *et al*., 2006). *Sutherlandia* hairy root cultures that were placed in the light failed to regenerate shoots, however green callus production was

observed on some of the roots (data not shown). In contrast, the tobacco hairy root cultures spontaneously regenerated shoots and these were excised to establish plantlet cultures. The advantage of spontaneous plant regeneration is that it is commonly not associated with a callus phase (Chaudhuri *et al*., 2006) and the shoots that are produced are genetically similar to the hairy roots that it was derived from. The use of plant growth regulators often induces a callus phase, prior to somatic embryogenesis and these plants may not necessarily be genetic clones due to somaclonal variation during this regeneration procedure. Successfully induction of regeneration of shoot or somatic embryos from *S. frutescens* (and tobacco) hairy root cultures was achieved by cultivation of roots on a medium containing the plant growth regulator, BA at both concentrations (0.5 and 1 mg L^{-1}) investigated (Figure 4.3.9). During regeneration the production of callus was observed as well as the appearance of meristemoidal tissue which could lead to the production of somatic embryos (Figure 4.3.9A and B). The shoots of both *S. frutescens* (Figure 4.3.9C) and tobacco (Figure 4.3.9D) which were produced were excised and successfully established on solid MS medium and continued to grow. The next step will involve more thorough investigation of the copy number of the *rol* genes which inserted into the genome as this can also significantly influence growth (and secondary metabolite production) of different hairy root clones. One of the features of hairy root cultures is the ease of regenerating whole transgenic plants (Oksman-Caldenty and Hiltunen, 1996). This is another distinguishing feature for transformation.

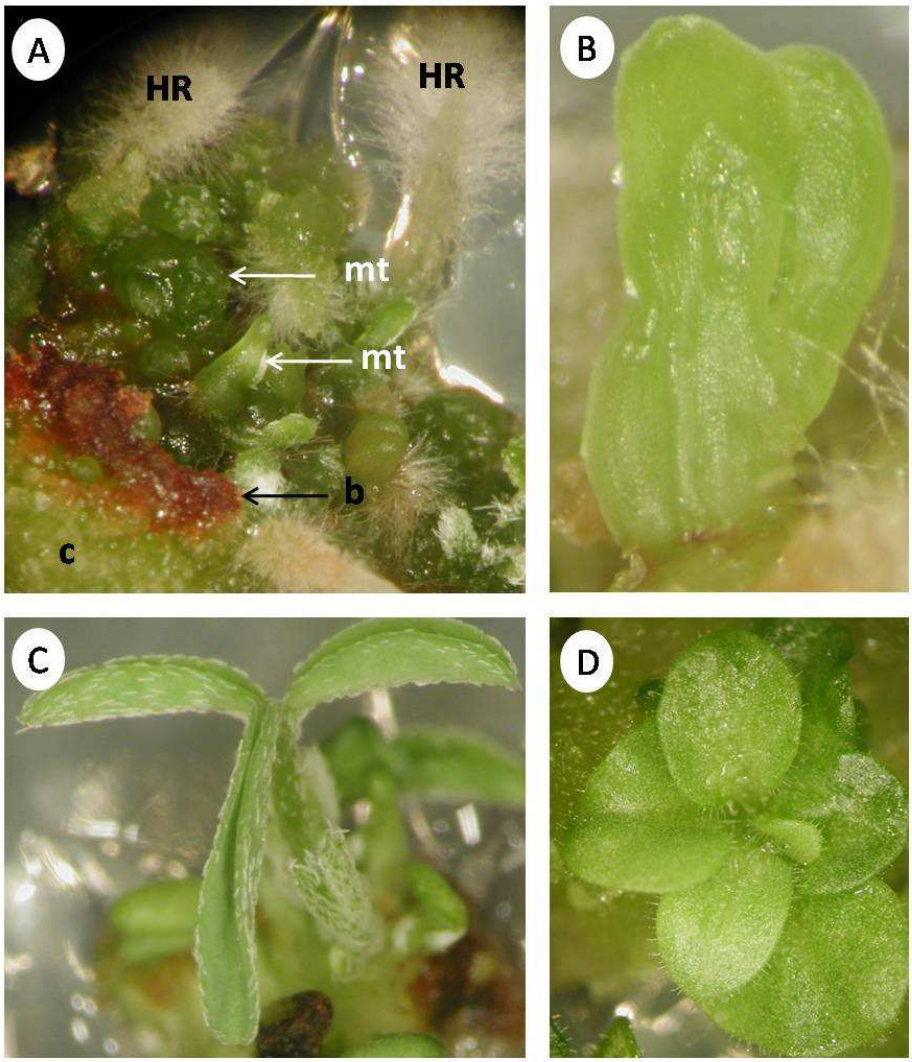


Figure 4.3.9: Regeneration of shoots of *S. frutescens* (A-C) and tobacco (D) from hairy roots using plant growth regulator BA. A) the appearance of meristemoidal tissue (mt), callus (c) and hairy roots (HR), during regeneration some of the callus also turned a brown-red color (b) which may be indicative of anthocyanin production; B) A closer inspection of some of the meristemoidal tissue; C) A regenerated *S. frutescens* shoot, and D) A regenerated tobacco shoot

4.4 CONCLUSION

Genetic transformation with *Agrobacterium* is species and explant dependent. Some plants are recalcitrant to transformation. During this study, *S. frutescens* could successfully be transformed using all three *Agrobacterium* strains and transformation was more dependent on

the type of explants used than the bacterial strain. The hairy root cultures were successfully established in both a liquid and solid culture system and they displayed typical hairy root characteristics such as anti-gravitropic growth and an increased growth rate. The establishment of liquid cultures is important as upscaling to bioreactors is vital for industrialization of this system. The molecular analysis using PCR amplification confirmed the presence of all three *rol* genes of the T_L-border in each of the four hairy root clones. Transgenic shoots were successfully regenerated from the hairy root cultures using a plant growth regulator in the cultivation medium. In future, further studies can be conducted to elucidate secondary metabolite synthesis and accumulation in liquid cultures during the growth cycle to investigate the phase during which metabolites are optimally synthesized. This result can benefit the development of methods for secondary metabolite extraction and product development. The protocol developed here is easy, simple and reproducible. It may thus be readily adopted for more intricate molecular genetic engineering. This may be regarded as a major achievement.

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CHAPTER 5:

Nitrogen stress in *S. frutescens* shoots and hairy root cultures

5.1 INTRODUCTION

5.1.1 The use of *in vitro* cultures to study stress biology

The study of the stress response on a whole plant level is complex due to the fact that the majority of tissue is made up of non-growing cells. This makes it challenging to investigate the response of biochemical processes in the actively growing cells/regions (Ahmad *et al*., 2007). *In vitro* organ and cell cultures are ideal to study the effect of stress for several reasons. Firstly, *in vitro* cultivation provides a controlled and uniform environment which can easily be manipulated to investigate the effect of stress on the biochemistry and physiology of the cultures at the cellular and organ level (Ahmad *et al*., 2007). Secondly, these cultures allow the accurate measurement of the growth and response to the applied stress (Shibli and Al-Juboory, 2002). During tissue culture, those lines which display enhanced stress tolerance can be identified, multiplied and used to establish whole plants with better tolerance to abiotic stress conditions in the field (Ahmad *et al*., 2007). The use of shoot cultures is preferred above the use of cell cultures as they are genetically more stable (Shibli *et al*., 2007) and can be rooted *in vitro*, whereas cell cultures can undergo somaclonal variation during the regeneration of shoots. The plants derived from cell cultures are thus not necessarily exact genetic replicates of the original cell from which they were derived (Rout *et al*., 2000).

5.1.2 The role of nitrogen in the plant

Nitrogen is a macronutrient and limited or excess nitrogen can thus exert an abiotic effect on the *in vitro* microplants. Nitrogen application is known to increase vegetative growth which can be beneficial when the whole shoot biomass is the product (Carrubba, 2008). However, nitrogen application can also negatively impact medicinal and aromatic plants (MAPs) causing for example a delay in flowering, interference with the production of essential oils and increased lodging (Carrubba, 2008). A nitrogen deficiency results in the accumulation of reactive oxygen species (ROS) and a concomitant increase in anti-oxidant enzymes to scavenge these compounds (Kováčik and Bačkor, 2007).

A plant cultivated in limited nitrogen conditions can respond in several ways such as a) enhanced development of roots to source nitrogen deeper in the soil matrix; b) relocation of the nitrogen in the plant to the actively growing regions (from the older leaves to the younger leaves and meristem); or, c) forming symbiotic relationships with micro-organism such as rhizobacteria to fix atmospheric nitrogen (legumes). Nitrogen availability can also affect important metabolic pathways. For example, the enzyme rubisco which is involved in the Calvin cycle (responsible for photosynthetic carbon reduction) accounts for more than half of the nitrogen in a leaf. A reduction in nitrogen can therefore also affect photosynthesis in the long run (Lambers *et al*., 1998).

5.1.3 Aims of this study

Since nitrogen is an important macronutrient, the effect of the nitrogen (NH_4NO_3 and KNO_3) availability in the cultivation medium on the synthesis of GABA, arginine, asparagine and canavanine as well as the physiological changes were investigated using *in vitro* shoot and hairy root cultures of *S. frutescens*. Although this study was not conducted to investigate the effect of abiotic stress on the interaction between the carbon and nitrogen metabolism, the results and discussion section will refer to some of these pathways to assist with the explanation of the results.

5.2 MATERIALS AND METHODS

5.2.1 Plant stock in a continuous culture system

The shoots used for this experiment were obtained from *in vitro* cultured plantlet stocks cultivated on MS medium (for details refer back to Section 3.2.2). Root tips from clone C58C1-g were obtained from cultures on solid 0.5xMS medium (for details refer back to Section 4.2.4). These cultures were subcultured on a monthly basis onto new media. To investigate the effect of nitrogen availability on the synthesis of canavanine, arginine, GABA and asparagine, shoot and root cultures were transferred onto modified MS (Murashige and Skoog, 1962) medium. The basic MS medium (referred to as 1x nitrogen) was adapted to contain twice the amount of nitrogen (3.8 g L^{-1} KNO_3 and 3.3 g L^{-1} NH_4NO_3; referred to as 2x nitrogen) or half of the amount of nitrogen (0.95 g L^{-1} KNO_3 and 0.825 g L^{-1} NH_4NO_3; referred to as 0.5x nitrogen), whilst all other compounds were maintained as stipulated (Table A2; Appendix). The following chemicals were added during the preparation of these media; 3% (w/v) sucrose, 0.1 g L^{-1} myo-inositol, pH adjusted to 6.0 using 1 M NaOH or 1 M HCl and

solidified with 0.8% (w/v) agar. Three meristematic shoots (2 cm each) were placed in a glass vessel containing 25 ml medium and these were placed in the light growth chamber (conditions as described in Chapter 3; Section 3.2.1.1). Three root tips (1 – 2 cm) of the hairy root clone C58C1-g were aseptically transferred onto the nitrogen modified media in a Petri dish. The hairy root cultures were transferred to the dark growth room. Upon harvesting, the shoots were weighed for fresh weight analysis and the rooting response was also noted. The plant material was freeze dried for three days and stored at -80˚C until metabolite analysis.

5.2.2 Extraction of canavanine, arginine, GABA and asparagine

The freeze dried material from each bottle or Petri dish was ground to a fine powder and approximately 0.06 g tissue was used for metabolite analysis. Methanol (in a ratio of 2 µl: 0.01 g tissue) was added to all samples and sonicated for 15 minutes prior to centrifugation. A 50 µl aliquot of the sample was placed into a glass hydrolysis tube and dried under vacuum for one hour. Thereafter, 20 µl of a methanol: water: triethylamine (2: 2: 1; v/v) solution was added to each sample to adjust the pH and samples were dried for one hour. The samples were derivatized by adding 20 µl of the derivitizing solution (methanol: water: triethylamine: phenylisothiocyanate (PITC); 7: 1: 1: 1 (v/v)) and the mixture was incubated at room temperature for 10 min. The samples were then dried under vacuum for 1.5 hours or until complete dryness, but for no longer than three hours. Finally the derivatized dried sample was dissolved in 200 µl of Picotag® sample diluent (Waters, Millford, MA, USA) and filtered through a 0.45 µM filter.

5.2.3 LC-MS analysis of canavanine

The analysis was conducted on a Waters Alliance HPLC coupled to a Waters API Quatro Micro-triple quadrupole mass spectrometer (Waters, Millford, MA, USA). Separation was achieved on a Waters Xbridge C18, 2.1 x 50 mm, 3.5 µm column using a 0.1% formic acid (solvent A) to acetonitrile (solvent B) gradient. The gradient consisted of a 1 minute isocratic step of 96% solvent A, followed by a linear gradient over 7 minutes to 40% solvent A, 60% solvent B and returning to the initial conditions for 7 minutes. The mass spectrometry (MS) method consisted of a MRM (multiple reaction monitoring) of 312.2 > 239 using a cone voltage of 15 V and collision energy of 15 for canavanine. A linear 4 point calibration curve was created using canavanine standard (Sigma, USA). The limit of detection of the method is 0.05 mg g^{-1} and the recovery (as determined by repeated extractions of the same sample) is 66.2%.

5.2.4 Data collection and statistical analysis

Fifteen bottles and Petri dishes, each containing three shoots or root tips were prepared for each medium (treatment). The shoots and hairy root cultures on different nitrogen media were harvested after 21 days. The data collected from the shoots from each treatment was the total fresh weight (g) and the percentage shoots producing roots. This experiment was repeated three times. For the metabolite analysis, seven samples were used to measure canavanine, GABA and asparagine and three samples were used to measure arginine in the shoots. For the hairy roots, four samples were used to measure all the metabolites. Quantitative analysis is represented as mean values and the reproducibility of the results is expressed as standard error. The normality of the data was determined using a Shapiro Wilk's W test prior to further analysis using a Post Hoc Tukey HSD test or a Kruskal-Wallis test depending on the normal distribution of the data. The data for the regeneration of roots from microshoots, recorded as a percentage, were arcsine transformed prior to analysis using the Kruskal-Wallis test. The statistical studies to determine the correlation between metabolite changes were determined using a Mann-Whitney U test. The differences between means reaching a minimal confidence level of 95% were considered as being statistically significant. All data were analyzed with the use of Statistica Release 8 program (Statsoft Inc. 2007).

5.3 RESULTS AND DISCUSSION

Nitrogen is one of the macronutrients which are important for the growth and productivity of plants (Noguchi and Terashima, 2006). A reduction in the total nitrogen and protein content in both the shoots and roots were observed in *Matricaria chamomilla* plants cultivated in limited nitrogen medium (Kováčik and Bačkor, 2007). When exposed to low nitrogen conditions, spinach leaf cells exhibited an increased carbohydrate content, which results in an increase in the carbon: nitrogen (C: N) ratio (Noguchi and Terashima, 2006). The increase and accumulation of carbohydrates can cause the suppression of photosynthesis due to a reduction in the amount of photosynthetic components such as rubisco (Noguchi and Terashima, 2006). During these conditions, the excess carbohydrates are metabolized by the TCA cycle and the components of the respiratory electron transport chain and this suppresses the rise in the C: N content (Noguchi and Terashima, 2006). This may explain that during limited nitrogen conditions enhanced nitrogen partitioning to the mitochondrial proteins and a reduction in the chloroplast proteins becomes more pronounced (Noguchi and Terashima, 2006). Additionally, increased consumption of carbohydrates may also prevent the production of ROS which would damage the cell (Noguchi and Terashima, 2006). The change in the nitrogen availability therefore influences several pathways (respiration and photosynthesis

amongst others) and more studies are required to determine the precise changes in these pathways which affect the nitrogen metabolism.

For quality control purposes during the production of commercial *Sutherlandia* pharmaceuticals, the four metabolites (canavanine, arginine, asparagine and GABA) amongst others (such as pinitol) are monitored. Quantifying the effect of medium additives in tissue culture on the synthesis of these metabolites is not only important for assessing their influence on nitrogen metabolism, but it also has crucial commercial implications. Currently, little is known about the nutritional requirements for commercial agricultural production of South African medicinal plants. Even though this system, here in this study, used *in vitro* manipulation of unrooted microshoots, any information on the response to mineral nutrient acquisition and their impacts on both the metabolome and growth, are valuable. Such strategies may be adopted and adjusted for the use in field cultivation.

5.3.1 Metabolite levels of *in vitro* shoots cultivated on different nitrogen media

From this study, it can be deduced that nitrogen has a significant impact on the free amino acid pool and that synthesis and subsequent accumulation of these metabolites is strongly dependent on nitrogen availability (Figure 5.3.1A-D). Arginine, canavanine, asparagine and GABA are representatives of the pool of free amino acids and amino acid analogues in plant tissues. The synthesis of three of these metabolites (asparagine, arginine and canavanine) appears to be directly related to the amount of nitrogen supplied to the shoots (Figure 5.3.1A-C). However, the opposite effect was noted for GABA, as less GABA accumulated with higher nitrogen availability (Figure 5.3.1D).

When nitrogen in the MS medium was low (0.5x nitrogen), canavanine (Figure 5.3.1A) was detected at a significantly decreased level (1.98 mg g^{-1} DW) than the control (1x nitrogen; canavanine; 7.8 mg g^{-1} DW). Exposure of plants to higher levels of nitrogen (2x nitrogen) did not have a significant impact on canavanine accumulation, possibly indicating a 'threshold' effect for canavanine metabolism. It is also possible that other nutrients or medium components may be important for canavanine synthesis. For example, in *Canavalia ensiformis* callus cultures, canavanine (0 – 3 mg g^{-1} DW) could only be detected in those cultures cultivated on Phillips and Collins (1979) (PC) medium, whilst cultures on Linsmaier and Skoog (1965) (LS) medium lacked canavanine (Ramirez *et al*., 1992). However, no attempt was made to identify the compounds contributing to this result (Ramírez *et al*., 1992).

From the medium composition in Table A1 (Appendix) it appears that the PC medium contains more phosphates than the LS medium and in the future the effect of this macronutrient on amino acid synthesis, can be investigated.

A pattern similar to where higher concentrations of NO_3/NH_4 in the medium directly correlated to increases in canavanine was observed for arginine. The arginine level was enhanced about 3.5-fold from 3.5 mg g^{-1} DW (0.5x nitrogen) to 12 mg g^{-1} DW in the control shoots. More nitrogen in the medium (2x nitrogen) resulted in an additional 3-fold increase with the arginine level approaching a value of 35 mg g^{-1} DW (Figure 5.3.1B). Arginine as a nitrogen storage or reserve molecule (Gao *et al*., 2009), in this instance, may act directly or indirectly (via nitric oxide or polyamines) as a signaling molecule to indicate the nutrient (or stress) status of the roots (Gao *et al*., 2009). It is therefore required at higher levels so that it can be transported to the other organs in a whole plant (previously summarized in Section 2.4.2.2). However, unrooted microplants were used for these experiments in this study and one of the disadvantages of such a system is that inter-organ cross-talk mechanisms are thus not operational.

A limited nitrogen level also significantly reduced the accumulation of asparagine in the shoots (0.5x nitrogen) (Figure 5.3.1C). As more nitrogen became available, the asparagine level increased 1.7-fold from 41 mg g^{-1} DW to 69.0 mg g^{-1} DW in the control medium. A further increase in the nitrogen content in the medium (2x nitrogen) resulted in the asparagine level being elevated to 90.8 mg g^{-1} DW. However statistical analysis indicated that this change was not significant. Like arginine, asparagine is also one of the amino acids produced during nitrogen assimilation. It also functions as a nitrogen storage compound which is transported in the plant and is used for protein synthesis (Section 2.4.2.1). The enzyme asparagine synthase (See Section 2.4.2.1; Literature review) which is responsible for asparagine synthesis is inhibited by high levels of carbohydrates. Thus since the carbohydrate levels are high during reduced nitrogen availability, it is expected that asparagine synthesis would be inhibited. As more nitrogen becomes available in the medium, the C: N ratio can be reduced as the carbohydrates are being used for the synthesis of amino acids, resulting in an increase in the asparagine synthesis.

