Effects of over-expressing the *AgGPPS2* gene in *Salvia stenophylla* (Burch. ex Benth) on terpenoid biosynthesis

by
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Declaration

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Abstract

Plant secondary compounds have been exploited as medicines, flavourants, incense and are widely used for different culinary purposes. Efficacy of herbal remedies is determined by the phytochemical profile which is dependent on the species, locality and growth conditions. *Salvia stenophylla* (Burch. ex Benth.) is a local aromatic sage growing in almost all South African biomes, with the volatile compound accumulation varying depending on season, locality and genotype. *Salvia stenophylla* extracts have proven anti-inflammatory, anti-plasmodial, anti-oxidant and anti-anxiety properties due to the presence of essential oils, flavonoids and caffeic acid derived phytochemicals. As a result, *S. stenophylla* extracts are increasingly being used for the formulation of pharmaceutical and cosmeceutical products. However, these industries largely depend on the wild populations for raw materials, and increased commercialization of sage-based natural products and medicine exerts harvesting pressure. This might reduce the sustainability of herbal medicines since there is no formal cultivation of *S. stenophylla* in South Africa. Medicinal plant biotechnology provides options for propagation and manipulation of herbal plant to increase synthesis of secondary compounds. The aims of this study were to develop tissue culture propagation system so as to provide an alternative to wild-harvesting, to improve essential oil quality and accumulation in *S. stenophylla* by applying third generation biotechnological tools to alter terpene biosynthesis via heterologous gene expression aided by *Agrobacterium*-gene transfer and to charaterize the resultant metabolite profiles using thin layer chromatography, gas chromatography mass spectrometry and liquid chromatography mass spectrometry. Initially, seeds were germinated *in-vitro* on one-tenth Murashige and Skoog medium (MS) (1962). Seeds were decontaminated by washing them in 3.5 % (w/v) hypochloride and were either scarified using 70 % (v/v) sulphuric acid, placed on media with smoke solution or both. Both scarification and smoke supplementation induced germination, but seeds subjected to both treatments, as a combination, showed very poor germination. Seedlings produced were used to establish an efficient tissue culture system for *S. stenophylla.* Shoot tips, nodal and basal explants were placed on MS media with different plant growth regulator (PGR) combinations and concentrations. The best plantlet regeneration and shoot elongation were observed on plants on medium

supplemented with 5.7 µM indole acetic acid (IAA) and 8.9 µM *N*-6-benzyladenine (BA), producing 4-6 shoots per explant with 6.67 cm mean length. Treatment with 4.5 µM 2,4 dichlorophenoxyacetic acid (2,4 D) did not produce shoots but had the highest biomass production, which was all in the form of callus. The resultant shoots from the micropropation system had similar metabolites as wild type plant and also they had some compounds that were not detected in their wild type counterparts. The effects of nitrogen, potassium, water stress, and phytohormones on metabolite accumulation were also studied in a *S. stenophylla* microplant system. Plant growth regulator free half strength MS medium encouraged the most prolific growth. Increasing potassium and nitrogen concentrations correlated to the accumulation of (-)-α-bisabolol whilst changes in other major compounds like β-bisabolene, α-muurolene, α-patchoulene, and D-limonene were insignificant. To investigate the effects of water stress on metabolite accumulation and profile, water stress was induced using sorbitol and polyethyl glycol. Reduced water availability only negatively affected rooting *in-vitro* whilst the chemical profile was not affected. The study also focused on terpene production in *S. stenophylla* by over-expressing the heterologous geranyl diphosphate synthase gene (*AgGPPS2*) from *Abies grandis,* using *Agrobacterium tumefaciens* (EHA105). The *AgGPPS2* gene was ligated to a pCAMBIA1301 vector which was cloned in EHA105 and this in turn was used to transform *S. stenophylla.* Resultant transgenic plantlets exhibited normal growth characteristics but showed variation in the metabolite accumulation. GC-MS analysis showed a 6 % increase in (-)-α-bisabolol accumulation whilst 3-δ-carene, α-pinene and camphor were lowered. Solvent extracts were analysed using LC-MS and these had rosmarinic acid in greater abundance in transgenic than in the wild type plants. However, the chemical profiles of the wild type and transgenic plants showed some similarities suggesting *AgGPPS2* expression only alters the abundance of some secondary compounds, whilst the overall integrity of the metabolome is maintained. Unlike most metabolite engineering studies elsewhere, this is the first attempt a local South African sage has been genetically engineered to enhance terpene biosynthesis. This is crucial in an economy that is increasing becoming plant based for pharmaceutical, industrial and food needs. Further studies are required to elucidate the efficacy of transgenic and *in-vitro* plant-derived extracts.

Opsomming

Plant sekondêre produkte word gebruik as medisinale-, geur-en reukmiddels en word ook as algemene bestandeel gebruik in kookkuns. Die doeltreffendheid van kruie middels word bepaal deur die fitochemiese profiel. Hierdie profiel is afhanklik van die spesie, ligging en groei kondisies. *Salvia stenophylla* (Burch. *ex* Benth.) is ‘n plaaslike aromatiese salie wat voorkom in byna al die biome van Suid-Afrika , waar die versameling van vlugtige verbindings varieer op grond van die seisoen, ligging en genotipe. *Salvia stenophylla* ekstraksies is bewys om anti-inflamatories, anti-plasmodiaal, anti-oksidant en anti-angs eienskappe te besit as gevolg van die noodsaaklike olies, flavonoïede en afgeleide kaffieksuur fitochemikalieë wat die plant besit. As gevolg word *S. stenophylla* ekstraksies meer en meer aangewend in die ontwikkeling van farmaseutiese en kosmetiese produkte. Hierdie industrieë benodig rou plant material vanuit die natuurlike populasies wat druk op die biome sit as gevolg van die verhoogde kommersialisering van salie-gebaseerde natuurlike produkte en medisyne. Gevolglik is die volhoubaarheid van medisyne afkomstig vanaf kruie soos salie in bedwang omdat geen formele kultivasie van *S. stenophylla* in Suid-Afrika al in plek gestel is nie. Medisinale plant biotegnologie bied moontlikhede vir kultiveering, voortplanting en manipuleering van kruie om sodoende die produksie van sekondêre verbindings in kruie te verhoog. Die doel van hierdie studie was om ‘n weefselkultuur voortplantingssisteem te ontwikkel om sodoende ‘n alternatief te bied vir wild oesting. Die studie het gefokus op die verbetering van noodsaaklike olie kwaliteit en akkumulasie in *S. stenophylla* deur die gebruik van derde generasie biotegnologiese gereedskap om terpeen biosintese te modifiseer. Hierdie is bereik deur weefselkultuur en heterologiese geen uitdrukking wat aangehelp is deur Agrobacterium geen oordrag. Die gevolglike metaboliet profiele was gekarakteriseer deur gebruik te maak van dun laag chromatogragie, gas chromatografie massa spektrometrie en vloeistof chromatografie massa spektrometrie. Aanvanklik is die sade ontkiem *in-vitro* op een-tiende Murashige en Skoog medium (MS) (1962). Dekontaminasie van die sade is gedoen deur die sade te was in 3.5 % (w/v) hipochloried waarna die sade óf geskarifideer is deur te was met 70 % (v/v) swawelsuur óf op medium geplaas is wat ‘n rook oplossing bevat óf albei. Beide skarifikasie en rook aanvulling het ontkieming aangehelp, maar sade

wat aan albei behandelings blootgestel is, het baie swak ontkieming getoon. Saailinge was gebruik om ‘n effektiewe weefselkultuursisteem vir *S. stenophylla* te produseer. Stingel punte, nodale en basale eksplante was op MS medium geplaas met verskillende kombinasies en konsentrasies van verskeie plant groei reguleerders (PGR). Die beste plant regenerasie en stingel verlenging was geobserveer met plante op medium aangevul met 5.7 µM indool asynsuur (IAS) en 8.9 µM N-6-bensieladenien (BA), waar dit 4 tot 6 stingels per plant met ‘n gemiddelde lengte van 6.67 cm gegroei het. Behandeling met 2,4 Dichlorophenoksiasynsuur (2,4 D) het nie stingels produseer nie, maar het die hoogste biomassa produksie getoon in die vorm van kallus. Die gevolglike stingels van die mikrovoortplantingsisteem het soortgelyke metaboliete as die wilde tipe plante, maar verbindings wat nie in die wilde tipe plante voorkom nie was ook ontdek. Die effek van stikstof, kalium, water stres en fitohormone op metaboliet akkumulasie was ook bestudeer in *S. stenophylla* mikroplantsisteem. Plant groei reguleerder vrye half sterkte MS medium het die produktiefste groei aangemoedig. Verhoging van kalium en stikstof konsentrasies het gekorreleer met die versameling van (-)-α-bisabolol en terwyl veranderinge in ander belangrike verbindings soos β-bisaboleen, α-muuroleen, α-patchouleen en D-limoneen onbeduidend was. Om die effek van water stres op metaboliet- akkumulasie en -profiel te ondersoek was water stres geinduseer deur sorbitol en poli-etielglikol te gebruik. Die verlaging van water beskikbaarheid het slegs ‘n negatiewe invloed op *in-vitro* wortelvorming gehad terwyl die chemiese profiel onveranderd gebly het. Die studie het ook gefokus op die terpeen produksie in *S. stenophylla* deur die ooruitdrukking van die heterologiese geranieldifosfaatsintase geen (AgGPPS2) vanaf *Abies grandis* met behulp van *Agrobacterium tumefaciens* (EHA105). Die *AgGPPS2* geen was geligeer in ‘n pCAMVIA1301 vektor wat geklooneer was in EHA105 en dit op sy beurt was getransformeer in S. stenophylla. Die gevolglike transgeniese plante het normale groei ten toongestel, maar het variase in metaboliet akkumulasie getoon. GC-MS analises het gewys dat ‘n 6 % toename in (-)-α-bisabolol versameling terwyl 3-δ-kareen, α-pineen en kamfor verlaag het. Opgelosde ekstraksies was geanaliseer deur VC-MS en het gewys dat roosmariensuur in groter hoeveelhede in transgeniese plante as wilde tipe plante akkumuleer. Nogtans het die chemiese profiele van die twee genotipes sommige ooreenkomste getoon, wat voorstel dat *AgGPPS2*

uitdrukking slegs sekere sekondêre verbindings menigte male vermeerder terwyl die algehele integriteit van die metaboloom gehoue bly. In teenstelling met die meederheid studies oor metaboliet ingenieuring wat elders plaasvind, is hierdie die eerste poging om ‘n plaalike Suid-Afrikaanse salie genieties te ingenieur met die oog om die terpeen biosintese te verbeter. Hiedie is krities in ‘n ekonomie wat voortdurend vorder om plant gebaseerde produkte te maak vir farmaseuties, industrieël en voedsel behoeftes. Verdere studies is nodig om helderheid te gee oor die effektiewiteit van transgeniese en *in-vitro* plant afkomstige ekstraksies.