An inverse relationship was noted with regards to GABA levels and increasing nitrogen levels (Figure 5.3.1D). For example, when the nitrogen level in the growth medium was halved (0.5x

nitrogen), GABA levels was significantly higher (4.8 mg g^{-1} DW) representing a 2.3-times increase from the control (2.2 mg g^{-1} DW). However, the GABA level of shoots in 2x nitrogen medium was slightly reduced below the control level (1.92 mg g^{-1} DW). Several ideas may explain the inverse correlation with regards to GABA and nitrogen concentrations in microshoots (Figure 5.3.1D). Firstly, changes in the TCA cycle may be related to the response in GABA synthesis. Limited nitrogen causes an upregulation of some of the TCA cycle enzymes which metabolizes the excess carbohydrates. This may induce a reduction in the use of GABA as a substrate for succinate production in the TCA cycle in the shoots (See Section 2.4.2.4), which would explain the increase in the GABA levels. Additionally GABA synthesis from glutamate is enhanced when glutamine synthesis is inhibited, protein synthesis is reduced or protein degradation is enhanced which describes the conditions during nitrogen stress (Shelp *et al*., 1999). At higher nitrogen concentrations, the metabolic pathways function normally (TCA, respiration and photosynthesis) and here the nitrogen may be fluxed into those pathways involved in the synthesis of other proteins causing a reduction in GABA levels. Alternatively, the reduction in carbohydrates may restore GABA's role for the production of intermediates for the TCA cycle, which would increase its degradation.

Therefore, the synthesis of the three amino acids is limited by low nitrogen levels and under these conditions the pathway for GABA synthesis appears to be favoured. This suggests that GABA may act as a nitrogen storage molecule during limited nitrogen conditions. At higher nitrogen levels, the flux towards GABA may be down regulated and the nitrogen assimilated by other basic metabolic pathways such as amino acid and protein synthesis. Excess nitrogen can cause some increase in the amino acid synthesis, but the excess nitrogen may be stored in other forms.

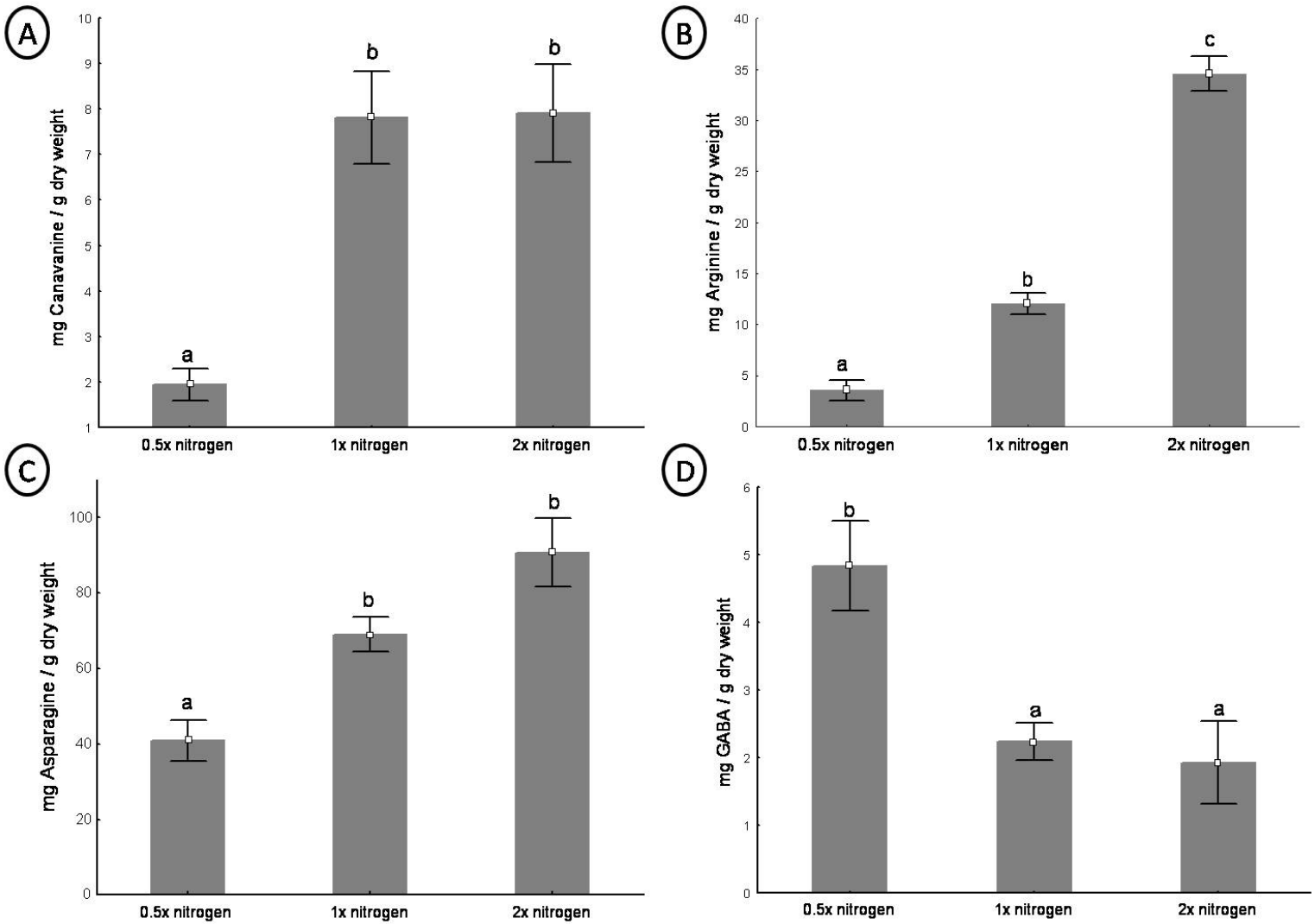

Figure 5.3.1: The canavanine, arginine, asparagine and GABA level in *in vitro* cultivated *Sutherlandia* shoots cultivated on MS medium containing different nitrogen levels (0.5x nitrogen; 1x nitrogen (control) and 2x nitrogen). The bars represent the mean ± standard error and different letters indicate a statistically significant difference at the 95% confidence level

5.3.2 Physiological response of *in vitro Sutherlandia* shoots cultivated on different nitrogen media

Overall, the composition of the medium has a strong influence on the growth capacity of *in vitro* shoots, exerting microdevelopmental changes. The general trend indicated that the fresh mass increased as more nitrogen became available (Figure 5.3.2A). Shoots cultivated in 0.5x nitrogen medium exhibited a significant reduction (1.2-times) in fresh mass (0.119 g) from the control shoots (0.146 g). This reduction could either be caused by a lowered water uptake and/or reduced accumulation of biomass. Plants cultivated in a limited nitrogen environment experience decreased root hydraulic conductivity, which in turn negatively influences water uptake. This may increase the inability of the leaves to maintain their turgor pressure as observed in the *M. chamomilla* shoots (Kováčik and Bačkor, 2007) which can result in reduced growth. The shoots on 2x nitrogen medium only displayed a moderate increase in fresh mass (0.160 g), which was not significantly different from the control cultures. However, it remains to be established whether the change in fresh mass is related to the change in the biomass accumulation (indicating possible changes in photosynthesis) or whether it is due to a change in water uptake. The measurement of dry weight would confirm which of these factors are responsible. However the dry mass was unfortunately not determined as the amount of tissue generated during tissue culture was limited and was solely used for metabolite analysis.

Roots spontaneously developed on the *in vitro* shoots cultivated in the MS medium (Figure 5.3.2B). Here, it was found that the nitrogen level had an impact on the root induction in the *in vitro* microshoots and that higher nitrogen levels, reduced the rooting capacity. In 0.5x nitrogen medium, 52% of the shoots rooted, whilst only 37% of the shoots on control medium and 29% of the shoots on 2x nitrogen medium rooted. An improved rooting frequency during limited nitrogen conditions, may be related to increased use of assimilates for root development thereby enlarging the absorption surface for nitrogen uptake. This in turn may lead to lowered fresh mass. In contrast, those shoots that were cultivated on a higher nitrogen medium invested more of their photo-assimilates into shoot growth, which may be translated into a higher biomass. It would be interesting to investigate whether the rooting response is improved when shoots are cultivated in a medium containing lower nitrogen levels (below 0.5x nitrogen) as this may assist with improving the rooting capacity of *in vitro* shoots.

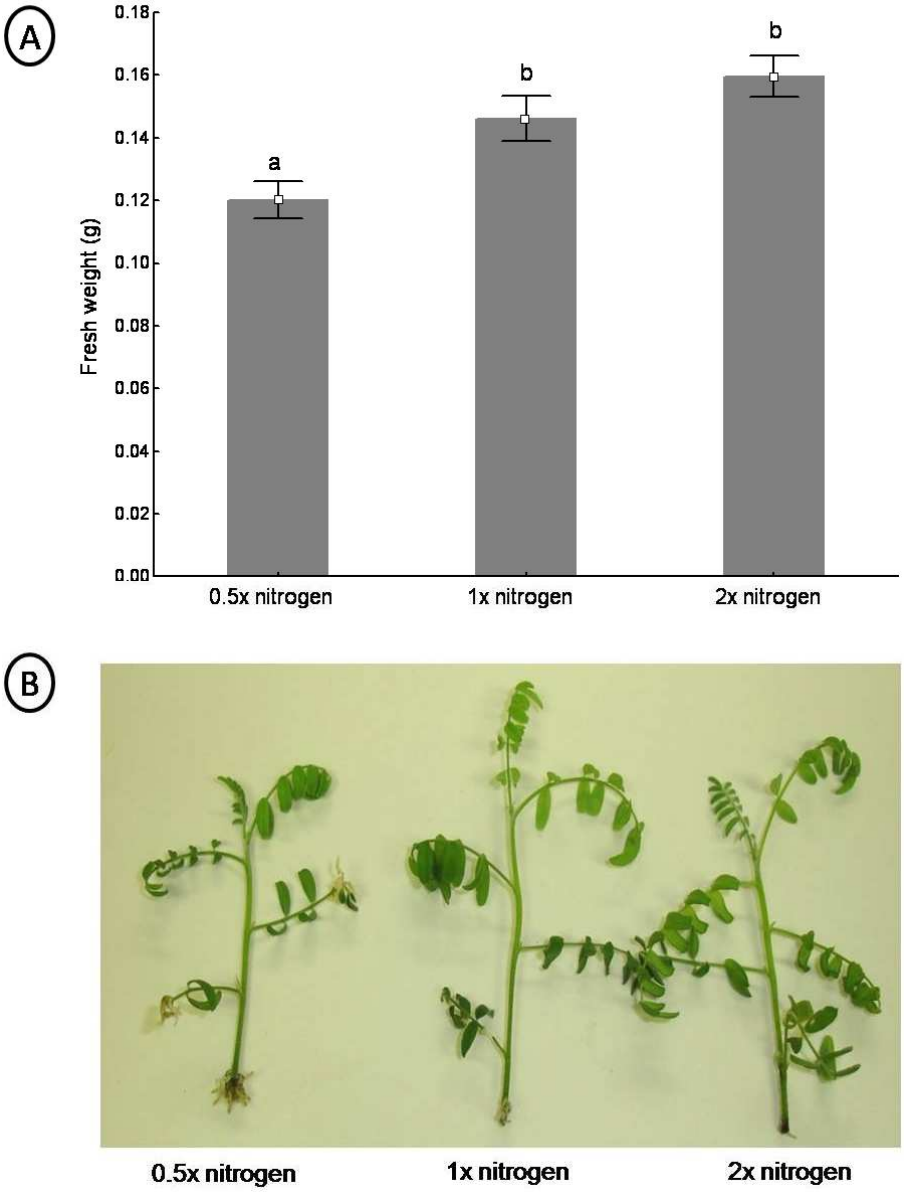

Figure 5.3.2: The morphological responses of *in vitro S. frutescens* shoots cultivated on MS medium amended to contain different nitrogen levels (0.5x nitrogen; 1x nitrogen (control) and 2x nitrogen). A) The accumulation of fresh mass (g) by *in vitro Sutherlandia* shoots cultivated on modified MS medium. The bars represent the mean ± standard error and different letters indicate a statistically significant difference at the 95% confidence level; B) Shoots cultivated on MS amended medium (from left to right): 0.5x nitrogen, 1x nitrogen, 2x nitrogen

5.3.3 Nitrogen availability in *Sutherlandia* hairy roots

Hairy roots have the ability to grow on a medium which does not contain any plant growth regulators. The presence of canavanine in the *in vitro Sutherlandia* shoot cultures, but absence in the hairy root cultures (results not shown) validates the results of Hwang *et al*.

(1996a) who also exclusively detected canavanine in the *in vitro* shoots of *Canavalia lineate*. Hwang *et al*. (1996b) also investigated canavanine synthesis in white and green *C. lineate* callus cultures and concluded that canavanine production was not favoured in the white callus. Canavanine synthesis therefore seems to require the differentiation of chloroplasts. In this study, canavanine was not investigated in callus, but dark-grown root cultures which are devoid of chloroplasts. This may be one reason for the lack of canavanine detection in these cultures. Alternatively, canavanine may have been present, but in such low levels that it was beyond the detection level of the LC-MS technique used. Another option is that canavanine synthesis was down-regulated in the transgenic hairy root cultures, due to the random insertion of the *rol* genes in the genome. It is also possible that canavanine (as well as other metabolites) were exuded in the medium and were therefore not detected in the root tissue. Due to the hairy root cultures lacking the ability to synthesize canavanine, further experimentation with stress effects on these cultures, was disregarded.

Hairy root cultures are cultivated in the dark and they therefore do not photosynthesize and the carbohydrate source for these roots is the sucrose added to the media. Since no additional carbon source is added during the cultivation period (21 days) the carbon source may become limiting towards the end of the cycle. Similarly to the shoots, limited nitrogen will (initially) result in a higher C: N ratio and the excess carbon is metabolized by the TCA cycle and components of the respiratory electron transport chain (Noguchi and Terashima, 2006). As the nitrogen content in the medium is enhanced, the C: N ratio is reduced and the free amino acid pool increases as the metabolic pathways return and function normally.

As in the shoots, arginine and asparagine levels in the hairy roots (for the C58C1-g clone) increased with a higher amount of nitrogen in the medium (Figure 5.3.3A and B respectively). The arginine level in 0.5x nitrogen cultivated roots (0.41 mg g^{-1} DW) significantly increased 3-fold, when the nitrogen content in the medium was doubled (control; 1.34 mg g^{-1} DW). Twice the nitrogen content in the control medium, also resulted in a significantly higher (1.6-fold more) arginine (2.14 mg g^{-1} DW) level in the roots. Arginine accumulation in the roots (and shoots) appears to be greatly dependent on the nitrogen availability. However, arginine accumulation in the roots did not occur to the same level as in the shoots (Figure 5.3.1B). Metabolites in hairy root cultures are often exuded into the medium. However, the cultivation medium was not investigated for its metabolite content and it is possible that these metabolites may have become root exudates in the medium for both shoot and root cultures.

Although not statistically significantly different, the asparagine level in the roots cultivated on 0.5x nitrogen medium doubled from 54 mg g^{-1} DW to 107 mg g^{-1} DW when the nitrogen content in the medium was doubled (control) (Figure 5.3.3B). However, contradictory to the shoots where the asparagine level continued to increase with more nitrogen, the asparagine level of roots on 2x nitrogen medium appeared to level off (111.6 mg g^{-1} DW). A reason for this may be that certain metabolic pathways, such as the TCA cycle which provides carbon skeletons (oxaloacetate) for the production of asparagine, may approach a steady state level. The asparagine content in the roots and the shoots were in a similar range (40 to 100 mg g^{-1} DW). From the correlation analysis conducted (Table A4; Appendix), the results indicated that the increase in arginine in the hairy roots was positively correlated with the increase in asparagine ($r = 0.8135$; $p = 0.0013$). The reason for this is likely due to their role as free amino acids which are synthesized during nitrogen assimilation.

The production of GABA in the roots (Figure 5.3.3C), did not follow a similar pattern as in the shoots (Figure 5.3.1D). Differences in GABA levels were observed, however this could not be verified through statistical analysis at the 95% confidence level. Modification of the medium resulted in approximately a 1.5-fold increase in the GABA level from 0.35 mg g^{-1} DW (0.5x nitrogen medium) to 0.53 mg g^{-1} DW (control). The opposite effect was observed in the shoots where the GABA level was halved when the nitrogen content was increased (0.5x nitrogen to control). However, a further increase in the amount of nitrogen available (2x nitrogen) caused almost a 2-fold reduction in the GABA level (0.25 mg g^{-1} DW). It is unclear from these results what the role of GABA in the roots is. GABA can act as a signaling molecule during stress conditions (oxidative stress) but in such situations, it does not always accumulate (Bouche and Fromm, 2004; Section 2.4.2.4). At normal nitrogen levels, GABA is thus able to accumulate, but under low nitrogen or high nitrogen levels, GABA may be shunted into other pathways or act rapidly as a signaling molecule. This results in the perceived lower concentration of this molecule (Figure 5.3.3C). Alternatively, GABA may play an important role in maintaining homeostasis in cultivated roots, and under high nitrogen, when carbon becomes more limiting, the rapid use of GABA in the TCA cycle may occur to provide for carbon skeletons. Additionally, the GABA level in the tissue may not necessarily represent the true values as translocation of GABA into the medium is a likely possibility. Furthermore, during transformation the incorporation of the transgenes may somehow interfere with GABA synthesis in the hairy roots. The GABA level in the hairy root cultures was below the level detected in the shoots.

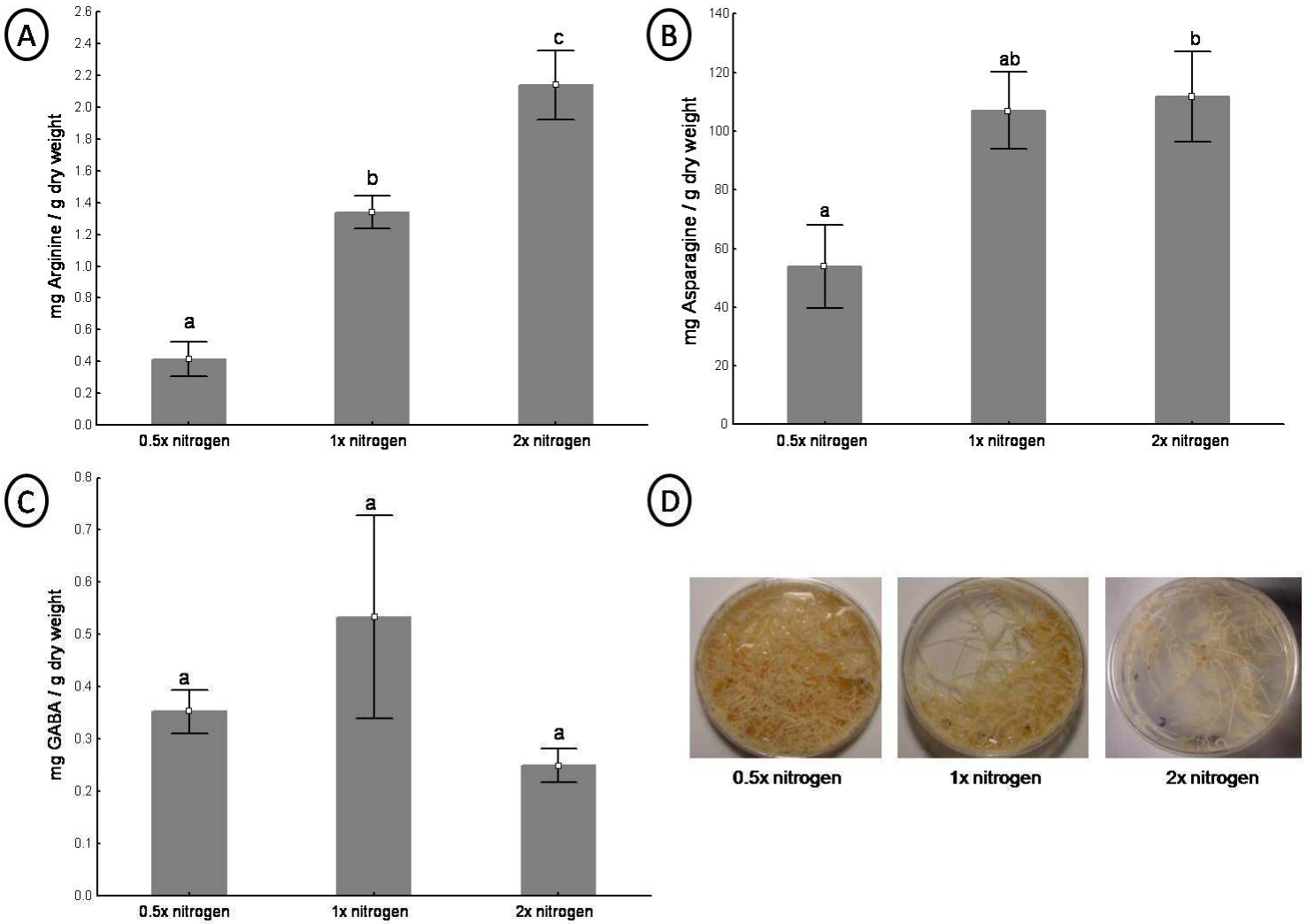

Figure 5.3.3: The metabolite level in *in vitro* cultivated *Sutherlandia* hairy roots of clone C58C1-g cultivated on different nitrogen levels (0.5x nitrogen; 1x nitrogen (control) and 2x nitrogen). The bars represent the mean ± standard error and different letters indicate statistically significant difference at the 95% confidence level

5.3.4 The physiological response of hairy roots to nitrogen availability

Previously (Chapter 4, Section 4.3.3; Figure 4.3.4A), it was indicated that hairy roots cultivated on various media had a difference in their growth index. A variation in the growth response was also observed in the roots cultivated on the different nitrogen amended media (Figure 5.3.3D). There appears to be an inverse relationship between the nitrogen content in the growth medium and hairy root growth. Hairy roots cultivated on a medium with low nitrogen content grew more expediently, and the increase in nitrogen content appeared to reduce growth. This is similar to the observation of the effect of nitrogen content on the production of roots in the microshoot. Rooting of *in vitro* shoots was higher when the nitrogen content in the medium was low (0.5x nitrogen). These results collectively indicate that rooting and root development is favoured by limited nitrogen supply, whilst higher levels of nitrogen may have a negative impact on this physiological response. This qualitative result (data not shown), is validated by the data of Kováčik and Bačkor (2007). These authors measured the root biomass of *in vitro M. chamomilla* plants under limited nitrogen conditions. Here, the increase in root growth was attributed to the preferential flux of photo-assimilates to the growth of the roots (Kováčik and Bačkor, 2007).

5.3.5 Comparison of metabolite levels of *in vitro* and *ex vitro* plants

Wild plants can accumulate canavanine levels between 0.42 – 14.5 mg g-1 DW, whilst commercial plants (SU1 variety) were found to accumulate between 1.6 – 3.1 mg g-1 DW (Van Wyk and Albrecht, 2008). Out of curiosity the plants growing in our glasshouse were also monitored for metabolite (arginine, canavanine, GABA and asparagine) levels. These plants (grown for 3 months and monitored during June 2009) accumulated approximately 3.88 mg canavanine g-1 DW (Table A9, Appendix). The canavanine level in the in vitro shoot cultures varied between 1.98 – 8.0 mg g-1 DW (depending on the nitrogen content) which is in the range reported by Van Wyk and Albrecht (2008). Interestingly, in vitro shoots had higher levels of asparagine (41 – 90.8 mg g-1 DW) compared to wild plants (1.6 – 35 mg g-1 DW; Van Wyk and Albrecht, 2008) or glasshouse plants (2.13 mg g-1 DW; Table A9, Appendix). For arginine, levels were low (0.15 mg g-1 DW; Table A9, Appendix) in glasshouse plants compared to in vitro shoots (3.5 – 35 mg g-1 DW) or wild plants (0.5 – 6.7 mg g-1 DW; Van Wyk and Albrecht, 2008). As for the GABA levels, plants in nature accumulated less GABA (0.23 – 0.85 mg g-1 DW; Van Wyk and Albrecht, 2008) than the in vitro shoots (1.92 – 4.8 mg g-1 DW), whilst the glasshouse plants accumulated approximately 2.42 mg GABA g-1 DW (Table A9, Appendix).

Taken as a whole, the results from these nitrogen experiments suggests that plants growing under limited nitrogen conditions in nature, would have reduced arginine, asparagine and canavanine content. The level of these metabolites can be enhanced by increasing the nitrogen availability to these plants. However, increased nitrogen might have the opposite effect on GABA levels. The reason for the variation in the data is that plants in nature are simultaneously exposed to a variety of stresses at a particular point in time. Seasonal and climatic changes are known to affect production of metabolites in plants (Chinkwo, 2005; Srivastava and Srivastava, 2007). It is possible that combinations of abiotic stresses and/or biotic stress can alter the metabolism in such a way to increase synthesis of the specific compounds to values higher than reported for the *in vitro* shoots and roots in this thesis.

5.4 CONCLUSION

In vitro propagation allows for rapid production of plants *en masse*, but for such a cultivation system to be commercially viable, once transplanted *ex vitro*, these plants should synthesize the metabolites at similar levels to wild plants. Tissue culture can lead to somaclonal variation which can have several impacts at both the biochemical and genetic levels. For this reason, I was interested in comparing the composition of propagated *S. frutescens* tissues to those growing in the wild. Although nitrogen may be limiting in nature, legumes such as *S. frutescens* have developed symbiotic relationships with rhizobacteria, which can enhance their nitrogen assimilation capability. Although nodulated cultures were not investigated, this would make for an interesting study in the future to elucidate the effect of nodulation on primary and secondary metabolism during stress conditions. In this study the four metabolites of interest, provided some preliminary insight into the metabolic response of primary metabolism as well as the physiological response to nitrogen stress. However, several secondary metabolites are also synthesized from primary metabolites. Examples include the shikimic acid pathway which requires PEP (from glycolysis) as a precursor or the terpenes which can be synthesized from pyruvate (Taiz and Zeiger, 2002). Changes in the secondary metabolite profile of *S. frutescens* require further scrutiny. Nutrient composition has significant impacts on nitrogen assimilatory compounds. Through the use of media manipulation, I was able to show promotory effects on canavanine, arginine and asparagine accumulation with higher nitrogen supplementation.

This study may serve as a scientific platform for future studies on 1) the elicitation of secondary metabolism, 2) the identification of compounds involved in the pharmacological

activity, and, 3) the development of a more comprehensive investigation into the stress response. The results presented in this chapter, can also be beneficial to the agricultural community such as farmers who would like to enhance the metabolic yield and quality of their product by the application of certain cultivation practices.

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CHAPTER 6:

Salt and water stress on *in vitro S. frutescens* shoots

6.1 INTRODUCTION

6.1.1 The influence of the adverse and beneficial effects of water and salt stress on the industry

The impact of stress on plants can be deleterious to some industries and beneficial to others. The harmful effects are observed when studying the response of food crops to stress. Stress can result in major losses in the crop yield. Additionally, stress can change the nutritional value and taste of fruits. For example, the accumulation of certain amino acids during salt stress can make strawberry fruit taste bitter and this is very unfavourable for the food industry and exporting of products (Keutgen and Pawelzik, 2008).

However, application of stress can be advantageous to the horticultural industry. An example is the application of an osmoticum to induce drought stress in the ornamental plant *Impatiens* which resulted in a reduction in growth. This response can be beneficial as it is cheaper to ship shorter plants (Burnett *et al*., 2005). The application of *in vitro* stress can also assist with the identification of tolerant lines which can be used to select and cultivate more stress tolerant plants. The *in vitro* application of stress, induced by moderate levels of osmoticum, can also increase the *ex vitro* survival of plants (Shibli and Al-Juboory, 2002). However, I was interested in investigating how the application of *in vitro* salt and water stress affect the synthesis of the four metabolites (GABA, canavanine, arginine and asparagine) as well as the influence it has on the physiology of these plants.

6.1.2 Effect of *in vitro* water stress on plant physiology and development

Plants exposed to drought exhibit a reduction in growth which can be attributed to a reduction in cell elongation and protein synthesis (Lambers *et al*., 1998). The shoots respond more notably than the roots during water stress and this may partly be due to the increase in transport of photo-assimilates to the roots. The increase in root development improves water sourcing and uptake capabilities of the plant, whereas the reduction in leaf size can decrease the area for water loss due to transpiration (Lambers *et al*., 1998). Drought stress also lowers

nutrient uptake in the roots and transport to the shoots, which is partly caused by the reduction in stomatal conductance and impaired membrane permeability (Hu and Schmidhalter, 2005). The wilting of plants observed during drought can possibly be correlated to a reduction in K^+ as this mineral is important for stomatal regulation, protein synthesis, homeostasis, osmoregulation, charge balance and the energy status of the plants (Hu and Schmidhalter, 2005). During water stress, plants respond by lowering the osmotic and water potential of the cells by the accumulation of solutes such as cations, anions, proline, free amino acids, soluble proteins, carbohydrates and phenols in the plant cells for continued water uptake (Ahmad *et al*., 2007).

6.1.3 Effect of *in vitro* salt stress on the general physiological and developmental responses

Plant species differ in their response to salt stress which impacts the yield, growth and nutrient acquisition of the plant (Shibli *et al*., 2003). Plants under salinity stress experience a reduction in growth due to a combination of physiological responses which include changes in their water status, photosynthetic efficiency as well as carbon allocation and utilization (Jaleel *et al*., 2007). However, the uptake of ions can have an additional deleterious effect on the plant when the ions accumulate to toxic levels. During salinity stress, the plant cell membranes become damaged due to the accumulation of ions and ROS which eventually interrupts plant lipid metabolism (Jaleel *et al*., 2007) subsequently causing lipid peroxidation.

These physiological changes in the shoot height, shoot dry weight, rooting percentage, root number and root length, have been observed during the induction of salt stress in several species such as Bitter almond (*Amygdalus communis* L.) (Shibli *et al*., 2003); 'Nabali' olives (*Olea europea*) (Shibli and Al-Juboory, 2002) and sunflowers (*Helianthus annuus* L.) (Dos Santos and Caldeira, 1999) to name a few. In the field, a farmer might identify the symptoms of salt stress as an increase in stunting, chlorosis, necrosis and flower abortion in the crop (Shibli *et al*., 2003). Plants subjected to salt stress also respond by decreasing their osmotic potential (osmotic adjustment) by synthesizing more dissolved solutes (as described in Section 2.5) (Shibli *et al*., 2007). The type of physiological and biochemical response can vary depending on the species, cultivar, genotype, the organ or type of tissue, developmental stage and tolerance to stress (Gaspar *et al*., 2002).

6.1.4 The use of *in vitro* cultures to study the effect of abiotic stress

As described earlier, the use of *in vitro* cultivated shoots to study the effect of stress is beneficial as it allows the change and study of single factors whilst keeping the other growth conditions optimal. This is important as the effect of salt or water stress is often amplified or obscured by other environmental conditions (Shibli *et al*., 2007).

6.1.5 Aim of this chapter

This chapter will focus on the effect of salt and water stress on the synthesis of GABA, canavanine, asparagine and arginine. The effect on the physiology of the shoots would determine whether the *in vitro* shoots respond similarly as other plants subjected to these stresses. The physiological factors investigated included measurement of the fresh mass, shoot elongation, the rooting frequency in microshoots and the number of roots produced per shoot. Since these two types of stresses (water and salt) are reported to decrease water uptake, I hypothesized that the stressed shoots would have a reduction in growth. An analysis of the rooting response, recorded by Cano *et al*. (1998), concluded that root growth can possibly be used to evaluate the salt tolerance of tomato species by using *in vitro* shoot apex cultures. The production of roots is important as it allows the exclusion of ions taken up by the medium, whereas unrooted shoots directly absorb all the components in the medium. Shoots which are unable to root, would therefore probably suffer more from ion toxicity than those apices that root (Cano *et al*., 1998).

6.2 MATERIALS AND METHODS

6.2.1 Plant stock in a continuous culture system

The shoots used for this material were obtained from *in vitro* cultured plant stocks cultivated on MS medium (Section 3.2.2). These cultures were subcultured on a monthly basis onto new medium. To investigate the effect of water and salt stress on the synthesis of canavanine, arginine, GABA and asparagine, shoot cultures were transferred onto MS (Murashige and Skoog, 1962; Duchefa Biochemie, Netherlands) medium containing the osmoticum to induce each stress. Water stress was induced by adding polyethylene glycol (PEG) 6000 at 3% (w/v), whilst the control did not have PEG. Polyethylene glycol was chosen as the osmoticum to induce water stress as it is an inert, non-ionic, non-penetrating and non-toxic compound which induces water stress by decreasing the water potential of the medium, without being absorbed by the plant (Ahmad *et al*., 2007). A mild and more severe salt stress was induced by adding either 50 mM or 100 mM NaCl to the medium. The control cultures were not exposed to NaCl.

Sodium chloride was selected to induce salt stress as it is an ionic and penetrating agent which produces specific ion toxicities in cultured plants (Ahmad *et al*., 2007).

6.2.2 Metabolite extraction and LC-MS analysis

The methods described in Chapter 5 (Section 5.2.2 and 5.2.3) were followed.

6.2.3 Data collection and statistical analysis

Fifteen bottles each containing three shoots were prepared for each medium. So, each medium was regarded as a separate treatment. The shoots on PEG and salt media were harvested after 28 days. The data collected from the shoots from each treatment was the total fresh weight (g); total shoot length (mm); the frequency of the shoots producing roots (recorded as a percentage) and the number of roots per shoot. This experiment was repeated three times.

For the metabolite analysis, seven samples were used to measure canavanine, GABA and asparagine and three samples were used to measure arginine in the shoots. Quantitative analyses are represented as mean values and the reproducibility of the results is expressed as standard error.

The normality of the data was determined using a Shapiro-Wilk's W test prior to further analysis. The results for the metabolites were analyzed using either a Post Hoc Tukey HSD test or a Kruskal-Wallis test depending on the normal distribution of the data.

The average number of roots for the regeneration of roots from shoots, recorded as a percentage, was arcsine transformed prior to analysis.

The fresh weight and total shoot length data for shoots cultivated on water stress medium were analyzed using a Post Hoc Tukey HSD test. The data for the percentage rooting response and the average number of roots produced by shoots subjected to water stress, was analyzed using a Mann-Whitney U test.

The fresh weight data for shoots cultivated on salt stress medium were analyzed using a Post Hoc Tukey HSD test. The percentage shoots producing roots, the average number of roots and the total shoot length, were statistically analyzed using the Kruskal-Wallis test.

The statistical studies to determine the correlation between metabolite changes were determined using two dimensional scatterplots function. Statistical analysis was conducted at the 95% confidence limit. All data were analyzed with the use of the Statistica Release 8 program (Statsoft Inc. 2007).

6.3 RESULTS AND DISCUSSION

6.3.1 The effect of water stress on metabolite levels

Water stress in *Sutherlandia* shoots was induced by including 3% (w/v) polyethylene glycol (PEG) in the growth medium. Addition of PEG in the medium reduces the water potential of substrates by forming hydrogen bonds with water in the medium (Burnett *et al*., 2005). To continue water uptake, plants have to lower and keep their water potential below the water potential of the medium (Taiz and Zeiger, 2002). The water potential of the plant can be lowered by osmotic adjustment, a process involving the uptake and subsequent accumulation of ions in the vacuole concomitant to the synthesis of compounds in the cytoplasm (Shibli *et al*., 2003). During water stress plants can also close their stomata to reduce water loss and this can result in a reduction in photosynthesis (Taiz and Zeiger, 2002). The carbohydrates derived from the metabolic pathways involved in photosynthesis are important as they provide substrates to replenish other cycles (for example the TCA cycle). However, *in vitro* shoots are supplied with a carbon source (sucrose) to prevent carbon becoming the limiting factor. Besides a minimized capacity for water absorption in the plant, water stress impacts negatively on the acquisition of macro- and micronutrients from the medium. Impaired nutrition may results in unpredictable genetic and biochemical changes which alter overall metabolism.

Water stress had little to no effect on metabolite production in *S. frutescens* (Figure 6.3.1A–D). However, the lack of statistically significant changes can be ascribed to the level of PEG (3% w/v), which may have been suboptimal for inducing a severe drought effect in propagated plants. These four metabolites can also serve to indicate the nitrogen status of the plant. With this in mind, when nitrogen is not limiting, synthesis of these compounds would thus not be significantly altered. Overall, the impact of reduced water availability in target tissue was virtually negligible with regard to canavanine as canavanine only reduced from 5.6 mg g^{-1} DW (control) to 5.16 mg g^{-1} DW (3% PEG) during water stress (Figure 6.3.1A). In the study by Ramirez *et al*. (1992), the effect of PEG (3% PEG 8000) on canavanine synthesis in *Canavalia ensiformis* (L.) DC callus, cultivated in PC (Phillips and Collins, 1979) medium was investigated. At the end of the growth cycle the water stressed *C. ensiformis* callus cultures contained slightly more canavanine than the control callus. Furthermore, the water stressed callus exhibited a reduction in the total amino acid content and an increase in the quaternary ammonium compounds at the end of the growth cycle (Ramírez *et al*., 1992). The appearance of quaternary ammonium compounds indicates the synthesis of compatible solutes.

The response of plants to environmental stress is extremely complex and requires intimate monitoring of a variety of factors which are implicated in the general stress response. Some of these are beyond the scope of this thesis. However it is still important to note that compatible solutes (for example proline, glycine betaine and polyamines to name a few) and nitric oxide (a signaling molecule) are amongst some of the changes that require careful consideration. For instance, arginine can assist with nitric oxide synthesis (Gao *et al*., 2009) and it is also implicated in the production of polyamines (putrescine, spermine and spermidine) associated with plant stress (Forde and Lea, 2007). The enhanced synthesis of these compatible solutes may explain the reduction in arginine content from 15.3 mg g^{-1} DW in the control cultures to 12.7 mg g^{-1} DW in the drought stress cultures (Figure 6.3.1B).

A slight increases in GABA (from 2.35 mg g^{-1} DW to 2.58 mg g^{-1} DW) and asparagine (from 66.7 mg g^{-1} DW to 78.96 mg g^{-1} DW) were noted when shoots grew on medium with PEG as an additive (Figure 6.3.1C and D respectively). The synthesis of GABA is related to the activity of GAD (2.4.2.4). In PEG treated rice calli the Ca^{2+} level increased as the water stress became more severe reaching a maximum at the highest level of (29%) PEG (8000) (Ahmad *et al*., 2007). High levels of Ca^{2+} interact with calmodulin which activates the enzyme GAD for GABA synthesizes. The enzyme GAD therefore appears to be regulated by Ca^{2+} in water stressed calli and this increased GAD activity may explain the increase in GABA levels in *Sutherlandia* shoots. Alternatively, the increase in GABA levels may be due to a reduction in degradation as observed in wheat leaves cultivated in medium containing 20% (w/v) PEG (Bartyzel *et al*., 2003). GABA degradation is linked to conversion of GABA to succinic semialdehyde which eventually leads to succinate (Bartyzel *et al*., 2003) (See Section 2.4.2.4). Stress conditions appear to increase the accumulation of succinic semialdehyde which may decreases the degradation of GABA by the GABA-T pathway (Bartyzel *et al*., 2003). The third role for GABA may be as a signaling molecule during water stress (Shelp *et al*., 1999). Such mechanisms may explain increased GABA levels in *Sutherlandia* shoots.

The increase in asparagine may emphasize its role as a nitrogen transporter molecule due to the rapid protein degradation during water deficiency, which increases this amino acid pool. Secondly, the enzyme asparagine synthase (AS) (detailed in Section 2.4.2.1) which is responsible for asparagine synthesis is inhibited by high levels of carbohydrates. If photosynthesis is inhibited, there will be a reduction in the amount of carbohydrates and this can enhance the activity of AS resulting in increased asparagine levels (Figure 6.3.1D). Asparagine is known to function in nitrogen transport from the site of synthesis (source) to

other tissue types (for instance the roots). With organ cultures, such intricate transport mechanisms may not be fully functional. Otherwise, deposition of nutrients as exudates in the medium may occur without the researcher being able to detect this. Analysis of the medium components post treatment was not considered in this study.

Statistical analysis of the changes indicated that the reduction in canavanine was correlated to the reduction in arginine ($r = +0.7260$; $p = 0.0414$). Similarly, the reduction in arginine was correlated to the increase in asparagine ($r = +0.7179$; $p = 0.0038$) and finally, the increase in asparagine was correlated to the increase in GABA ($r = +0.5904$; $p = 0.0262$). From these results, it appears that other metabolic pathways (photosynthesis, hormone metabolism and anti-oxidants) may be more important in the response to water stress. The lack of significant change in the metabolic response was compensated by the morphological response as major changes to growth capacity of plantlets became apparent with water stress.