Scientific publications from the dissertation

Journal Papers (Addendum I)

1. Musarurwa HT, Koegelenberg L, Makunga NP (2012) Chemical variation in essential oil profiles detected using headspace solid phase microextraction gas chromatography spectrometry in response to potassium, nitrogen and water available to micropropagated plants of *Salvia stenophylla* (Burch. ex Benth)" *stenophylla, Journal of Plant Growth Regulation* 31: 206-220

2. Musarurwa HT, van Staden J, Makunga NP (2010*) In-vitro* seed germination and cultivation of the aromatic medicinal *Salvia stenophylla* (Burch. *ex* Benth.) provides an alternative source of epi-α-bisabolol, *Plant Growth Regulation* 61: 287-295

3. Musarurwa HT, Ndimande GS, Makunga NP (in preparation) Effects of over-expressing the AgGPPS2 gene in *Salvia stenophylla* (Burch. *ex* Benth.) on terpenoid biosynthesis". *Metabolic Engineering*

Conference presentations (Addendum II)

Oral

1. **Musarurwa HT,** Koegelenberg L, Makunga NP (2011) *In-vitro* increase of potassium and nitrogen availability improves (-)-α-bisabolol accumulation in *Salvia stenophylla* (Burch. ex Benth; Family Lamiaceae) microplants International Organization for Chemical Sciences in Development Symposium, 12-16 January 2011 Cape Town

2. Makunga NP, **Musarurwa HT,** Koegelenberg L, Ndimande GS (2011) Altered volatile compounds of *in-vitro* microplants *S. stenophylla* (Burch. ex Benth) *South African Association of Botanists Conference,* 16-22 January, Rhodes University, Grahamstown

Posters

3. **Musarurwa HT,** Makunga NP (2010) Effect of tissue culture, genetic and nutrient manipulation of secondary compound profile of *Salvia stenophylla*, *Analitika* December 2010, Stellenbosch

4. **Musarurwa HT**, Ndimande GS, Makunga NP (2010) Conservation of a South African Sage using Biotechnology. *Student Conference on Conservation Science*, American Museum of Natural History, New York City

5. **Musarurwa HT**, Makunga NP (2009) Establishing a tissue culture system to increase secondary metabolite production: Over-expression of geranyl diphosphate synthase to up-regulate production of terpenes in *Salvia stenophylla, South African Journal of Botany* 75: 437

6. **Musarurwa HT**, Ndimande SG, Makunga NP (2009) Genetic Improvement of *Harpagophytum procumbens* to improve metabolite yield: a tool for increasing iridoids and reducing harvesting pressure. *Greengold Symposium, 14-16* September, Pretoria

Dedication

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Table of contents

Table of figures

List of tables

List of acronyms

%	Percent
℃	Degree Celsius
µCi	Mircrocurie
2,4D	2,4-Dichlorophenoxyacetic acid
AgGPPS2	Geranyl diphosphate synthase gene from *Abies grandis*
ANOVA	Analysis of variance
BA	Benzyladenine
BLASTN	Basic local alignment tool (nucleotides)
bp	Base pairs
CamV35S	Cauliflower mosaic virus 35S promoter
cDNA	Complimentary ribonucleic acid
Ci	Curie
cm	Centimetres
CTAB	Cetyl trimethylammonium bromide
DCM	Dichloromethane
DF	Degree of freedom
dH_2O	Distilled water
DMAPP	Dimethylallyl diphosphate
DNA	Deoxyribonucleic acid
dNTPs	Deoxynuleoside triphosphate
DXP	Deoxyxylulose phosphate
DXR	1-deoxyxylulose-5-phosphate
EDTA	Ethylenediaminetetraacetic acid
ESI	Electron spray ionization
EtBr	Ethidium bromide
EtOH	Ethanol
g	Grams
GC-MS	Gas chromatography mass spectrometry
GPP	Geranyl diphosphate

GPPS	Geranyl diphosphate synthase
HEPES	4-(2-Hydroxyethyl)-1-piperazine-N'-(2-ethanesulfonic acid)
HPLC	High pressure liquid chromatography
HSD	Honest significant difference
HS-SPME	Headspace solid phase microextraction
h	Hour(s)
IAA	Indole acetic acid
IPP	Isopentenyl diphosphate
kPa	Kilopascals
LB	Luria-Bertani
LC-MS	Liquid chromatography mass spectrometry
m	Metres
M	Molar
m/z	Mass to charge ratio
MEP	2-C-methyl-D-erythritol 4-phosphate
min	Minutes
Mj cm^{-2}	Millijoules per square centimetre
mL	Millilitres
mM	Millimolar
mm	Millimetres
MS	Murashige and Skoog (1962)
MS	Mass spectrometry
MS	Mean sum of squares
MVA	Mevalonic acid
NAA	Napthalene acetic acid
ng	Nanogram
NIST	National institutes of standards and technology
NMR	Nuclear magnetic resonance
OD	Optical density
PCA	Principal components analysis
PCR	Polymerase chain reaction