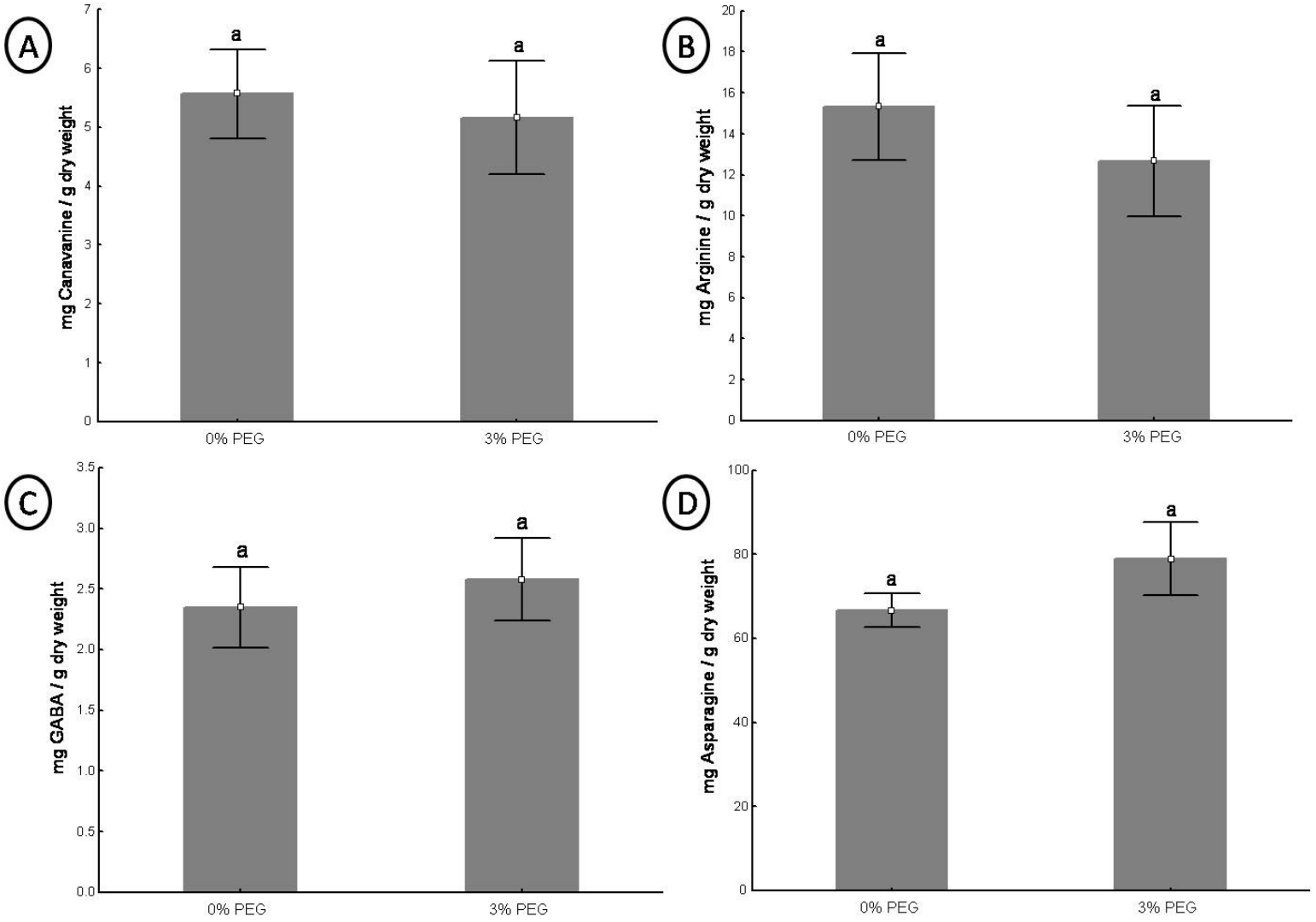

Figure 6.3.1: The effect of water stress induced by including 3% (w/v) PEG in the medium on the accumulation of A) canavanine, B) arginine, C) GABA, and, D) asparagine in *in vitro Sutherlandia* shoots cultures (0% PEG is the control medium). Bars represent the mean ± standard error. Different letters indicated statistically significant difference at the 95% confidence limit

6.3.2 The effect of water stress on the physiology of *in vitro* shoots

The shoots under water stress displayed a significant reduction in the mean fresh mass (0.233 g), representing an approximate 2.4-times reduction from the control shoots (0.557 g) (Figure 6.3.2A). This reduction in fresh mass may be due to a reduction in water uptake, nutrient uptake or due to the reduction in biomass accumulation. Burnett *et al*. (2005) found that drought stress decreased the general pool of nitrogen in Impatiens (*Impatiens walleriana* Hook. F.) foliage with an increase in PEG 8000 concentration in the medium. However, this reduction in nitrogen did not have a detrimental effect on growth. These authors emphasized that nutrient uptake is not directly related to water uptake since most nutrients are actively absorbed. Here, the reduction in growth was rather attributed to the reduction in water availability due to the application of PEG. The contribution of these factors (reduced water uptake and biomass accumulation) to reduced growth can be determined by the analysis of the dry mass as this is a better indication of growth.

A second physiological impact observed included the reduction in total shoot length in the PEG treated shoots (28 mm) below the control shoots (69 mm) (Figure 6.3.2B). These drought stressed shoots (Figure 6.3.2D) also exhibited a reduced leaf size. Similarly, Impatiens plants treated with PEG, were smaller (smaller leaves and shorter plants) and less compact (with fewer leaves per shoot height) than the control plants (Burnett *et al*., 2005). In these plants the reduction in morphological characters was partly attributed to a decrease in the leaf water potential (Burnett *et al*., 2005).

The final physiological impact was the negative effect of water stress on the rooting response which resulted in a significant decrease in the rooting frequency of the PEG treated shoots (results not shown). Very few drought stressed shoots (5%) produced roots compared to 53% of the shoots on the control medium which produced roots. There was also a significant decrease in the number of roots produced, with almost no roots observed on the water stressed microshoots whilst the control shoots had approximately four roots per shoot (Figure 6.3.2C). To reiterate, rooting *in vitro* in *Sutherlandia* occurs spontaneously without requiring an additional PGR treatment. Shoots were unable to maintain a high rooting frequency when growing on 3% PEG medium. This may imply that a reduction in H_2O availability severely affects endogenous auxin levels indicating phytohormonal imbalances and synthesis of other stress related phytohormones (such as ABA) in propagated tissues. Abscissic acid, which is synthesized by the roots, is believed to act as a signaling molecule which helps to reduce water loss through transpiration by closing the stomata in the leaves (Taiz and Zeiger, 2002).

The increased levels of ABA during stress may also enhance leaf senescence in plants (Taiz and Zeiger, 2002). ABA also causes an elevated cytosolic Ca^{2+} level as well as a stimulated synthesis of nitric oxide (NO) in the guard cells which induces stomatal closure (Taiz and Zeiger, 2002). The effect of ABA on Ca^{2+} levels and NO synthesis may also partly contribute to metabolite changes. A direct (strong) correlation with respect to growth performance (characteristics) due to water stress was revealed by statistical analysis.

Even though few changes in the primary metabolites of interest were noted in this study, water stress would clearly impact on crop yield in a field situation as the organogenesis potential of *S. frutescens* is clearly dependent on water absorption. For future reference, it would be wise to measure the water potential of both the medium and the shoots prior to stress induction as well as at the end of the stress period. It is suggested that the permanent wilting point of several plants is about -1.5 MPa. Therefore, plants growing under conditions which exceed this value may be incapable of reducing their water potential below this point without risking death (Taiz and Zeiger, 2002). Measurement of the water potential will thus provide some insight into the stress response of these plants. Additionally, plants in nature are simultaneously exposed to several kinds of abiotic stress in conjunction to biotic factors (pathogen attack). However, manipulation of several different factors at the same time complicates the experimental system and design. Effects may thus be amplified and obscured through simultaneously changing a variety of conditions (Shibli *et al*., 2007). However, there is great value in monitoring several environmental conditions in tissue culture as this is more reflective of true-to-life situations which plants face, providing a better understanding of fundamental processes connected to the stress response and adaptation.

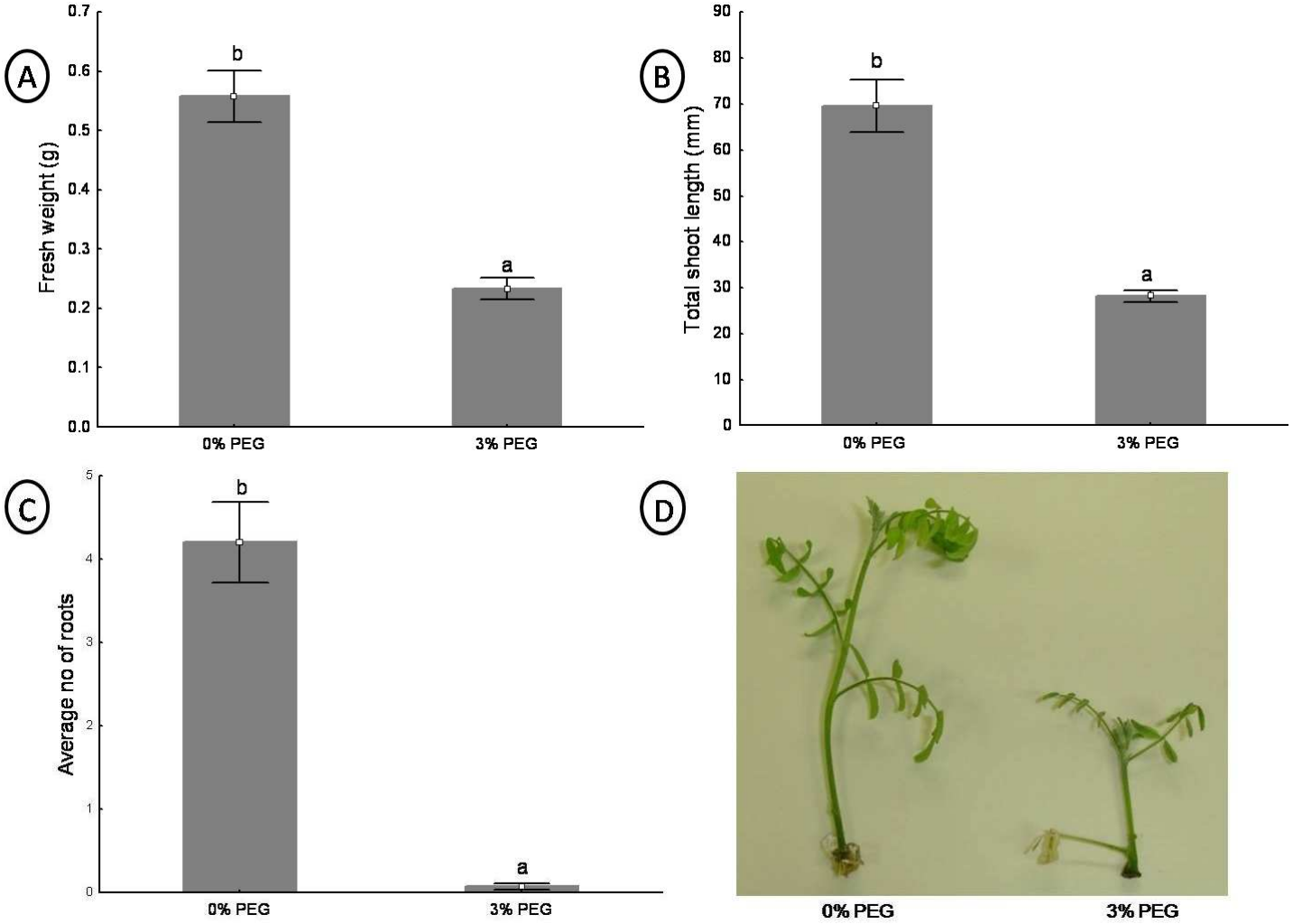

Figure 6.3.2: The physiological responses observed during the induction of water stress on the *in vitro* shoots. A) The accumulation of fresh mass (FW; g) and B) the total shoot length (mm) of shoots cultivated in medium containing no PEG (control) or containing 3% (w/v) PEG. The bars represent the mean ± standard error and different letters indicate statistically significant difference at the 95% confidence level; C) the average number of roots produced per shoot for each treatment; and D) a photo of a shoot cultivated in the control medium and 3% PEG medium

6.3.3 The impact of salt stress on metabolite levels

Salinity appears to interfere with mineral (micro- and macronutrients) acquisition (Shibli *et al*., 2003). The nitrogen content of bitter almond (*Amygdalus communis* L.) shoots cultivated in NaCl medium was reduced as salinity in the medium increased (Shibli *et al*., 2003). This reduction in nitrogen uptake can be attributed to either the interference of NO_3^- translocation and uptake across the root plasma membrane due to Cl^- antagonism (Shibli *et al*., 2003). Alternatively, salt stress can reduce the activity of nitrate reductase, which is involved in nitrogen uptake (Shibli *et al*., 2007). A reduction in nitrogen uptake by the plant might explain the reduction in the nitrogen and soluble protein content observed in tomato plants cultivated in medium containing NaCl (Shibli *et al*., 2007). Furthermore, those tomato plants also had a reduced sulphur content which further contributes to the reduction in protein biosynthesis (Shibli *et al*., 2007). Alternatively, the decrease in soluble protein content can also indicate enhanced protein hydrolysis which would result in an increase in the amino acid levels (Shibli and Al-Juboory, 2002). Due to the reduced nitrogen uptake and increased protein degradation it is challenging to predict the metabolic response in these plants.

Changes in the uptake and accumulation of macro- and micronutrients in tomato plants under salt stress indicated that potassium (K^+) and calcium (Ca^{2+}) concentration are reduced, whilst the sodium (Na^+) ions accumulates in the cells (Shibli *et al*., 2003; Shibli *et al*., 2007). The reduction in Ca^{2+} is also attributed to replacement of Ca^{2+} in the cell membrane by Na^+ ions which result in a reduction in the membrane stability (Shibli *et al*., 2007). Calcium and K^+ are both important to maintain the cell-membrane integrity (Hu and Schmidhalter, 2005). Plants usually try to maintain a high K^+/Na^+ ratio as it improves the resistance to salinity (Hu and Schmidhalter, 2005). However, since salts damage the cell membranes, it can increase K^+ and other solute leakage from the cells (Santos *et al*., 2001). Excess Na^+ ions also inhibits K^+ uptake due to an antagonistic relationship (Shibli *et al*., 2003). The change in these minerals are apparently more sensitive to higher levels of salt as it was indicated that several plants are tolerant to low levels of salt (Shibli *et al*., 2003). The uptake of micronutrients such as Cu, Mn and Zn increased during salt stress, whilst iron (Fe) uptake was significantly reduced by salinity stress (Shibli *et al*., 2003). The effect of salt on mineral uptake therefore changes the nutrient content of the medium.

Salt stress in the *in vitro* shoots was induced by adding sodium chloride (NaCl) to the cultivation medium. The changes in metabolite levels in the *in vitro* shoots exposed to salt stress did not appear to follow exactly the same metabolic response as the water stressed

shoots and this may be due to the additional ionic effect which relates to ion toxicity in plants (Jaleel *et al*., 2007). Similarly to water stress, NaCl levels resulted in insignificant metabolic changes to canavanine, GABA and asparagine (Figure 6.3.3 A, C and D). During this study, only the addition of 100 mM NaCl to the medium, resulted in an increase in canavanine synthesis (7.0 mg g^{-1} DW) above the control and 50 mM NaCl conditions (5.4 mg g^{-1} DW) (Figure 6.3.3A). The only metabolite which was significantly affected by the increasing salt concentration was arginine with plants showing elevated arginine levels (38.33 mg g^{-1} DW) when NaCl supplementation increased to 100 mM NaCl compared to the controls (10.4 mg g^{-1} DW) and 50 mM NaCl (34.7 mg g^{-1} DW) (Figure 6.3.3B). The increase in canavanine at the highest salt level may be due to a general increase in the metabolic pathway involved in canavanine and arginine synthesis (See Section 2.4.2.2 and 2.4.2.3). This result was interesting, considering that water stress previously appeared to reduce canavanine and arginine synthesis. However, it is possible that the reduction in water potential of the medium upon 100 mM NaCl inclusion differs from the reduced water potential induced by 3% PEG. It is also possible that the ionic effect of NaCl activates a different response than water induced stress. Higher amounts of free arginine may ensure that there is enough substrate for the synthesis of compatible solutes and nitric oxide which may be involved in the stress response to increasing salt.

Addition of 50 mM or 100 mM NaCl resulted in a reduction in GABA synthesis with the increasing level of salt in the medium (Figure 6.3.3B). Since salt stress may reduce the amount of water available to the plant, the plant responds by closing its stomata, to limit water loss. However, closing the stomata can result in reduced photosynthesis and thus a decrease in the carbohydrate levels. Reduced carbohydrates, which are partly used to replenish the TCA cycle, can therefore indirectly impact the TCA cycle pathways. The reduction in GABA levels may therefore suggest that GABA is rapidly being degraded to provide intermediates (succinate) to replenish the TCA cycle. An alternative theory to increased degradation would be a reduction in GABA synthesis. The enzyme GAD in the cytosol is activated by Ca^{2+}-calmodulin. Ahmad *et al*. (2007) found that (in contrast to water stress) the Ca^{2+} level of the salt treated rice calli was reduced as the salt intensity in the medium increased and that the reduction was most severe at the highest salt intensity tested. The reduction in Ca^{2+} levels can indicate that less GAD is being activated (See Section 2.4.2.4) which may have caused the reduction in GABA levels observed in *Sutherlandia* shoots.

A minor decrease in asparagine was observed (Figure 6.3.3D). A reduction in asparagine content due to salt stress was also observed by Di Martino *et al*. (2003) in salt stressed spinach (*Spinacia oleracea*) plants. The reduction in free asparagine can be due to the negative impact of salt on the TCA cycle which decreases the intermediates (oxaloacetate) necessary for asparagine synthesis. Furthermore since asparagine is a nitrogen transport molecule the reduction can indicate that less nitrogen is being assimilated due to the negative impact of ion accumulation on enzyme activity.

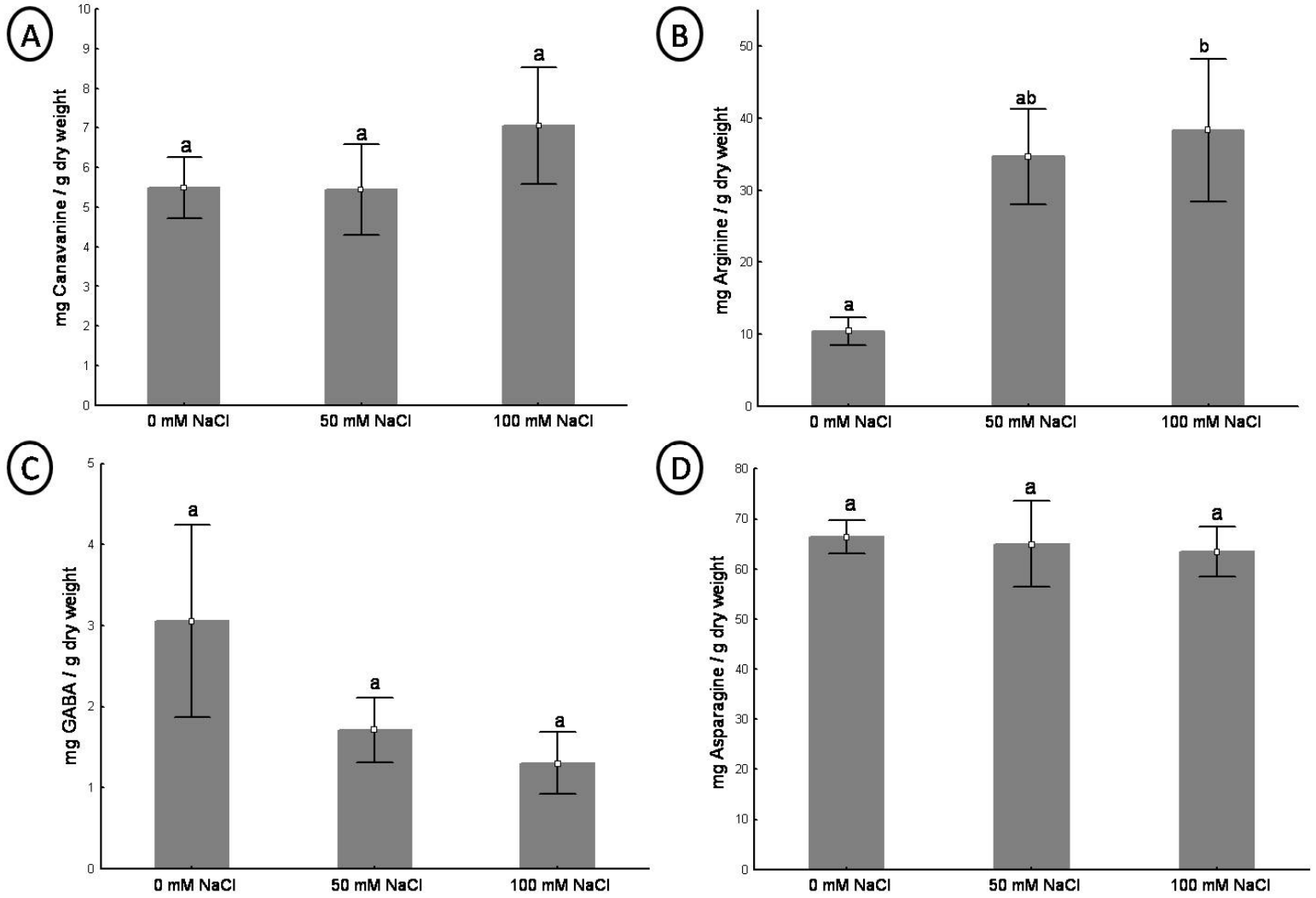

Figure 6.3.3: The A) canavanine, B) arginine, C) GABA, and, D) asparagine level in *in vitro* cultivated *Sutherlandia* shoots cultivated on MS media containing 50 or 100 mM NaCl levels (the control contained no sodium chloride). The bars represent the mean ± standard error and different letters indicate statistically significant difference at the 95% confidence level

6.3.4 The impact of salt stress on the physiology of the *in vitro* shoots

Initially salt stress does not appear to reduce the biochemical and photochemical capacity of the leaves. However, prolonged exposure to high levels of salt can impair the photosynthetic electron transport chain and carbon metabolism thus affecting photosynthesis negatively (Di Martino *et al*., 2003). Long term exposure to salt stress can also reduce rubisco activity, as well as the chlorophyll content in leaves which will cause a further reduction in photosynthesis (Di Martino *et al*., 2003). Furthermore, plants under salt stress can close their stomata to reduce water loss and this can decrease stomatal and mesophyll conductance which would inhibit photosynthesis due to a restriction in CO_2 diffusion (Di Martino *et al*., 2003). Salt also inhibits the efficiency of the translocation and assimilation of photosynthetic products (Jaleel *et al*., 2007).