PDA	Photodiode array
PEG	Polyethylene glycol
PGR	Plant growth regulator
Pi	Inorganic pyrophosphate
Poly (A^+) RNA	Polyadenylated ribonucleic acid
PVPP	Polyvinylpolypyrrolidone
Ri	Root inducing
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
s	Seconds
SS	Sum of squares
SSC	saline-sodium citrate solution
Taq	*Thermus aquaticus*
TBE	Tris-borate-EDTA
TBS	Dimethyl-(*tert*-butyl)-silyl
TE	Tris EDTA buffer
Ti	Tumour inducing
TIC	Total ion chromatogram
TMS	Trimethyl-silyl
T-NDA	Transfer deoxyribonucleic acid
TOF	Time of flight
Tris	Tris(hydroxymethyl)-amino methane
UPLC	Ultra pressure liquid chromatography
YMB	Yeast mannitol broth
λPst	Lamda DNA *Pst* digest
µg	Microgram
µL	Microlitre
u	Units

Chapter One

General introduction

1.1 Medicinal plant use

In South Africa, traditional medicine (sometimes referred to as complementary, alternative or non-conventional medicine) is still an important component of the primary health delivery system and most of the medicines used are of plant origin with more than 80 % of the population using herbal preparations for their health care needs (Van Wyk et al. 1997, Jäger and Van Staden 2000). This widespread use of plant medicines led the South African government to enact the Traditional Health Practitioners Act 22 of 2007, a law giving a legal framework for traditional healers and at same time formally acknowledging the importance of complementary medicine in the primary health sector. The herbal pharmacopeia varies with different cultures and geographical location. For instance, the Zulu herbal medicine system has 1032 plants being used in KwaZulu Natal, Xhosa healers in the Eastern Cape use 166 floral species, and the Cape bush doctors ('bossiedokters' in Afrikaans) who are mainly Rastafarians (Cape coloureds practicing the Rastarian religion and lifestyle) work with at least 181 plants (Philander 2011). Over 170 plants are on the record as being regulars in the Khoi-San health system in the Western Cape (Hutchings et al. 1996, Dold and Cocks 2002, Cocks and Dold 2006, van Wyk 2008).

Plant medicinal preparations usually comprise more than one plant species. There is a common belief in most cultures, that has also been proven scientifically, that phytomedicine works better when it is a cocktail of herbs since there are synergistic interactions (van Vuuren and Viljoen, Philander 2011). Traditionally plant medicines are prepared in water and taken in as decoctions, infusions, teas, and are sometimes powdered or used as ash for oral application or to be taken as a tea (Masika and Afolayan 2003, Philander 2011). Different kinds of plants are used for extraction purposes but herbs and shrubs are the most popular (Tabuti et al. 2003).

To date, there are over 300 000 known plant species (Giam et al. 2011), making a total of about 200 000 known individual chemical compounds with South Africa having an estimated 3000 indigenous medicinal plants that are commonly used (Van Wyk et al. 1997).

Plant metabolites are categorized into two types depending on their function in the plant's life cycle. The primary metabolites are essential in the plant's day to day activities as they contribute to the function and anatomy, and their absence may hinder important growth processes like photosynthesis and respiration. Compounds that contribute to colour, aroma and anti-herbivory characteristics to plants are called secondary compounds (Hare 2011, Heldt and Piechulla 2011, Kroymann 2011) and are derived from primary compounds that are shunted into secondary metabolism. Secondary compounds do not directly affect the plant growth pattern but their presence facilitate plant-environment communication and thus it is not surprising that they exhibit a lot of diversity within and between different plants (Kroymann 2011). It follows that the production of secondary metabolites is controlled by the environment and there is no uniformity in accumulation in plants of the same species.

By nature, secondary compounds are structurally complex and due to chirality, artificial synthesis is nearly impossible and often uneconomic (Balandrin et al. 1985). Also, secondary metabolic pathways are not that well understood to easily allow *ex-vivo* production. At present, the most viable option is to enhance the metabolic machinery to produce more of the compound using metabolite engineering. One way of working towards such a goal is through genetic modification of key enzymes involved in the biosynthesis process. This does not only result in the production of needed compounds, but may also promote *ex-situ* conservation of the plant species as reliance on wild growing plants becomes less. This technology also forms the basis of commercial molecular farming.

Mankind has, since antiquity, found uses for secondary compounds as food preservatives and additives, medicines, beauty preparations and also in religious occasions as incense. Of late, the demand has been on the rise placing naturally growing plants, used for industrial production, at risk of extinction (Li 1998).

Synthesis of secondary metabolites is thus limited in plants (Chappell 2002) and yet the need for the same compounds has been increasing. Our limited understanding of the function of secondary metabolites in living organisms (Burke and Croteau 2002) only manages to explain their effect to the external environment. It is well documented that they are the active constituents in most natural products and herbal medicines (Kappers et al. 2005), and manipulation of their production pathways has industrial value (Burke et al. 1999, Light et al. 2005).

1.2 Medicinal *Salvia*

Salvia stenophylla, commonly known as the mountain sage, is a member of the Lamiaceae family. Generally sages are used worldwide as traditional medicine (Tepe et al. 2004), food preservatives (Weng and Wang 2000) and for culinary purposes (Lu and Yeap Foo 2001). The *Salvia* genus is one of the largest genera on earth having about 900 known species worldwide (Ulubelen et al. 2000). A number of sages have been cultivated for ornamental and aromatherapy purposes. The *Salvia* genus got its name from the Latin word *salvare* meaning to heal. Most of species from the sage family are remedies to a wide range of illnesses and world over have been documented to have anti-inflammatory, anti-bacterial, anti-plasmodial and anticancer action (Kamatou 2006, Kamatou et al. 2008). Increased demand for some *Salvia* species, especially in Europe and Asia, resulted in field cultivation and micropropagation to improve supply of the herbs. However, the limiting factor in *Salvia* production has been the secondary metabolites biosynthetic pathways of, which only allows limited accumulation of the compounds.

Plant secondary compounds are not produced in large quantities. Their role is usually to mediate plant-environment interactions and most of the time they are often not required in large amounts. However, the constantly increasing demand for natural additives in the pharmaceutical and cosmeceutical industries is exerting pressure on natural ecosystems as about 67 % of different medicinal plants in use are harvested from the wild (Canter et al. 2005). Wild *Salvia stenophylla* is harvested in the eastern parts of South Africa (the Cape, Free State and Natal) where it is more

abundant (Jäger and van Staden, 2000). It has a high percentage of (-)-α-bisabolol and its demand by the natural products industry is increasing tremendously. As such, there is need to find other alternative ways of supplying the desired compounds without threatening natural populations and at the same time without altering the chemical composition of the active metabolites. Also, the genetic potential of most organisms is not often reached due to the prevailing environmental conditions and optimization of such variables can enhance plant metabolite production. Growth systems and nutrient availability are usually limiting. Also, genetic modification is known to improve secondary metabolite biosynthesis (Lange et al. 2011) and thus the technique can be used to improve the production of secondary compounds. In summary, this study is aimed at determining the effect of *AgGPPS2* over-expression on gene expression. In parallel, a tissue culture system was developed and manipulated to optimize metabolite synthesis and accumulation.

1.3 Outline of the thesis

Chapter 1 gives a general overview of medicinal plant use, cultivation and the industry in South Africa with specific reference to *Salvia* species. It is here where a brief review of metabolite engineering in medicinal plants is given in an attempt to justify the use of third generation biotechnology in improving metabolite quality and quantity in *Salvia stenophylla.*

Chapter 2 describes in detail, the botany of S. *stenophylla*, its distribution and phytochemistry. It is a presentation of literature on medicinal plant biotechnology from tissue culture to recombinant technology. These methods are critically examined to show their strength and gaps in knowledge. At the end of the chapter, a comprehensive statement of the over–arching aim and objectives of the study is provided.

Chapter 3 addresses the first objective. It is a synopsis of how an efficient tissue culture system for *S. stenophylla* was developed from seed germination to plant

regeneration *in-vitro*. Results from the initial GC-MS analysis of *in vitro* plants are also presented.

Chapter 4 reports on the optimization of two marco-nutrient (nitrogen and potassium) and phytohormone requirements in *S. stenophylla* micropropagation and their subsequent effect on metabolite accumulation. Changes in the accumulation of secondary compounds are shown when using different concentrations of nitrogen and potassium. The influence of varying ratios of cytokinins and auxins is also presented.

Chapter 5 describes the transformation process so as to assess the effect of *AgGPPS2* over-expression on *S. stenophylla* metabolite accumulation. Transformation is achieved using the *Agrobacterium* gene delivery system. Details of this experiment are summarised in this chapter.

Chapter 6 documents the effect of transgenesis on volatile and and non-volatile metabolites using both GC-MS and LC-MS.

Chapter 7 provides the conclusions of the study by summarizing the findings and developments. It ends with suggestions for areas that need future research.

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Chapter Two

Literature review

2.1 *Salvia stenophylla* (Burch. ex Benth.)

Salvia stenophylla is a member of the Lamiaceae family belonging to the sage genus used worldwide as traditional medicine (Tepe et al. 2004), food preservatives (Weng and Wang 2000) and for other culinary purposes (Lu and Yeap Foo 2000, Lu and Yeap Foo 2001). It is one of the approximately 26 sages endemic to southern Africa (SANBI). The *Salvia* genus is one of the largest genera on earth having about 900 known species worldwide (Ulubelen et al. 2000), with a number of them being cultivated for ornamental and aromatherapy purposes.

The blue mountain sage is found in the Cape Provinces, Gauteng, KwaZulu-Natal and the Free State Province of South Africa, and exists in neighbouring countries: Lesotho, Namibia and Botswana (Jäger and van Staden 2000, Germishuizen 2006). It is more prevalent in the central interior of South Africa and especially in the Free State where most of the populations are found (**Figure 2.1**). It generally grows on calcareous sandy soils and is often found along roadside, disturbed and wastelands (Viljoen et al. 2006).

The flowers are blue to mauve with a white patch at the centre (**Figure 2.2**). Flowering begins from late July and stretches to late February with each flower producing four viable brown seeds with an average diameter of 1 mm. *Salvia stenophylla* grows upright, reaching an average height of 50 cm at maturity if undisturbed. It exhibits apical dominance which may be broken when the apical

meristem is browsed or when it senesces and falls resulting in adventitious growth. The stem is squared, a typical feature of the sages, with sparse hairs and the leaves are linear-oblong to oblong-lanceolate and are also lightly hairy with the length varying from 5 cm to 14 cm (**Figure 2.2**). The leaves have specialized storage organs called trichomes in which essential oils collect (Kamatou et al. 2005, Kamatou et al. 2008a).