Sutherlandia shoots cultivated in increasing levels of NaCl displayed typical physiological responses similar to other plants subjected to salt stress. Increasing salt significantly impacted the accumulation of fresh mass in the *in vitro* shoots (Figure 6.3.4A). A moderate increase in salt in the medium induced with 50 mM NaCl significantly increased the fresh mass accumulated (0.187 g) above the control (0.155 g). However, a further increase in NaCl caused a slight reduction in the fresh mass (0.174 g). Mild stress was found to be beneficial to some species and can result in an increased growth. However, the tolerance to a specific level of stress (concentration of salt in the medium) varies with species (Shibli and Al-Juboory, 2002). This can explain the significant increase in fresh mass of shoots subjected to 50 mM NaCl (Figure 6.3.4). At this level of salt, ions are taken up which lowers the water potential of the plant and causes an increase in fresh mass due to increased water uptake (Figure 6.3.4A). Therefore, *Sutherlandia* shoots appear to be tolerant to mild salt stress and at 50 mM NaCl, the Na^+ and Cl^- did not reach a toxic level. Protective mechanisms are thus presumed to be functional at this stress level with plants maintaining their active sequestration of ions (Na^+ and Cl^-) to the vacuole. At higher levels of salt (100 mM NaCl), ion toxicity appears to become a larger problem to the plant.

A second physiological response observed was a reduction in the rooting frequency of plantlets with increasing salt in the medium. The rooting response was not affected by a moderate increase in salt as 64.5% of the shoots in the control medium and 50 mM NaCl medium produced roots (Table 6.3.1). However, with 100 mM NaCl treatments the percentage rooted shoots dropped to 39% (Table 6.3.1). A reduction in the total number of roots produced per shoot was also noted (Figure 6.3.4B). Although insignificant, the shoots on 50 mM NaCl had slightly fewer roots (5 roots per shoot) than the controls (7 roots per shoot).

Clearly, high salt treatment (100 mM NaCl) further exacerbated the decline in the rooting potential of *in vitro* plants. The statistical analysis of the data strongly supported the deleterious effects of moderate to high salt supplementation on microplant growth performance ($r = 0.7333$; $p = 0.000$ for the number of roots per shoot). Additionally the change in fresh mass was correlated to a lowered capacity for shoots to elongate ($r = +0.299$; $p = 0.0043$).

Table 6.3.1: The effect of salt on the rooting response (% shoots producing roots)

Treatment	% shoots producing roots
0 mM NaCl	64.5 ± 5.5
50 mM NaCl	64.5 ± 6.2
100 mM NaCl	38.8 ± 4.8

Apart from ABA-regulated rooting, a decrease in phosphate (P) uptake as observed in the bitter almond (Shibli *et al*., 2003) is postulated to be responsible for the reduction in rooting with increasing salinity. Phosphate acts as a major element for root growth and development (Shibli *et al*., 2007). A similar response in rooting capacity was observed with *in vitro* shoot apices of different tomato (*Lycopersicon*) species treated with salt. Generally the percentage of shoot apices producing roots decreased as the salt level in the medium increased (Cano *et al*., 1998). However, this effect may be species dependent as *L. pennellii* displayed less sensitivity to salt than *L. esculentum* (Cano *et al*., 1998).

The final physiological response investigated during this study was the effect of increasing salt level on the elongation of the *in vitro* shoots (Figure 6.3.4C). A reduction of the total shoot length was apparent with high salt levels. The addition of moderate levels of salt significantly decreased the total shoot length in 50 mM NaCl (49 mm) whilst the control shoots were approximately 61 mm long. The reduction in length was more pronounced in shoots cultivated on the 100 mM NaCl medium (36 mm). Additionally, the shoots on 50 mM NaCl also had a yellow colour (Figure 6.3.4D). In contrast, the leaves of shoots on 100 mM NaCl medium did not appear yellow; however, they were reduced in size (Figure 6.3.4D). A lowered plant vigour including the production of shorter plants with smaller leaves and fewer roots concluded the negative response to elevated salt in the growth medium. A reduction in shoot elongation *in vitro* in response to salinity is well supported by data on the tomato (Shibli *et al*., 2007);

Madagascar periwinkle (*Catharanthus roseus*) (Jaleel *et al*., 2007) and olive plants (*Olea europea*) (Shibli and Al-Juboory, 2002). This reduction in growth was attributed to factors such as 1) a decrease in water uptake due to a reduction of the water potential of the NaCl-containing medium (Jaleel *et al*., 2007), and, 2) the reduction in growth is also dependent on the time period during which plants are exposed to saline growth conditions emphasizing that different mechanism are important during different growth periods (Munns, 2002). Plants subjected to salinity stress respond by lowering the cell osmotic potential by the synthesis of compatible solutes in the cytoplasm which results in a reduction in the cell sap osmotic potential (Shibli and Al-Juboory, 2002). However these compounds are also synthesized to combat the deleterious effects of the ions which accumulate during severe salinity stress as several of the plants that were investigated were tolerant to mild levels of NaCl.

The yellow appearance of the leaves of the shoots on 50 mM NaCl is a diagnostic feature of chlorosis (Figure 6.3.4D). A similar response was observed in *in vitro* olive plants, *in vitro* shoots of the bitter almond and tomato microshoots treated with increasing levels of NaCl (Shibli and Al-Juboory, 2002; Shibli *et al*., 2003; Shibli *et al*., 2007, respectively). In these studies the response was attributed to a lowered capacity for Fe uptake which is associated with an antagonistic reaction between Fe and Na^+ (Shibli *et al*., 2003). Reduction in Fe uptake negative impacts the plant proteins and enzyme synthesis and activity. This also affects the chloroplasts resulting in a reduction in photosynthesis. Leaf yellowing also serves to indicate a decrease in the macronutrients (such as nitrogen) in the leaves as salt in the medium interferes with uptake of nutrients (Shibli *et al*., 2003).

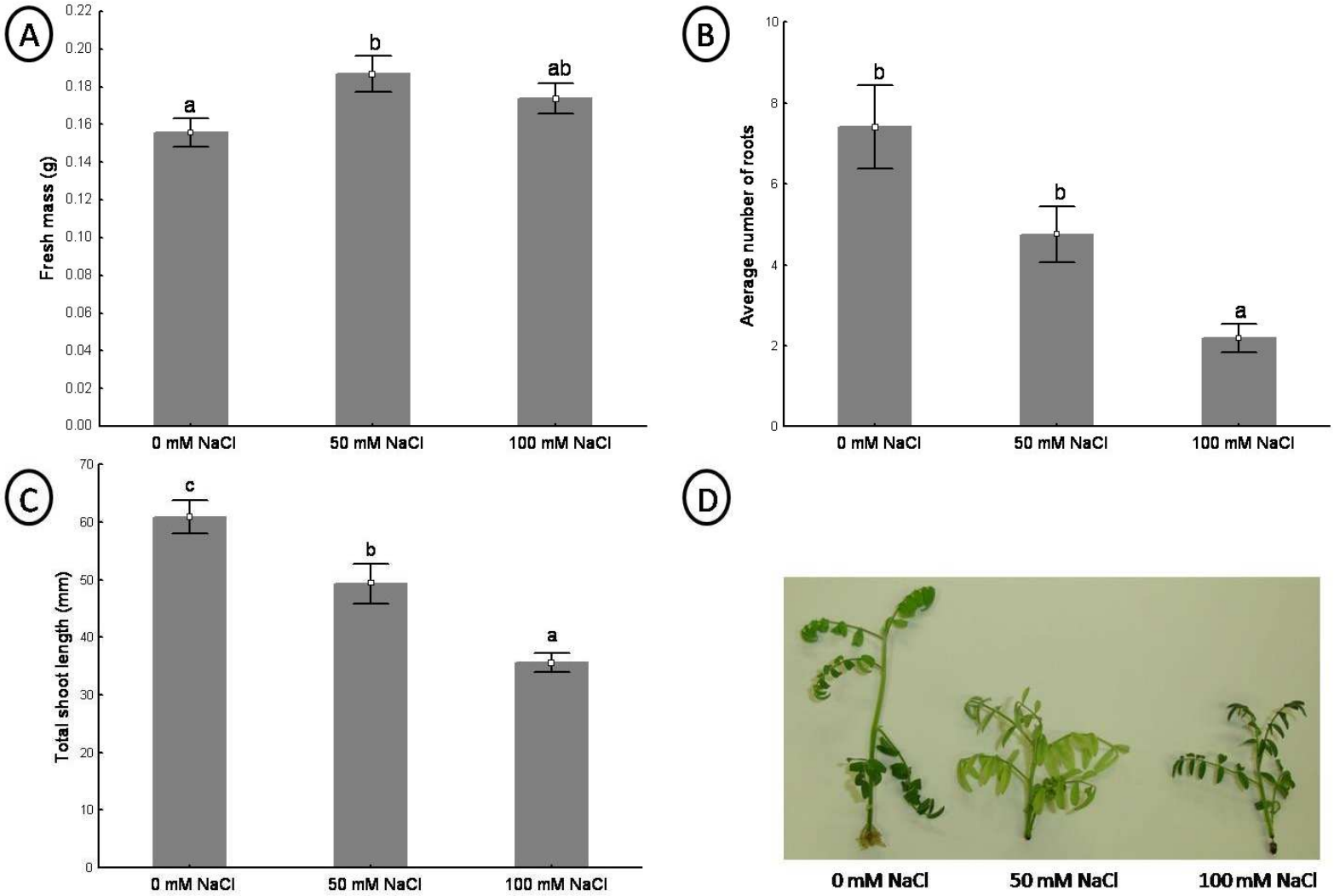

Figure 6.3.4: The total amount of fresh mass accumulated by the shoots cultivated in media in the absence (0 mM NaCl; control) or presence of 50 mM or 100 mM NaCl. The bars represent the mean ± standard error and different letters indicate statistically significant difference at the 95% confidence level

6.3.5 Comparison of *in vitro* studies to *ex vitro* analysis for all abiotic stresses

Similar to the effect of nitrogen availability, one can now compare the effect of salt and water stress on metabolite synthesis to the level of metabolites observed in wild plants. The highest level of canavanine (14.5 mg g^{-1} DW) is reported for plants collected from nature (Table A9, Appendix; Van Wyk and Albrecht, 2008). The glasshouse cultivated plants, the shoots cultivated on 0.5x nitrogen medium (Figure 5.3.1A) and commercial plants (Table A9, Appendix; Van Wyk and Albrecht, 2008) appear to synthesize similar canavanine levels (1.6 – 3.88 mg g^{-1} DW). The canavanine level did not vary significantly in the shoots on control medium (Figure 6.3.1A and Figure 6.3.3A), 3% PEG (Figure 6.3.1) and 50 mM NaCl (6.3.3A). However, an increase in the canavanine level was induced by higher levels of nitrogen (Figure 5.3.1A) and salt (100 mM NaCl) (Figure 6.3.3A) in the medium. Salt and high nitrogen also resulted in a significant increase in arginine synthesis (Figure 6.3.3B and Figure 5.3.1B respectively). The arginine level of the shoots cultivated on 0.5x nitrogen (Figure 5.3.1B) was in the range reported for the wild harvested plants (1.6 – 35.0 mg g^{-1} DW) (Table A9, Appendix). Whereas, the highest asparagine level was observed with 2x nitrogen treated shoots (Figure 5.3.1C) followed by water stressed shoots (Figure 6.3.1D). Finally, the lowest GABA level was observed in the wild harvested plants (0.23 – 0.85 mg g^{-1} DW) whereas the highest level (4.8 mg g^{-1} DW) was observed in 0.5x nitrogen medium (Figure 5.3.1D).

6.4 CONCLUSION

Salt stress does not significantly change canavanine, asparagine and GABA synthesis but it increases arginine synthesis. Currently a significant problem is that the secondary metabolites of *Sutherlandia* plants have not been resolved, therefore the effect of abiotic stress on the synthesis of secondary metabolites will require screening of extracts to compile a good secondary metabolite profile. Apart from the suggestions mentioned previously (Chapter 5, Conclusions) this work can be followed up with pharmacological assays to examine the contribution of abiotic stresses to secondary metabolism. To extend this current study, the use of rooted whole plants instead of organ shoot cultures, may assist with elucidating tissue-specific, inter-organellar mechanisms related to source-sink interactions involved in stress responses. Once more, data derived from this study may have application to the agricultural sector informing on agronomic factors for consideration in commercial *S. frutescens* cultivation.

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CHAPTER 7:

Phytochemical and anti-microbial analysis

7.1 INTRODUCTION

There are a growing number of scientific papers on the pharmacological activity of *Sutherlandia frutescens*. Although these studies all speculate on the compounds which are responsible for the biological activity, few have tried to isolate the compounds involved. Tai *et al*. (2004) used both gas chromatography (GC-MS) and liquid chromatography (LC-MS) to investigate some of the compounds in the herbal products which are sold commercially. Methyl and propyl paraben (used as preservatives), hexadecanoic acid, γ-sitosterol, sigmast-4-en-3-one and a few long chain alcohols were identified using GC-MS (Tai *et al*., 2004). In addition to canavanine, asparagine, arginine and GABA, the presence of the following amino acids; alanine, proline, isoleucine or leucine, phenylalanine and tryptophan was confirmed in the *Sutherlandia* herbal extract (Tai *et al*., 2004). Pinitol and inisitol were also detected during LC-MS analysis (Tai *et al*., 2004). If the pharmacological property of *S. frutescens* is attributed to the synergistic activity of several compounds, as suggested by Tai *et al*. (2004) and Prevoo *et al*. (2008), it may explain the difficulty in isolating single bioactive metabolites.

Today, several methods are available to study the secondary metabolite composition of plant extracts. Thin layer chromatography is a simple, fast and cheap method which is easy to run and the results are reproducible (Marston and Hostettmann, 1999). Furthermore, if TLC is combined with a bioassay (eg. anti-microbial, anti-fungal or anti-oxidant) it provides a valuable tool for the identification and isolation of active constituents in the extract (Marston and Hostettmann, 1999). However, the type of bioassay has to be chosen and designed in such a way that it is simple, inexpensive and can be performed rapidly to allow a large number of samples to be screened efficiently (Marston and Hostettmann, 1999). The bioassay should also be sensitive enough to reduce the number of false positives and to allow the detection of active compounds which can be present in low levels in the extracts (Marston and Hostettmann, 1999). This can be followed up by the use of HPLC, LC-MS and GC-MS for the efficient separation of the metabolites, as these techniques are very selective and have a high sensitivity for the detection of metabolites (Marston and Hostettmann, 1999). The use of mass spectrometry is favourable as it provides information on the molecular weight of compounds and can also be used to identify the structure of the metabolites (Marston and Hostettmann, 1999).

In this study, extracts were prepared from different plant organs (roots and shoots) from both *in vitro* and *ex vitro* sources. These extracts were used to conduct an anti-microbial screening using both Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) and Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) bacteria. The results from this assay may provide some insight on the location (roots or shoots) of the anti-bacterial compounds and may also indicate whether the *in vitro* cultures can be used as a source of these active metabolites. Secondly, phytochemical profiles of the *in vitro* hairy root cultures were compared with untransformed *in vitro* and *ex vitro* roots and shoots using TLC and LC-MS techniques. The aim for this experiment was to assess if the hairy root cultures produce the same metabolites as the untransformed plants, as suggested by Bourgaud *et al*. (2001).

7.2 MATERIALS AND METHODS

7.2.1 Plant material and chemicals

The standards used for the secondary metabolite analysis included; flavonoids (quercetin, kaempferol (Fluka, Switzerland), rutin and myrecetin); phenolic acids (caffeic acid and chlorogenic acid) and coumarins (coumaric acid). The standards for primary metabolites included; asparagine, canavanine, arginine, serine, glutamic acid, urea and betaine. These standards were all purchased from Sigma-Aldrich (Germany), unless otherwise indicated. Stock solutions (1 mg ml^{-1}) of all standards were prepared using 70% ethanol. The hairy root and untransformed root and shoot material was obtained from *in vitro* cultivated liquid and solid cultures (for details refer back to Section 3.2.2 and 4.2.4).

7.2.2 Extraction of secondary metabolites

After harvesting, the plant material was placed in brown paper bags and dried in a 55˚C oven for two days and then weighed to obtain the dry mass. Once dry, 1 g material of each sample was ground to a fine powder using a mortar and pestle and transferred to glass tubes (15 mm x 150 mm). Fifteen milliliter methanol (MeOH for extraction of moderately polar compounds) was added to each sample and the tubes were covered with tinfoil caps to reduce evaporation. All the samples were sonicated in an ultrasound bath (Bransonic 220; 50/60 kHz) for 45 min (this extraction was repeated twice). After sonication the MeOH extracts were filtered using a millipore filter unit (0.2 µm; Lasec, South Africa) and a 10 ml syringe. This was followed by an extraction using 15 ml dichloromethane (DCM for extraction of lipophilic compounds). The samples were sonicated once more for 45 min (the extraction with DCM was also repeated twice). These extracts were filtered through filter paper (Whatman® no.1). The MeOH and

DCM extracts of a sample were pooled together in a buchi flask and the solvents were evaporated under vacuum at 40˚C (using a Buchi 461 waterbath; Rotavapor RE 111 and Buchi 011). The dried residues were weighed and resuspended in MeOH: DCM (1:1; v/v) to a concentration of 100 mg ml^{-1} for TLC analysis or in 5% (v/v) DMSO (prepared in autoclaved dH_2O) to a concentration of 40 mg ml^{-1} for anti-microbial studies. All the dried samples that were not used immediately were closed with a tinfoil cap which was sealed with a 1.5 cm parafilm strip. These samples were stored in the fridge (5˚C) in the dark until further analysis.

7.2.3 The minimum inhibitory concentration (MIC) of extracts

7.2.3.1 Cultivation of bacterial strains

The bacterial test organisms (*Staphylococcus areus* (Gram+; ATCC 12600), *Klebsiella pneumoniae* (Gram–; ATCC 13883), *Esherichia coli* (Gram–; ATCC 11775) and *Bacillus subtilis* (Gram+; ATCC 6051)) from glycerol freezer stock solutions (kindly donated by Professor Johannes van Staden from the University of Kwazulu-Natal, Pietermaritzburg) were inoculated on autoclaved Müller-Hinton (MH) agar in Petri dishes (90 mm x 15 mm) by dilution streaking. The bacteria were incubated overnight in a 37˚C incubator. Single colonies were selected from plates and inoculated in 50 ml liquid MH medium in (100 ml) Erlenmeyer flasks, closed with a cotton wool plug and covered with a tinfoil cap. The flasks were shaken overnight at 37˚C on a rotary shaker at 60 rpm until the bacterial suspension reached the saturated phase (OD_{600} = 1.9 – 2.0). A 100-times dilution was prepared of the bacterial cultures using MH broth to obtain an OD_{600} = 0.4 – 0.6.