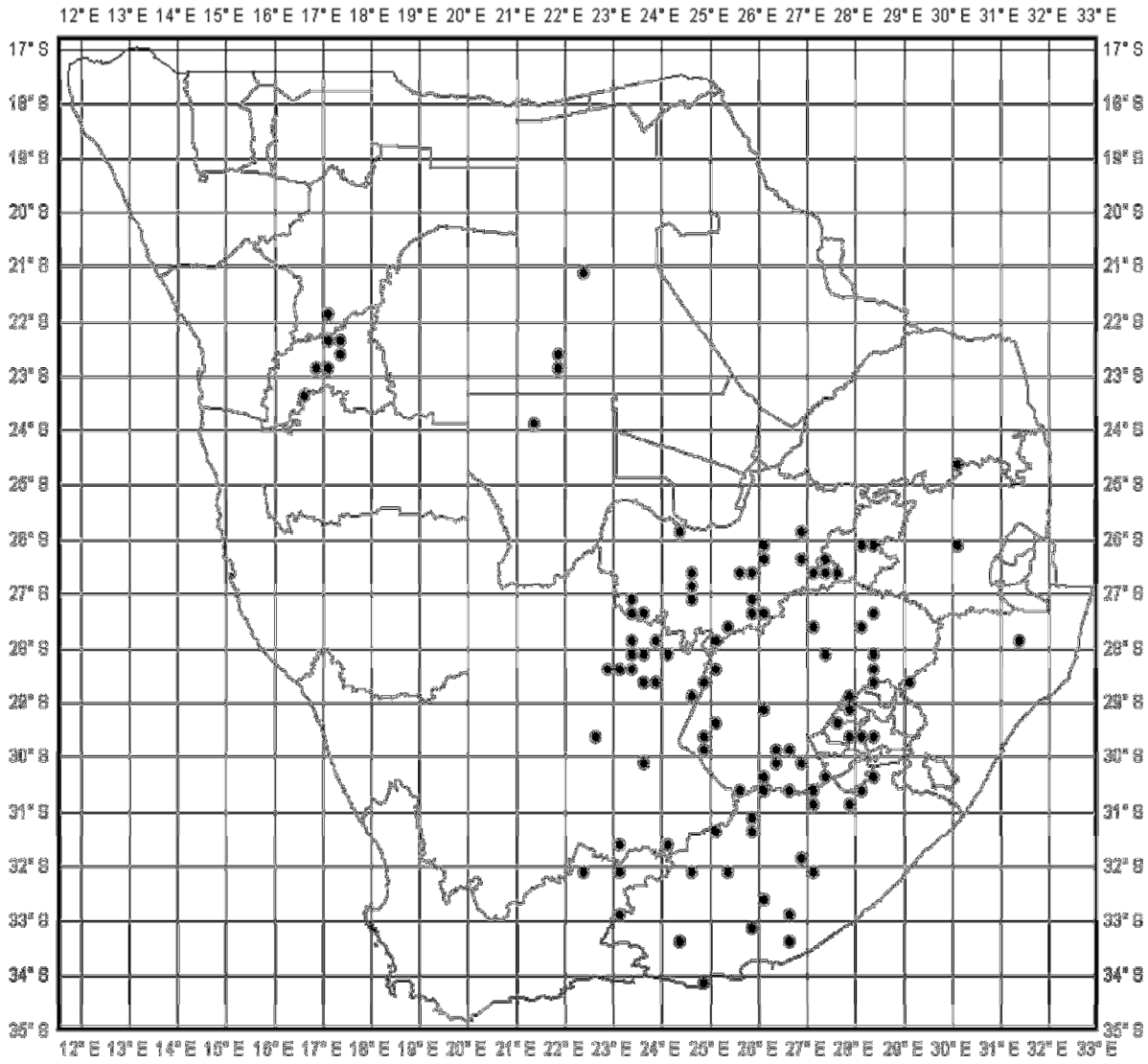


Figure 2.1 Spatial distribution of *S. stenophylla* in southern Africa (reproduced with kind permission of the South African National Biodiversity Institute (SANBI), Pretoria Computerised Information System (PRECIS)

Figure 2.2 Some typical morphological characters of *Salvia stenophylla*. Tissue culture-derived plants at 4 weeks after glasshouse acclimatization (**A**), mature leaf picked from flowering plants (**B**) and hairy square stem bearing 7-day old flowers (**C**).

An ability to produce aromatic pharmaceutically important secondary compounds is the major reason *Salvia* species are of medicinal significance. These are some of the small molecular weight compounds that are synthesized by plants to enhance their survival in an ecosystem (Aharoni et al. 2006) but are not crucial to the plant's daily metabolism. The secondary compounds are in the form of aromatic essential oils or polar extracts belonging to different chemical classes and their function is dependent on the chemical profiles. The oils have anti-stress, anti-bacterial, anti-inflammatory and anti-depressant activity (Seol et al. 2010) and are dominated by a family of chemicals collectively known as isoprenoid. *Salvia* isoprenoids are responsible for olfactory perception of plants by pollinators and herbivores (Kessler and Baldwin 2004, Hare 2011, Hare and Sun 2011), and also renders the plant vital for aromatherapy (Kessler and Baldwin 2004, Hare 2011). Most common water soluble components of the secondary metabolome include flavanoids, phenylpropanoids, caffeic acids derivatives and alkaloids. These are usually anti-oxidants, analgesics and in some cases anti-plasmodial, thus making *Salvia* extracts essential in detoxification, pain relieving and anti-malarial medicines.