7.2.3.2 Microtitre assay

The serial dilution microtitre assay as described by Eloff (1998) was used to investigate the minimum inhibitory concentration (MIC) of plant extracts. The dried extracts were resuspended in 5% (v/v) DMSO to a stock concentration of 40 mg ml^{-1}. After dilution, the concentration of the extracts in the wells was as indicated in Table 7.2.1.

Table 7.2.1: The concentration of the extracts in the wells after serial dilution using dH_2O

Microtiter plate well	Extract concentration after serial dilution (mg ml^{-1})
A	10
B	5
C	2.5
D	1.25
E	0.625
F	0.313
G	0.156
H	0.078

One hundred microliters of the extracts and streptomycin (40 mg ml^{-1}) were serially diluted two-fold with 100 µl distilled H_2O. Two columns were used per extract tested (Figure 7.3.1). There were four controls for this experiment. Three of the controls were used to monitor that the solvents or reagents used, do not interfere with the colour reaction and these include: 1) one well containing only 100 µl MH broth (no extract or bacteria) to ensure that the cultivation medium does not influence the colour reaction, 2) one well where the extract was substituted with 100 µl water (containing the bacterium) to ensure that the dH_2O used for dilution, does not influence the colour reaction, and, 3) one well containing 100 µl of the solvent (5% (v/v) DMSO) used to dissolve the extracts to ensure that it does not interfere with the colour reaction or with the growth of the bacteria. The fourth control was a serial dilution of the antibiotic streptomycin (positive control; stock, 10 mg ml^{-1}).

After dilution, 100 µl of the bacterial culture was added to each well. Separate microtiter plates were prepared for each bacterial strain that was investigated. The plates were covered with the lids, sealed with parafilm and placed inside a plastic container on top of slightly damp tissue paper to create a humid environment and prevent evaporation of the extracts. All the plates were incubated overnight in the 37˚C incubator. To indicate bacterial growth, 40 µl of 0.2 mg ml^{-1} INT (p-iodonitrotetrazolium violet; Sigma) was added to each well and the plates were incubated at 37˚C for 20 min. During incubation, the actively growing bacteria metabolize the colourless tetrazolium salt in INT to a red colour (McGaw *et al*., 2000). Therefore, the wells which displayed no colour change (indicating little to no bacterial growth), represent the extracts with anti-bacterial activity, whilst those wells with a red colour point to

the absence of significant anti-bacterial activity. The MIC was recorded as the lowest concentration of the plant extract which inhibited the growth of the bacteria (Eloff, 1998). For this study an MIC value of less than 1 mg ml^{-1} was considered to show significant anti-microbial activity. This experiment was repeated twice and the plates were also photographed to collect data.

7.2.4 Secondary metabolite analysis

7.2.4.1 Thin layer chromatography (TLC)-analysis

The chemical composition of the extracts was investigated according to the conditions as described by Katerere and Eloff (2005). A stock solution of the flavonoid standards (1 mg ml^{-1} in methanol) and extracts (50 mg ml^{-1} in 1: 1 (v/v) MeOH: DCM) were prepared. Thirty microliter root aliquot and 20 µl of the standards were applied as 1 cm bands on a silica aluminium TLC sheet (Merck, Kieselgel 60 F_{254}). Three solvent systems, as described by Katerere and Eloff (2005), were tested and these included: 1) hexane: ethyl acetate (2: 1; v/v); 2) chloroform: ethyl acetate: formic acid (20: 16: 4; v/v), and, 3) ethyl acetate: methanol: water (40: 5.4: 4; (v/v) – for the analysis of polar compounds such as glycosides (Wagner and Bladt, 2001)). The solvents were poured in three separate square glass containers (230 mm x 85 mm x 230 mm), which were sealed with a glass plate (100 mm x 240 mm). The TLC plates were placed in the containers and removed when the solvent front was about 1 cm from the top of the TLC plate. After removing the TLC plates the solvent front was quickly marked with a pencil and the plates were kept in the fumecabinet until all the solvent had evaporated. All the TLC plates were stored between tissue papers in a dark cupboard until further analysis.

During the visualization steps, different methods were selected to study the inter-clonal variation between the extracts. After development, some of the dried plates containing the flavonoid and coumarin standards were sprayed with a Natural product-polyethylene glycol (NP/PEG) solution (no.28, Wagner and Bladt, 2001) prior to visualization under UV light (365 nm) and the fluorescent compounds were marked. The second method involved spraying the TLC plates with an anisaldehyde stain solution (0.5 ml anisaldehyde, 10 ml glacial acetic acid, 85 ml MeOH and 5 ml concentrated sulphuric acid) (Wagner and Bladt, 2001). These plates were heated for 10 min at 100˚C and evaluated and photographed in visible light. The third method involved staining the plates with the amino acids and biogenic amine standards with the ninhydrin reagent (30 mg ninhydrin, 10 ml n-butanol and 0.3 ml 98% acetic acid) (no.29,

Wagner and Bladt, 2001). These plates were heated for 10 min at 100˚C and visualized and photographed under visible light.

7.2.4.2 Liquid chromatography-mass spectrometry (LC-MS)

The LC-MS analysis of extracts was performed using a Waters API Q-TOF Ultima. A capillary voltage of 3.5 kV was applied with a source temperature of 100˚C. The cone voltage was set at 35 V. Four microliter samples were injected and separation was achieved on a Waters BEH C18, 2.1 x 50 mm, 1.7 µm column using a gradient from 100% solvent A (de-ionized water containing 0.1% formic acid) to 100% solvent B (acetonitrile). The gradient consisted of a 1 min isocratic step of 100% solvent A, 2 min 78% solvent A and 22% solvent B; 4 min 56% solvent A and 44% solvent B, 5 min 100% solvent B, followed by re-equilibration for the final 2 min with 100% solvent B. A flow rate of 350 µl min^{-1} was applied during the analysis. During a separate analysis, the injection of the triterpenoid saponin standard SU1 (Figure 2.1.5; Literature review) previously isolated from *S. frutescens*, was used to screen for its presence in all the extracts investigated.

7.3 RESULTS AND DISCUSSION

7.3.1 The anti-microbial assay of extracts

The micro-dilution method is one of the bioassay techniques that can be used to investigate the anti-microbial activity of the extracts. The advantage of initially conducting a bioassay is that the extracts can be screened prior to *in vivo* analysis to identify those extracts which may contain active compounds (Houghton *et al*., 2007). Van Vuuren (2008) suggested that extracts with MIC values below 8 mg ml^{-1} indicate that the extracts have anti-microbial activity; however, those extracts with MIC values below 1 mg ml^{-1} are noteworthy.

Using the suggestion by Van Vuuren (2008) as a guideline, the results indicated that none of the hairy root extracts displayed noteworthy anti-bacterial activity as these extracts only exhibited activity at the higher (> 1 mg ml^{-1}) concentrations (Table 7.3.1 and Figure 7.3.1A). Of the hairy root clones, the extract from clone C58C1-g exhibited the best activity (2.5 mg ml^{-1}) against all the bacterial strains except *K. pneumoniae*. Clone A4T displayed the least amount of anti-bacterial activity, only having activity against *E. coli* at a concentration of 5 mg ml^{-1} (not significant).

The untransformed roots cultivated *in vitro* did not exhibit any anti-bacterial activity at the highest concentration tested (Figure 7.3.1B). The extract from the *ex vitro* harvested roots only displayed activity (5 mg ml^{-1}) against *K. pneumoniae* (Table 7.3.1). This result suggests that transformation may have improved the anti-bacterial activity of the hairy roots as they displayed more activity against the other bacterial strains. Lindsey *et al*. (2006) found that the anti-bacterial activity of extracts from *in vitro* cultivated roots was about three times lower than that obtained from the *ex vitro* root-bark extracts for *Maytenus senegalensis*. This was attributed to the *in vitro* cultures producing the bioactive metabolites at reduced levels compared to field cultivated plants.

Table 7.3.1: Anti-microbial activity (MIC, milligram per milliliter) of *Sutherlandia* extracts

Extract	*K. pneumoniae* Gram-	*E. coli* Gram-	*B. subtilis* Gram+	*S. aureus* Gram+
Hairy root clone A4T	10	5	10	10
Hairy root clone C58C1-g	5	2.5	2.5	2.5
Hairy root clone C58C1-e	5	5	5	5
Hairy root clone LBA	10	5	5	10
In vitro untr‘ roots	N/A	N/A	N/A	N/A
Ex vitro untr‘ roots	5	10	10	10
In vitro shoots	5	5	5	5
Ex vitro shoots	1.25	1.25	1.25	1.25
Streptomycin (control)	0.156	0.156	0.156	0.156

N/A – no activity

Untr’ – untransformed roots

The *in vitro* cultivated microshoots had the same activity (5 mg ml^{-1}) against all the bacteria investigated, whilst the extracts prepared from the *ex vitro* harvested shoots had the highest activity (1.25 mg ml^{-1}) against all four bacteria (Table 7.3.1 and Figure 7.3.1B). Plants growing in nature are exposed to various abiotic and biotic factors which may alter the synthesis of key metabolites (Sudha and Ravishankar, 2002) and some of these may have anti-microbial activity. The lack of activity in the hairy roots and other *in vitro* cultures can therefore be an

indication that the bioactive compounds are only present in low levels. Since the *in vitro* cultivated plants are cultured under sterile conditions, they may not have been stimulated to synthesize these metabolites. This may explain why the extracts from *ex vitro* material displayed a higher activity than the *in vitro*-derived plant extracts. Future studies can therefore include the use of the abiotically as well as biotically-stressed *in vitro* cultures for the investigation of changes in the anti-microbial activity. Furthermore, these results also suggest that the active compounds are not being synthesized in the roots and that they are only present in the shoot material or that they are present in higher levels in the shoot material.

The anti-microbial activity of *S. frutescens* commercial herbal tablets was also investigated by Katerere and Eloff (2005). These researchers investigated two different extraction methods (sequential extraction and single solvent extraction) using different solvents. The sequential extraction was conducted using hexane, dichloromethane, ethyl acetate and acetone and the single solvent extraction included the use of acetone, ethanol or water. The bacteria used for the assay included *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*. The bacterial strain *S. aureus* was the most sensitive to all the extracts prepared using sequential extraction, except for the acetone fraction (MIC > 10 mg ml^{-1}). The hexane extract exhibited the best anti-microbial activity against *S. aureus* and *E. faecalis* with a MIC of 0.31 and 1.25 mg ml^{-1} respectively. This result confirmed the non-polar nature of the active compounds of *S. frutescens* (Katerere and Eloff, 2005). The single solvent extracts were generally not active against the bacteria, except for the acetone fraction which had activity against *E. coli*, *P. aeruginosa* and *E. faecalis* with an MIC of 1.25 mg ml^{-1}.

The anti-bacterial activity of the non-polar fraction (hexane) of *S. frutescens* against *S. aureus* makes it useful as a topical application (Katerere and Eloff, 2005). Optimization of extraction methods is often crucial to ensure optimal extraction of pharmacologically important compounds during herbal product preparation. For example, Katerere and Eloff (2005) made an important suggestion that the preparation of a product with anti-staphylococcal activity should include the use of non-polar solvents for extraction of non-polar compounds and lipophilic formulations to prepare the product to maintain the structure and activity of the compound(s).

Water and ethanol, would most likely represent the solvents that are generally used by the public for preparing extracts from plants. However, the results from Katerere and Eloff (2005) suggest that these solvents would not be ideal to extract the active anti-bacterial metabolites as these extracts exhibited the least anti-bacterial activity compared to the acetone (single

solvent extraction) extract and the solvents used during the sequential extraction. During this investigation the methanol and DCM extracts were pooled together and therefore contained both polar and non-polar compounds. However, the results for the MIC (Table 7.3.1) for *S. aureus* were not as good as those reported by Katerere and Eloff (2005) when the sequential extraction method was used. It is likely that DMSO used here was not totally effective as a solvent to dissolve all isolated compounds.

During the investigation of the pharmacological activity of plant extracts there are a few important factors to keep in mind. Examples include: testing fresh or dried plant material or using different plant parts (leaves, flowers, bark, roots or fruits). Secondly, procedures for extraction, which include the type of solvent (acetone, methanol, ethanol, ethyl-acetate to list a few) or the extraction techniques (maceration or percolation) may play an important role (Cos *et al*., 2006). It is also important to choose the most appropriate bioassay to test for the relevant activity (Cos *et al*., 2006). For example although *Bacillus subtilis* was used as a representative of the Gram-positive bacteria during this study, Van Vuuren (2008) suggests that the use of this organism should be avoided as it is not pathogenic and is therefore not ideal to screen for anti-infective activity. Future work should therefore include the use of bacteria which may be more representative of human pathogens (for examples of test organisms for anti-bacterial, anti-fungal, anti-viral and anti-parasitic organisms for *in vitro* screening refer to Cos *et al*. 2006).

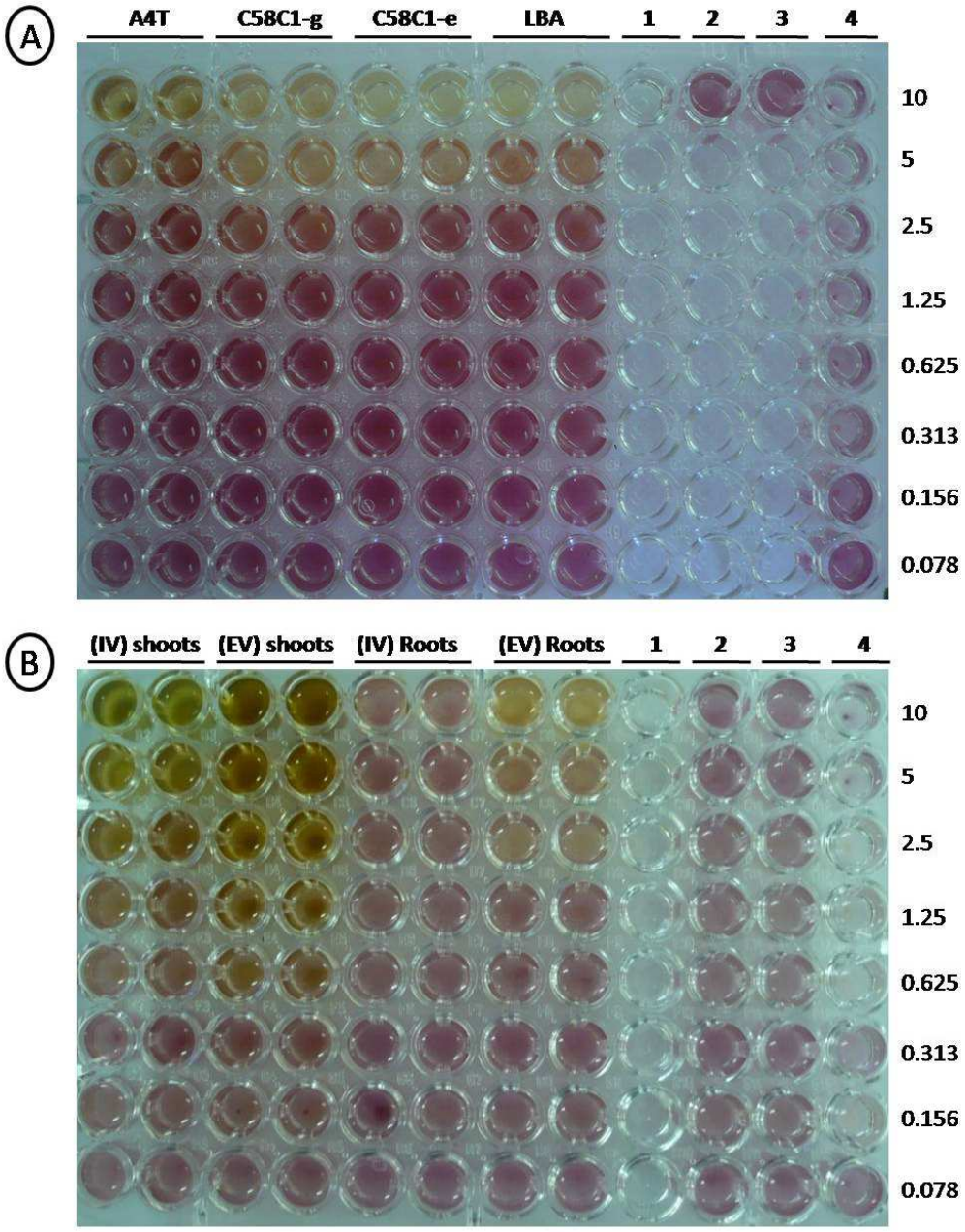


Figure 7.3.1: Anti-microbial assay of extracts prepared from hairy roots and untransformed tissue. A) hairy root clones (from left to right: wells 1 and 2; clone A4T; wells 3 and 4; clone C58C1-g; wells 5 and 6; clone C58C1-e; wells 7 and 8; clone LBA) and B) untransformed root and shoot extracts (from left to right: wells 1 and 2; *in vitro* (IV) shoots; wells 3 and 4; *ex vitro* (EV) shoots; wells 5 and 6; *in vitro* (IV) roots and wells 7 and 8; *ex vitro* (EV) roots. The controls for both experiments where: (1) MH broth without bacterium, (2) extract replaced with water (3) the highest concentration of solvent (5% DMSO) used, and, (4) streptomycin antibiotic (positive control)

7.3.2 TLC analysis of extracts

Methanol was used for the extraction of polar compounds whilst non-polar compounds were extracted with dichloromethane. Since the extracts were pooled together, they contained both polar and non-polar compounds. Three solvent systems were investigated for separation of compounds. The standards were selected to represent compounds from flavonoids, terpenoids, amino acids and coumarins. The TLC analysis was just a preliminary screening to investigate the inter-clonal differences between the hairy root clones and the untransformed roots.

During visualization, the TLC plates were firstly viewed using long wave UV light (365 nm). This method is usually applied to screen for the presence of anthraglycosides, coumarins, flavonoids, phenolcarboxylic acids and some types of alkaloids (Wagner and Bladt, 2001). Prior to visualization, the TLC plates were sprayed with a NP/PEG reagent (Natural products-Polyethylene glycol) and those compounds which fluoresce with a bright blue zone under UV light are reported to be coumarins. Additionally, according to Wagner and Bladt (2001), the PEG in this reagent increases the sensitivity for detection of flavonoids from 10 µg to 2.5 µg.

Of the three solvent systems tested, the polar system (ethyl acetate: methanol: water) worked best for the separation of the compounds in the extracts (Figure 7.3.2). However, the weak blue bands at the site of application may be flavonoids, as this particular solvent system does not provide a good separation for the flavonoid glycosides (Wagner and Bladt, 2001). These authors suggest using an ethyl acetate: formic acid: glacial acetic acid: acid water (100: 11: 11: 26) solvent system for the investigation of flavonoids.

From the phytochemical profiles, it was apparent that the hairy root clones were more complex than the untransformed *in vitro* and *ex vitro* roots. The bands marked with ‘a’ (indicated with the arrow, Figure 7.3.2) were more intense in the hairy root clones (slightly reduced in clone LBA) than in the untransformed roots. The zone marked with a ‘b’ (indicated with the bracket, Figure 7.3.2) contained noticeably more bands in the hairy root lanes than the untransformed root extracts. However, it is not clear whether these compounds are absent from the untransformed roots or whether they are present in such low levels that they were not detected. In the past, it has been shown that the synthesis of some metabolites is altered during transformation due to the random insertion of T-DNA genes and the copy number of the inserted T-DNA genes may also have an influence (Dhakulkar *et al*., 2005). Furthermore, in some instances, these roots may also be able to synthesize novel compounds that do not occur in the untransformed tissue (Dhakulkar *et al*., 2005).