2.2 Production of secondary compounds

The role of secondary metabolites is to enhance the interaction between plants and their environment and they are synthesised to assist with plant pollination, herbivory defence and stress responses (Verpoorte et al. 2000). Unlike primary metabolites, secondary compounds are present in low quantities with occasional intraspecific variation due to genetic, phenological and physiological mechanisms regulating their synthesis. Consequently, space and time limit metabolite synthesis and accumulation; within and between plant species, making them available under specific conditions (Pichersky and Gang 2000, Dewick 2002). Secondary metabolites are thus sometimes used as taxonomic markers to differentiate between species as they result in colour, aroma and flavour specific characters (Bennett and Wallsgrove

1994). Secondary compounds are terpenoids, alkaloids, flavanoids, steroids, quinones, and lignans.

In South African sages, Kamatou et al. (2008a) had shown that seasonality and location affect essential oil composition and total extract yield and subsequent biological activity in three South African *Salvia* species (*S. africana-lutea, S. africana-caerulea* and *S. lanceolata*). In some species, secondary compounds are synthesised only after a long growth period. As a result, dependence on plants as a source of secondary compounds is not only unreliable but also an environmental risk (Canter et al. 2005) leading to possible endangering and extinction (Mooney and McGraw 2007). The low secondary metabolite yield from plants is also insufficient for commercial harvesting often making cultivation of medicinal plants uneconomical (Verpoorte et al. 2002).

Chemical synthesis is usually viewed as a viable option but is not feasible due to the complex nature of secondary metabolites (Hughes et al. 2004). Synthetic analogues of natural compounds are fast losing their popularity as most people using herbal drugs are trying to avoid synthetic remedies because of their numerous side effects. With 12.5 % of the over 422 000 known floral species in the world being used as medicinal plants but only a few hundreds of these are under cultivation (Schippmann et al. 2002). Domestic cultivation has been cited by Canter et al. (2005) as a possible solution to increase secondary compound availability for the natural products market. It provides a platform for standardizing medicinal plant production culminating in homogenous metabolite output at low cost (Pank 1993).

2.3 Medicinal Properties of *Salvia*

The genus *Salvia* is known to cure heart diseases, hepatitis, stomach pains among other ailments. The genus has proven antimicrobial, anti-viral, anti-oxidant and ant-inflammatory properties (Dweck 2000) to name a few. Activity is sometimes explained by the great terpene richness in the sage essential oil, rendering it potent against a broad spectrum of microbes because of its lipophilic properties, its functional groups and its solubility in water (Knobloch et al. 1988). In other cases efficacy is a result of phenolics, phenolic diterpenes and flavonoids. Kamatou et al. (2008a) confirmed anti-malaria and anti-cancer properties. *Staphylococcus aureus* is one of the most notorious ubiquitous bacteria and is responsible the development of boils and rashes. Sages, *S. radula, S. aurita, S. dolomitica* and *S. chameleagnea* impede growth of *S. aureus* when the MIC is 0.06 mg mL^{-1}, providing justification for use as a traditional medicine by the Sotho people of South Africa to treat a variety of bacterial infections (Kamatou 2006, Kamatou et al. 2008b). Extracts from *Salvia* species exhibited anti-malarial activity against the chloroquine-resistant *Plasmodium falciparum.*

2.3.1 Pharmacology and phytochemistry of *S. stenophylla* essential oil

Salvia stenophylla is a source of a commercially sought after essential oil. The oil is produced by glandular peltate trichomes that are found in abundance in most *Salvia* species (Hoelscher 2003). *Salvia stenophylla* together with other related species (*S. repens* and *S. runcinata*) have a high level of toxicity against cancer cell lines. It also has anti-oxidant and anti-inflammatory activities (Kamatou et al. 2005, Kamatou 2006, Kamatou et al. 2008a, Kamatou et al. 2008b, Kamatou et al. 2010,). Anti-cancer activity is ascribed to the monoterpenes and sesquiterpenoids common in *Salvia* plants. The volatile component of the *S. stenophylla* metabolome is largely composed of monoterpenes (52 %) and sesquiterpenes (21 %) (**Figure 2.3B**) as was summarized by Kamatou (2006). Compounds most common in high concentration are (-)-α-bisabolol, δ-3-carene, manool, β-bisabolene, α-pinene, β-pinene and D-limonene.

(-)-α-Bisabolol, also known as levomenol (Kamatou et al. 2006), is a non-toxic monocyclic oxygenated sesquiterpenoid with anti-inflammatory, analgesic, antibiotic and gastric-protective properties (Jakovlev and Von Schlichtegroll 1969, Isaac and Thiemer 1975, Moreno-Fenandes et al. 1992), occurring in nature as four stereoisomers (**Figure 2.3**) with (-)-α-bisabolol being most common in *S. stenophylla.* It induces apoptosis and reduces perception of acute pain, making it important in the clinical treatment of highly malignant tumours known as glioma (Cavalieri et al. 2004, Alves et al. 2010). Hartmann and Kohler (1989) discovered the stabilization effect of (-)-α-bisabolol on hydrogen peroxide containing cosmetics and unlike menthol, thymiol and camphor which are 5-10 times less effective, it does not leave products with a sharp odour. It is a good skin conditioning agent at concentrations as low as 0.01-1 % in underarm deodorants and increases the skin permeability to drugs (Andersen 1999). At a concentration of 1 mg mL^{-1}, (-)-α-bisabolol is biocidal to *Leishmania infantum* promastigote, making it a good contender for the prevention of leishmaniasis (Morales-Yuste et al. 2010). Of the 26 indigenous southern African sages, only *S. stenophylla, S. runcinata, S. muirii* and *S. radula* are known to synthesize (-)-α-bisabolol in significant amounts (Kamatou 2006) resulting in characteristic superior anti-tumour activity. Jequier et al. (1980) and Viljoen et al. (2006) analysed *S. stenophylla* oil and α-bisabolol accounted for 40.98 % of the oil. Later, Brunke and Hammerschmidt (1985) reported the α-bisabolol proportion as 28.9 % of the total oil in the same species.