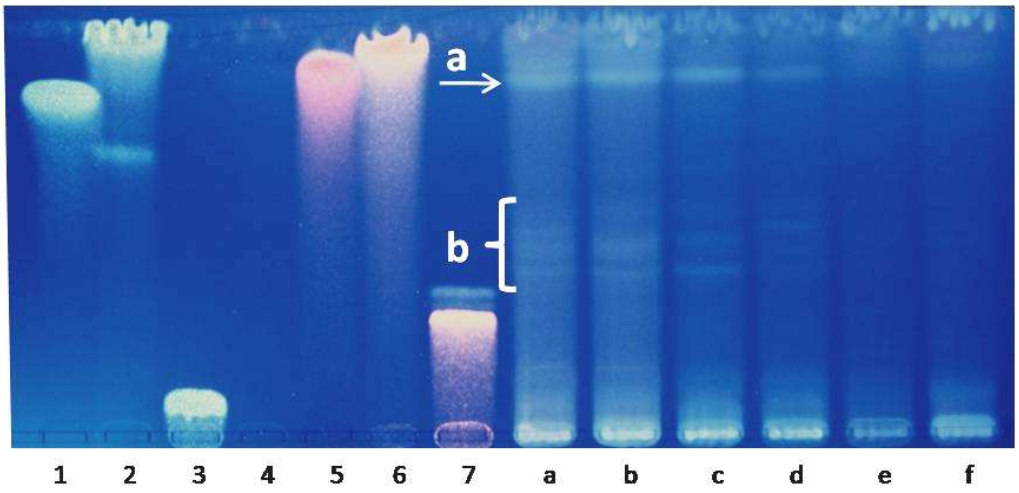


Figure 7.3.2: Thin layer chromatography plate of hairy root and untransformed root extracts visualized under UV_{365nm}. The extracts were separated with the ethyl acetate: methanol: water solvent system. The standards used included 1) caffeic acid; 2) kaempferol; 3) chlorogenic acid; 4) coumaric acid; 5) myrecitin; 6) quercetin, and, 7) rutin. The samples were loaded as follows: a) A4T, b) C58C1-g, c) C58C1-e, d) LBA, e) *in vitro* untransformed roots, and, f) *ex vitro* untransformed roots

The visualization of amino acids was achieved by spraying the TLC plates with ninhydrin (Figure 7.3.3). Some of the standards, arginine (lane 1), canavanine (lane 4), glutamic acid (lane 5) and serine (lane 6) changed to a violet colour (magenta for canavanine). It is therefore difficult to distinguish between these compounds in the extracts using this reagent. Asparagine turned an orange-brown colour. Urea, one of the products from canavanine and arginine degradation, remained a clear band (lane 7) after spraying. Visualization of inter-clonal differences was further impeded as the compounds remained on the application zone and did not separate well with any of the solvent systems investigated. In Figure 7.3.3, the ethyl acetate: methanol: water was used as the solvent system, the violet zones of all the samples remained at the site of application.

The only conclusion that could be derived from this result was that the violet zones of the hairy root extracts (lanes a, b, c and d) appeared to contain more amino acids than the *in vitro* and *ex vitro* untransformed root samples (lanes e and f respectively). The presence of an orange-brown band (R_f = 0.80; indicated with the arrow marked 'I') in clone A4T (lane a) closer to the solvent front on the TLC plate, was not detected in any of the other extracts. The reason for this may either be complete absence from the other transgenic and non-transformed roots or alternatively it was only synthesized at reduced levels. However, a band with a similar colour

(R_f = 0.09; the arrow marked with 'II') was visible closer to the application site of the *ex vitro* root extract (lane f). In this instance, it is unclear whether this compound was absent from the other extracts as this band in the adjacent zones may have been concealed by the violet zone. Better resolution of amino acids may be achieved with other staining methods. These include the Sakaguchi reagent, which stains arginine and its derivatives a scarlet colour, or Fearon's PCAF reagent, which stains canavanine a magenta colour (Harborne, 1998).

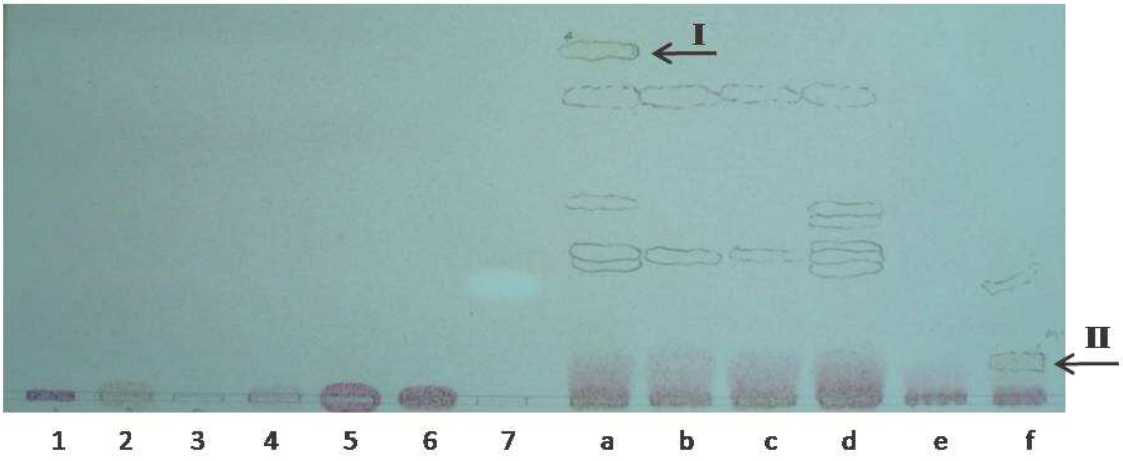


Figure 7.3.3: Thin layer chromatography plate of hairy root and *in vitro* and *ex vitro* untransformed root extracts visualized with the ninhydrin spray. Extracts were separated with the ethyl acetate: methanol: water solvent system and the TLC plates were sprayed with ninhydrin to visualize amino acids, amines or protein-like compounds. The standards used for this system included 1) arginine, 2) asparagine, 3) betaine, 4) canavanine, 5) glutamic acid, 6) serine, and, 7) urea. The samples were loaded as follows a) A4T, b) C58C1-g, c) C58C1-e, d) LBA, e) *in vitro* untransformed roots, and, f) *ex vitro* untransformed roots

Finally, the plates were also stained with anisaldehyde, which is often employed to detect terpenoids, propylpropanoids, pungent and bitter principles and sapononins (Wagner and Bladt, 2001). The TLC plate in Figure 7.3.4 was developed in the chloroform: ethyl acetate: formic acid solvent system prior to staining.

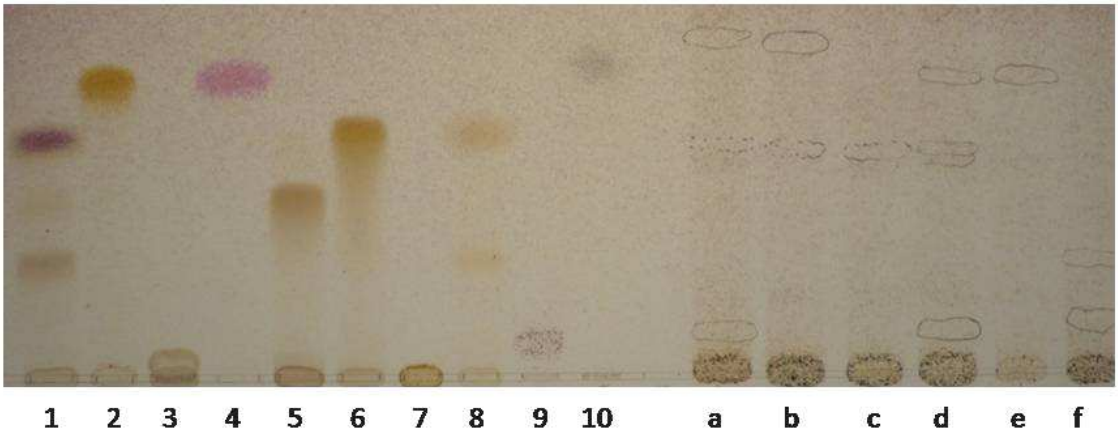


Figure 7.3.4: Thin layer chromatography plate of hairy root and *in vitro* and *ex vitro* untransformed root extracts visualized after anisaldehyde spray. The extracts were separated with the chloroform: ethyl acetate: formic acid solvent system. The standards used for this system included: 1) caffeic acid, 2) kaempferol, 3) chlorogenic acid, 4) coumaric acid, 5) myricetin, 6) quercetin, 7) rutin, 8) scopoletin, 9) shikimic acid, and, 10) vanillin. The samples were loaded as follows a) A4T, b) C58C1-g, c) C58C1-e, d) LBA, e) *in vitro* untransformed roots, and, f) *ex vitro* untransformed roots

The compounds that were stained in the extracts were detected on the application site and they had a yellow-black colour (Figure 7.3.4). Due to their location it was difficult to determine whether there were differences between the hairy root clones and untransformed roots, however, the *in vitro* untransformed roots (lane e) did appeared to contain less compound(s) compared with the hairy root and *ex vitro* root extracts. The TLC system provides a preliminary view of the metabolite profiles of the extracts but it is not a very powerful technique, therefore, this work was followed up with LC-MS analysis.

7.3.3 Liquid chromatography mass spectrometry (LC-MS) analysis of extracts

During the LC-MS screening of the hairy root, untransformed root and shoot extracts (*in vitro* and *ex vitro* material) the presence of the secondary metabolite SU1 (Van Wyk and Albrecht, 2008) was detected particularly in the shoot extracts (Figure 7.3.5; Table 7.3.2).

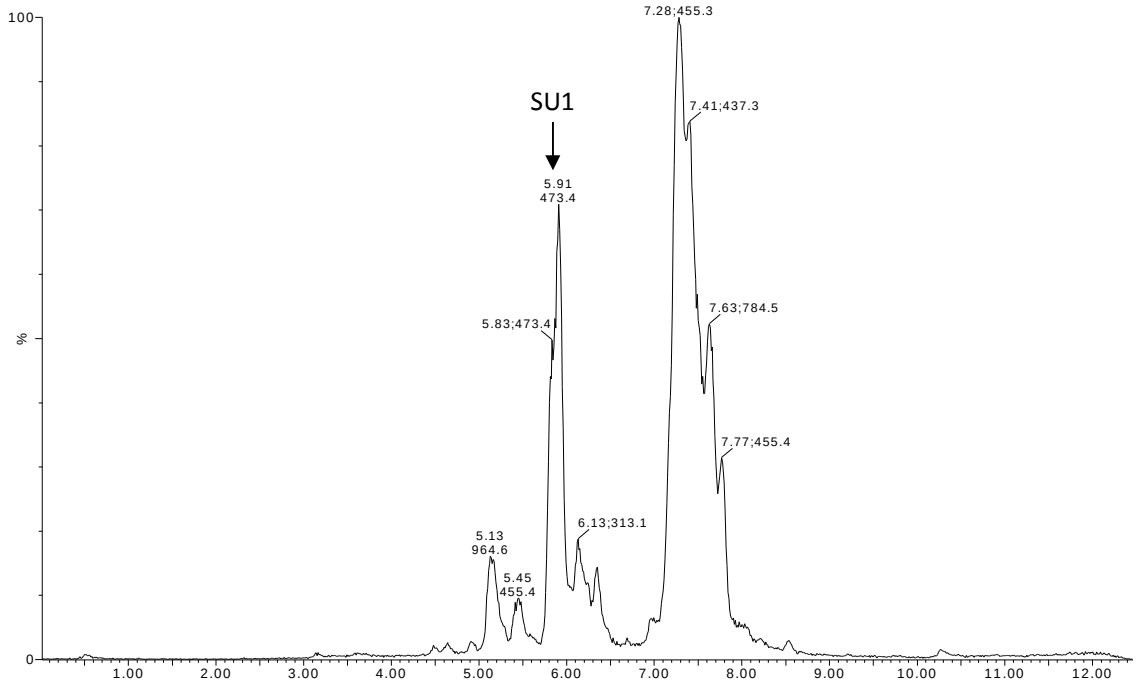


Figure 7.3.5: The detection of SU1 in the extract prepared from the *ex vitro Sutherlandia* shoot material using LC-MS analysis

Although the concentration of this compound was not investigated a comparison of the areas underneath the peaks for each extract could be made. Table 7.3.2 indicated that root extracts contained very little to no SU1. Although the *in vitro* shoots contained a high levels of SU1, this compound was most abundant in the *ex vitro* shoots.

Table 7.3.2: SU1 concentration in various extracts prepared from *in vitro* and *ex vitro* material

Extract	**Area for SU1**
A4T	Not detected
C58C1-g	Not detected
C58C1-e	65
LBA	76
In vitro untransformed roots	147
Ex vitro untransformed roots	64
In vitro S. frutescens shoots	1269
Ex vitro S. frutescens shoots	4830

A preliminary LC-MS profilling of the phytochemistry of the extracts was conducted. Unfortunately, due to a lack of information on the identity of the secondary metabolites of *S. frutescens*, it could not be determined which compounds were different (or similar) between the *in vitro* and *ex vitro* material or between the transgenic and untransformed plants. In future, this problem can be solved by generating a comprehensive secondary metabolite library to screen the extracts.

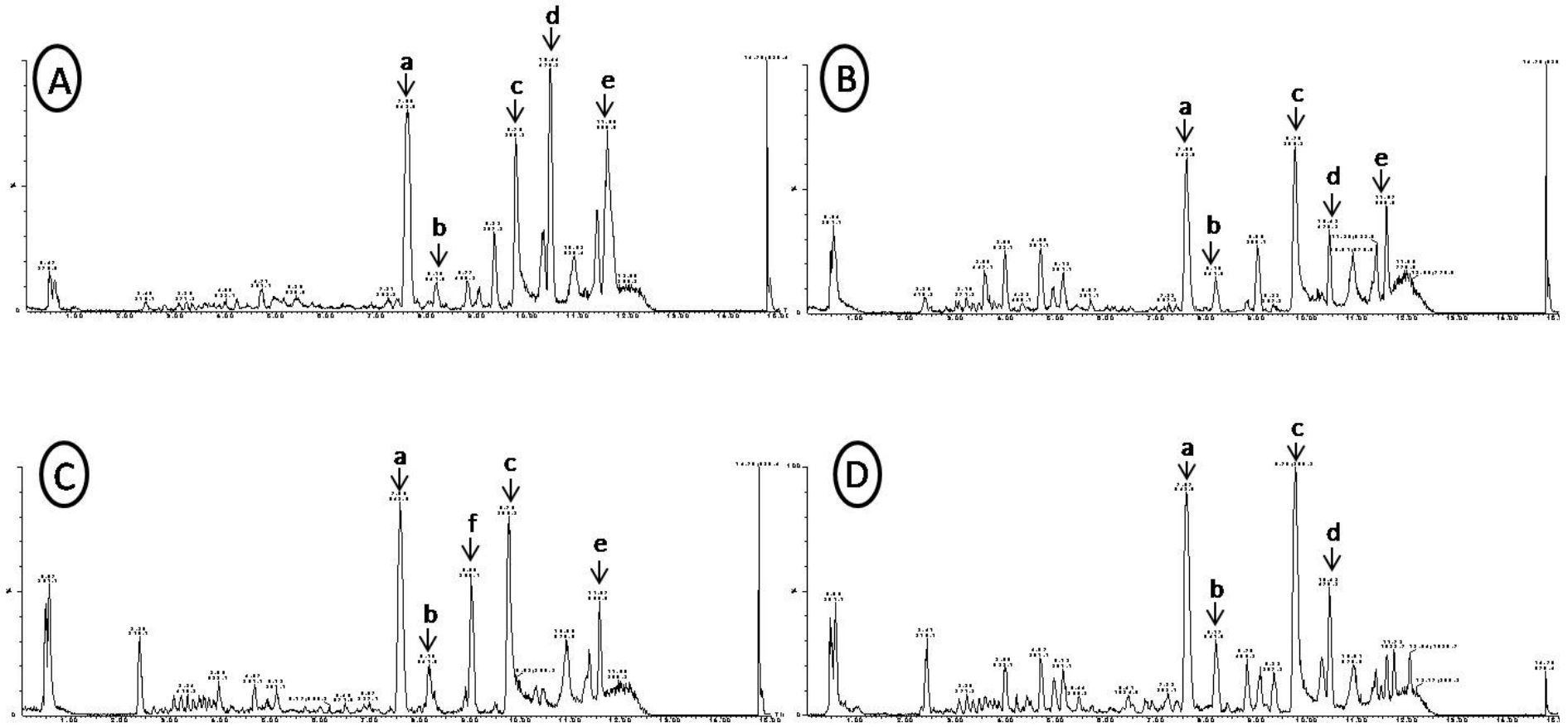
The comparison of the profiles indicated that there were inter-clonal differences between the hairy root clones (Figure 7.3.6A-D). Some of the peaks were present in all four hairy root clones, however other peaks, such as the one marked 'd' (retention time (R_t) = 10.44 and a molecular mass (M_r) = 479.2) was present in all the clones except clone C58C1-e (Figure 7.3.6C). Another observation from this peak was that it was more abundant in clone A4T (Figure 7.3.6A), whilst it occurred in a lower percentage in clones C58C1-g and clone LBA (Figure 7.3.6B and C respectively). Variation in the secondary metabolite level of hairy root clones was also indicated in *Gmelina arborea* Roxb. hairy root clones which accumulated different verbascoside levels (Dhakulkar *et al*., 2005). It must be re-emphasized that genetic transformation via *Agrobacterium* is untargeted with the transgene being preferentially incorporated into active parts of the genome, but this effect is still random. Positional effects and alterations to the transgene (duplications or inverted repeats) as well as deletion of endogenous genes can influence gene expression, causing altered metabolite synthesis (Dhakulkar *et al*., 2005).

The comparison of the hairy root extracts with untransformed root extracts revealed that certain peaks, such as the one marked 'f' (R_t = 9.00 and M_r = 365.1), were present at very low levels in the *in vitro* and *ex vitro* untransformed root extracts (Figure 7.3.6E and F). This peak was absent from all the hairy root clones except clone C58C1-e, in which it appeared at an increased percentage (Figure 7.3.6C). It would therefore appear that the synthesis of this metabolite was down-regulated or disrupted in the other three clones.

Another observation is that some of the peaks in the *ex vitro* roots were more abundant than in the *in vitro* untransformed root culture. Peak 'b' (R_t = 8.16 and M_r = 941.5), for example, was present in high levels in the *ex vitro* roots (Figure 7.3.6F) but was not detected in the *in vitro* untransformed roots (Figure 7.3.6E). Therefore, differences between *in vitro* and *ex vitro* untransformed roots were also present.

A comparison of the root profiles with the shoot profiles indicated that several of the peaks (a, b and d for example) in the roots were absent from the shoots. Differences in the secondary metabolite yield between the hairy root cultures and *ex vitro* shoot material was also clear. A similar observation was made in *Ruta graveolens* (L.) hairy root, cultures which contained higher levels of certain coumarins than the shoot culture extracts, additionally. these hairy roots also synthesized compounds which were never detected in this species before (Sidwa-Gorycka *et al*., 2009).

However, the reverse was also true; the *Sutherlandia* shoot extracts contained peaks which were not present in the root (transgenic and untransformed) profiles. An example is peak 'g' (R_t = 6.83 and M_r = 489.4), which was not only more abundant in the *ex vitro* shoot extracts than the *in vitro* cultivated shoots, but it was also absent from all the root extracts. These results suggest that the *ex vitro* material contain higher levels of certain secondary metabolites than the *in vitro* cultured plants. In *Saussurea involucrate* the aerial parts (leaves and flowers) collected from wild plants contained higher rutin levels than all the *in vitro* cultures that were analyzed (Fu *et al*., 2006). As suggested previously, plants growing in nature may experience various environmental stresses and this pressure can result in the upregulated synthesis of specific metabolites.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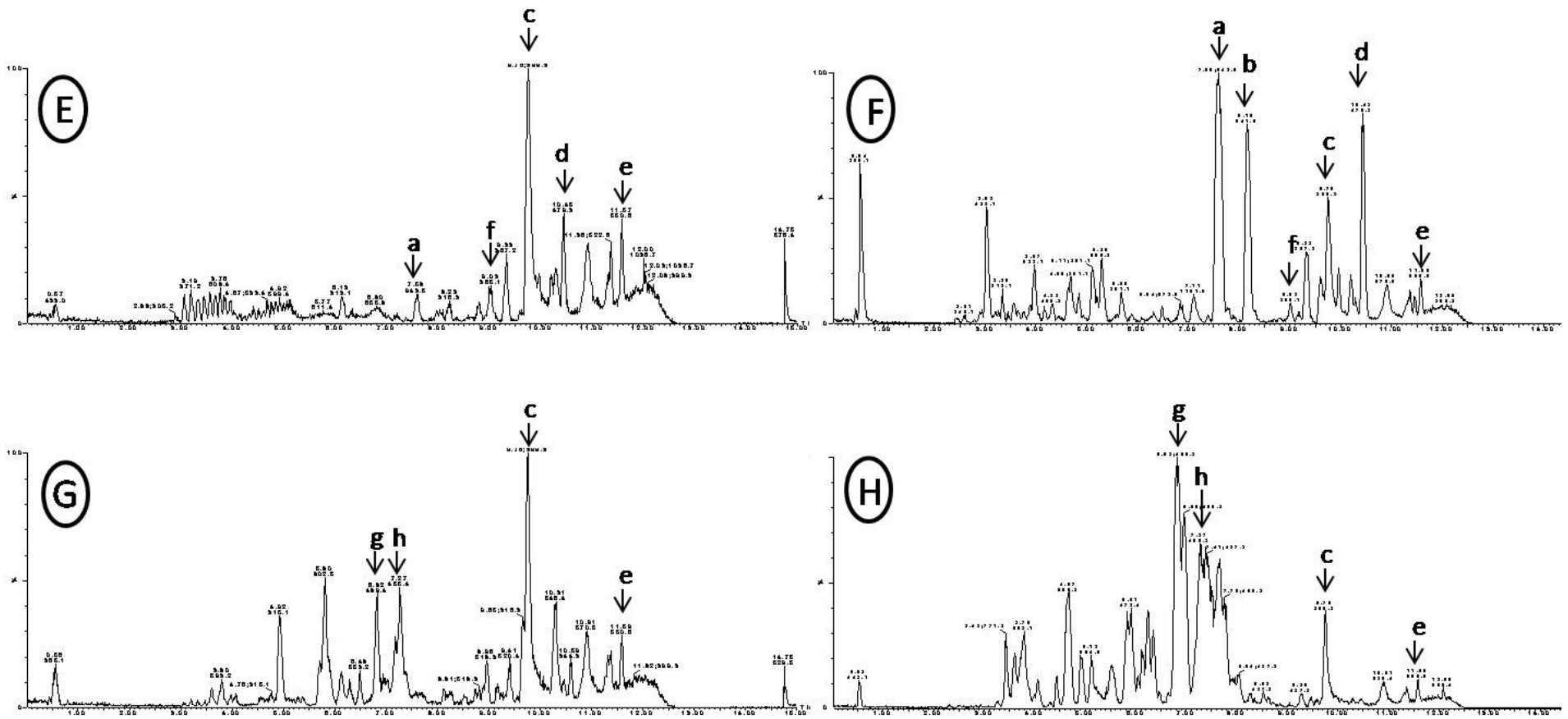

Figure 7.3.6: Liquid chromatography mass spectrometric (LCMS) profiles of extracts. A) Hairy root clone A4T, B) C58C1-g, C) C58C1-e, D) LBA, E) *in vitro* untransformed roots, F) *ex vitro* untransformed roots, G) *in vitro* cultivated *S. frutescens* shoots, and, H) *ex vitro* derived *S. frutescens* shoots. Peaks with the same retention time (R_t) and molecular weight (M_r) are indicated by similar letters in all the profiles

7.4 CONCLUSION

The preliminary investigation of the anti-bacterial activity indicated that the hairy root cultures displayed more activity than the untransformed (*ex vitro* and *in vitro*) roots, but the activity was still highest in extracts prepared from the shoots harvested from the wild. The differences in the secondary metabolic profiles suggested that the synthesis of certain compounds in the hairy roots were upregulated, which may have contributed to improving the anti-bacterial activity of the hairy root extracts. However, the phytochemical analysis also indicated that the shoots contained compounds which were not present in the root extracts and these can be further investigated as leads for active principles. These metabolites in the shoot extracts were also higher in the *ex vitro*-derived material. Plants in nature interact with the environment which can alter their metabolism and result in the production of higher levels of the active metabolites. This work will therefore be followed up with phytochemical analysis and pharmacological analysis of abiotically-stressed plants. The differences observed in the types of metabolites and specific metabolites levels in the phytochemical profiles of transgenic and untransformed *in vitro* and *ex vitro*-derived material could not be fully elucidated. Therefore, more work is required to discover what these compounds are and if they can assist with the identification of the metabolites responsible for perceived anti-bacterial activity. It can be concluded that variability in the type of secondary metabolites and the levels of these metabolites exist between hairy root clones, also between transgenic and non-transgenic tissues. There is also considerable variation between plant tissue extracts from *in vitro* propagules and plant material growing *ex vitro*.

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CHAPTER 8:

Conclusion and future prospects

Sutherlandia frutescens is one of South Africa's renowned medicinal plants with a long standing history of traditional use. Increase in the commercial demand and interest in the phytochemistry of this plant provides a strong incentive to apply scientific techniques to ensure its sustainable use in the future and to validate the medicinal value of this plant. The use of a biotechnological approach is key to accomplishing the goals of this project as it provides an important platform not only for conducting pharmacological and phytochemical research, but it also allows the use of genetic tools to alter key metabolic pathways for the synthesis of valuable medicinal products in the future.

Micropropagation of medicinal plants is useful as it serves aims of *in vitro* conservation and it is generally a prerequisite for molecular genetic transformation using *Agrobacterium*. The seed germination experiment confirmed previous results indicating that *S. frutescens* seed germination is dependent on the removal of the seed coat. An alternative treatment (flaming seeds) which also removes the seed coat was equally effective for seed germination. The micropropagation system described in Chapter 3 has several advantages. It is easy to establish using seedling material, with little contamination of explants occurring. In the future, the system described here could easily be substituted for use with leaf foliage. The current protocol had a high regeneration rate for shoot multiplication and elongation, where over 15 000 plants can be produced over a six month cycle on medium without plant growth regulators, thus reducing the costs of tissue cultured propagation. This is an added advantage particularly for adopting it for a commercial tissue culture setup, more especially seeing that rooting was spontaneous (occurring at a frequency of 85%) and acclimation could be achieved easily.

The study by Chinkwo (2005) indicated that extracts prepared from plants collected from the Western Cape region had higher apoptotic activity than those extracts from other regions (Free State and Bloemfontein) in South Africa. The difference in the activity was attributed to variation in the climatic and environmental conditions in which these plants grow which may induce variation in the phytochemical patterns of the extracts. During this study, three stress treatments; nitrogen availability, salinity stress and water stress were investigated to determine

the effect on the synthesis of four metabolites (canavanine, arginine, asparagine and GABA). These three factors represent typical abiotic stresses which these plants (which naturally occur near the coastal regions) may experience which include nutrient deficiency, limited water supply and high salt deposits in the soil. The four compounds were selected to monitor the effect of stress on primary metabolism and these compounds are also measured during the commercial preparation of herbal products.

Stable genetic transformation of *S. frutescens*, confirmed via PCR and Southern hybridization analysis, has been achieved for the first time in this species (Chapter 4). This is one of the major contributions of this study, because some medicinal plants show recalcitrance to genetic transfer. The frequency of genetic transformation was highest with strain A4T and this study also validates the use of cotyledon and hypocotyl explants. The most amenable tissue to transformation was the hypocotyls, with 93% of the explants producing roots with strain A4T. Some researchers find inherent difficulties in producing true hairy roots, even when they have been able to show that gene transfer has occurred through molecular biological techniques. Added to this, transgene silencing may lead to hairy root cultures losing their regenerative ability over extended periods in culture. The strategy described here (Chapter 4) produced root cultures displaying typical symptoms of the hairy root syndrome such as an anti-gravitropic growth and a high growth rate. Together with normal shoot cultures, these provided a useful tool to study the effect of abiotic stress on the synthesis of GABA, asparagine, canavanine and arginine (as demonstrated in Chapters 5 and 6). This was followed by a comparison of the phytochemistry and anti-bacterial activity of organ cultures (*in vitro* transgenic roots and untransformed microplants) as well as *ex vitro*-collected plant material (Chapter 7). Through LC-MS profiling, changes in the secondary metabolite pool was evident in different hairy root clones.

One of the core objectives was to use the generated organ cultures to study the influence of abiotic stresses. In a brief summary, nitrogen availability had a significant influence on the primary metabolites of interest. When nitrogen levels increased in the growth medium, all the metabolites, with the exception of GABA, accumulated to a higher level. It can be assumed that the observed increase is related to the fact that synthesis of these metabolites, which are free amino acids, is directly correlated with nitrogen availability. On the other hand, when GABA is degraded it can supply intermediate products to TCA metabolism (Chapter 5), it also plays a role in regulating pH and acts as signaling molecule during environmental stress in plants. Therefore, during stress conditions, GABA may be rapidly utilized to maintain

homeostasis and thus the GABA pool becomes lowered. The hairy root cultures were capable of synthesizing only three of the metabolites (GABA, asparagine and arginine), whilst no canavanine was detected in the hairy root cultures. This result corroborates work by Hwang *et al*. (1996) where it was suggested that roots are incapable of synthesizing canavanine. Interestingly enough, prolific root development became apparent when nitrogen was limited in the medium and this may be related to an attempt to increase the surface area for macronutrient absorption. The symbiotic relationship with rhizobacteria in *Sutherlandia* is an important physiological trait for nitrogen fixation. Tissue culture propagation requires exclusion of microbes in order to allow for prolific organogenesis under aseptic conditions. In this case, the nitrogen is provided by the medium. In future, it may be beneficial to assess the effect of nodulation in whole plants to generate a holistic perspective on the response to abiotic stress.

Water availability greatly influenced the growth characteristics with plants being smaller under water stress conditions and *in vitro* rooting became hampered. This is related to the importance of water to general plant growth and development. Reduced water content in the medium had little to no effect on the primary metabolic pathways related to the metabolites of interest. As for salinity stress, at 50 mM NaCl, observed changes to shoots included an increase in water uptake and chlorosis but shoot length was reduced and rooting was inhibited. The increased water uptake may be related to a reduction in the osmotic potential of the plant. NaCl treatment can also lead to ion toxicity (Liu and Van Staden, 2001) and this became more obvious at a 100 mM NaCl when it resulted in significant physiological changes. Of the metabolites monitored, arginine was the only metabolite that increased with increased salinity (Chapter 6). Although not tested in this study, it can be predicted that salt is likely to induce polyamine (spermine, spermidine and putrescine) and proline synthesis, as these compounds are essential for osmotic adjustments in the plant during conditions of stress. This is one effect that needs to be investigated further.

In Chapter 7, *ex vitro* material was compared to *in vitro* propagules. Propagated tissue displayed a lowered anti-bacterial activity against Gram-positive and Gram-negative bacteria. Plants growing in nature are exposed to various climatic and seasonal variations which alters secondary metabolism. In tissue culture, conditions are kept optimal for growth and this may result in reduced levels of pharmacologically active metabolites. Hairy root cultures displayed better activity than untransformed root cultures but this effect did not match the activity of the microplants. This further confirms the preference for use of leaf tissues for traditional purposes. In future, acclimatized microplants may be compared to wild plants for biological

activity. It would be interesting to test for other pharmacological-activities such as anti-oxidant, anti-fungal, anti-proliferative and central nervous system assays for both hairy root cultures and stressed *in vitro* plants. Changes in the chemical constituents in solvent extracts were detected after TLC and LC-MS profiling, with hairy root cultures showing enhanced levels of secondary metabolites. Overall, the hairy root extracts were more complex than the untransformed roots.

FUTURE RESEARCH:

Work conducted as part of this Masters study has established a platform for future research. It is envisage that this project will continue and will focus on several new questions. These include examining other methods for acclimation of *in vitro* plants (Chapter 3) which would then be followed by a comparative analysis of the phytochemistry and pharmacology to wild plants. This could be achieved through LC-MS, GC-MS and NMR profiling. Several *in vitro* assays are also envisaged to screen for other pharmacological activity. During this study, several different hairy root clones were produced and these clones may be further studied at the metabolic and molecular levels. Regenerated plantlets from transgenic hairy root cultures were easily generated (Chapter 4) and the expression of *rol* genes through reverse transcriptase-polymerase chain reaction (RT-PCR) may be monitored. This will further inform us on the effect of Ri transformation on the morphological traits of the transgenic plants. A more comprehensive examination into the metabolite profiles of these transgenic plants will follow.

Chapters 5 and 6 focused on the effect of several stresses (nitrogen, water and salt) on *in vitro* cultures. Stress in plants is complex and several new questions pertaining to the general response may be followed as part of a PhD. It would be interesting to analyze other primary metabolites such as carbohydrates, amino acids (proline, spermidine, spermine and putrescine), pinitol, and glycine betaine. Methods which include High Performance Liquid Chromatography (HPLC) may be used to assess the accumulation of these metabolites. During stress, reactive oxygen species (ROS) such as H_2O_2, O_2^- and OH^- can cause a high degree of oxidative damage in the plant. One of the mechanisms by which plants deal with this type of stress, is through upregulating enzymes that scavenge these harmful oxygen radicals. Some of these enzymes include superoxide dismutase (SOD) and catalase (CAT) and they react by scavenging ROS, thereby reducing the damage to lipids, proteins and nucleic acids (Dasgupta *et al*., 2008). The activity of these enzymes is generally monitored

through enzyme assays (Banowetz *et al*., 2004; Raza *et al*., 2007). Further work will also include using cDNA-AFLP analysis coupled to Fourier transform mass spectroscopy (FT-MS) to correlate the genes involved in the stress response with the types of molecules that are synthesized. Changes in metabolic flux will further inform on metabolome-transcriptome networks functioning in response to stress (Goossens *et al*., 2003). This work will be conducted in collaboration with researchers from the University of Ghent.

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APPENDIX

The components of the media used in this study are listed in Table A1 and A2. The medium was adjusted for the nitrogen experiments according to table A2.

Table A1: Media composition of MS, Gamborg B5, White's, Linsmaier and Skoog and Phillips and Collins tissue culture media

Components	MS[a]	Gamborg B5[b]	White[c]	Linsmaier and Skoog[d]	Phillips and Collins[e]
	Amount (mg L^{-1})				
Macronutrients					
$MgSO_4.7H_2O$	370.0	250.0	737.0	370.0	435.0
KH_2PO_4	170.0	-	-	170.0	325.0
KNO_3	1 900.0	2 500.0	80.0	1 900.0	2 100.0
NH_4NO_3	1 650.0	-	-	1 650.0	1 000.0
$(NH_4)_2SO_4$	-	134.0	-	-	-
$Ca(NO_3)_2{\cdot}4H_2O$	-	-	288.0	-	-
$CaCl_2.2H_2O$	440.0	150.0	-	-	600.0
$CaCl_2{\cdot}H_2O$	-	-	-	440.0	-
$Na_2SO_4{\cdot}10H_2O$	-	-	460.0	-	-
$NaH_2PO_4{\cdot}H_2O$	-	150.0	19.0	-	85.0
KCl	-	-	65.0	-	-

Micronutients					
H_3BO_3	6.2	3.0	1.5	6.2	5.0
$MnSO_4.4H_2O$	22.3	-	6.65	22.3	-
$MnSO_4 \cdot H_2O$	-	10	-	-	15.0
$ZnSO_4 \cdot 7H_2O$	8.6	2.0	2.67	8.6	5.0
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	-	0.25	0.4
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.001	0.025	0.1
$CoCl_2.6H_2O$	0.025	0.025	-	0.025	0.1
KI	0.83	0.75	0.75	0.83	1.0
$FeSO_4 \cdot 7H_2O$	27.8	27.8	27.8	27.8	-
Na_2EDTA	33.6	33.6	33.6	37.3	25.0
MoO_3	-	-	0.0001	-	-
Organic supplements					
Thiamine-HCl (vitamin B_1)	0.1	10.0	0.1	0.4	2.0
Pyridoxine-HCl	0.5	1.0	0.1	-	0.5
Nicotinic acid	0.5	1.0	0.5	-	-
Myo-inositol	100.0	100.0	-	100.0	250.0
Glycine	2.0	-	3.0	-	-
Sucrose	30 000	20 000	20 000	30 000	25 000

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Table A2: Murashige and Skoog medium (1962)

Components	Amount (mg L^{-1})	½ nitrogen (mg)	2x nitrogen (mg)
Macronutrients			
$MgSO_4.7H_2O$	370		
KH_2PO_4	170		
KNO_3	**1900**	950	3800
NH_4NO_3	**1650**	825	3300
$CaCl_2.2H_2O$	440		
Micronutients			
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		

The Tables indicated below summarize the statistical data for the nitrogen experiments (Table A2).

Table A3: Investigation of the correlation between metabolite changes of *in vitro* shoots cultivated on different nitrogen levels

Nitrogen availability (shoots)	**Arginine**		**GABA**		**Asparagine**	
Canavanine	r = -0.2316	p = 0.4688	r = -0.0147	p = 0.9496	r = 0.2501	p = 0.2742
Arginine	-	-	r = -0.2848	p = 0.3696	r = -0.2648	p = 0.4055
GABA	-	-	-	-	r = 0.1824	p = 0.4286

Table A4: Investigation of the correlation between metabolite changes of *in vitro* hairy roots cultivated on different nitrogen levels. Clone C58C1-g was used for these experiments

Nitrogen availability (hairy roots)	**Arginine**		**Asparagine**	
GABA	r = 0.0411	p = 0.8990	r = -0.0532	p = 0.8697
Asparagine	**r = 0.8135**	**p = 0.0013**	-	-

Tables A5 and A6 indicate the statistical data for the PEG (3%; w/v) experiments.

Table A5: Investigation of the correlation between metabolite changes of *in vitro Sutherlandia* shoots cultivated on media containing no PEG (control) or 3% PEG for the induction of water stress in the shoots

Water stress	**Arginine**		**GABA**		**Asparagine**	
Canavanine	r = 0.7260	p = 0.0414	r = 0.3009	p = 0.2958	**r = 0.7179**	**p = 0.0038**
Arginine	-	-	r = 0.1546	p = 0.7148	r = 0.4567	p = 0.2553
GABA	-	-	-	-	r = 0.5904	p = 0.0262

Table A6: Investigation of the physiological changes between *in vitro Sutherlandia* shoots cultivated on media containing no PEG (control) or 3% (w/v) PEG for the induction of water stress in the shoots

Water stress	Fresh mass		Shoot elongation		% shoots rooting	
Average no of roots	r = 0.3590	p = 0.0009	r = 0.8068	p = 0.000	r = 0.7552	p = 0.000
Fresh mass	-	-	r = 0.9325	p = 0.000	r = 0.5376	p = 0.000

Tables A7 and A8 represent the statistical data for the salt experiments.

Table A7: Investigation of the correlation between metabolite changes of *in vitro Sutherlandia* shoots cultivated on media with no salt (control) or in media containing 50 mM or 100 mM NaCl to induce salt stress in the shoots

Salt stress (shoots)	Arginine		GABA		Asparagine	
Canavanine	r = -0.2316	p = 0.4688	r = -0.0147	p = 0.9496	r = 0.2501	p = 0.2742
Arginine	-	-	r = -0.2848	p = 0.3696	r = -0.2648	p = 0.4055
GABA	-	-	-	-	r = 0.1824	p = 0.4286

Table A8: Investigation of the correlation between the physiological changes of *in vitro Sutherlandia* shoots cultivated on media with no salt (control) or in media containing 50 mM or 100 mM NaCl to induce salt stress in the shoots

Salt stress (shoots)	Fresh mass		Shoot elongation		% shoots rooting	
Average no of roots	r = 0.0350	p = 0.7435	r = 0.1771	p = 0.0969	**r = 0.7333**	**p = 0.000**
Fresh mass	-	-	**r = 0.2996**	**p = 0.0043**	r = 0.0651	p = 0.5424
Shoot elongation	-	-	-	-	r = 0.1599	p = 0.1344

Data represented in Table A9 indicates metabolites levels in non-micropropagated plants.

Table A9: Summary of the metabolite levels (mg g^{-1} DW) in *ex vitro* plants

	Canavanine	**Arginine**	**Asparagine**	**GABA**
Wild plants[a]	0.42 – 14.5	0.5 – 6.7	1.6 – 35.0	0.23 – 0.85
Commercial plants[a]	1.6 – 3.1	-	-	-
Glasshouse cultivated[b]	3.88 ± 1.8	0.15 ± 0.05	2.14 ± 1.1	2.42 ± 0.3

[a]Values obtained from Van Wyk and Albrecht (2008). Van Wyk B-E, Albrecht C (2008). A review of the taxonomy, ethnobotany, chemistry and pharmacology of *Sutherlandia frutescens* (Fabaceae). Journal of Ethnopharmacology 119: 620 – 629

[b] These results were not subjected to statistical analysis