The presence of D-limonene could also contribute to the anti-plasmodium activity as the compound impairs the terpenoid biosynthesis pathway in *P. falciparum*. The pathway is responsible for production of the crucial coenzyme Q needed by the parasite in its intraerythrocytic stage (Boyom et al. 2011). β- and α-pinene are also present in noticeable volumes in *S. stenophylla* oil. These compounds are used for the synthetic production of camphor, an important fragrant compound in cosmetics and perfumery industry. Camphor is a known noxious compound, also naturally

present in blue mountain sage and accumulates at an average rate of about 3.2 % of the total essential oil (Kamatou 2006). Another significant quality altering constituent of *S. stenophylla* oil is α-thujone. It is a monoterpene with a menthol odour and is usually present in low quantities. High abundance of α-thujone is not favourable, regardless of its antinociceptive properties (Höld et al. 2000), because it is toxic and can induce spontaneous abortions (Albert-Puleo 1978).

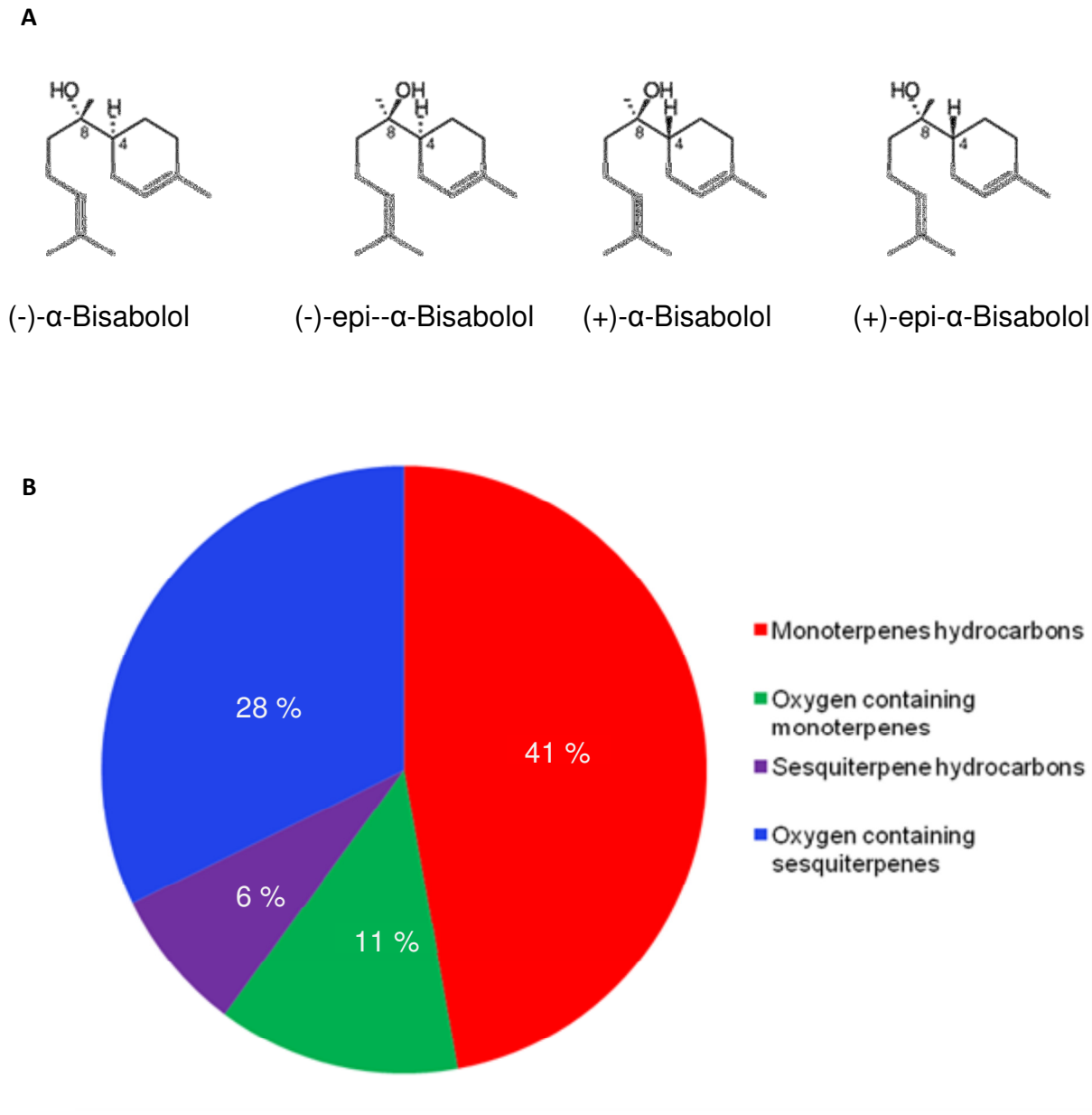


Figure 2.3 Four known stereoisomers of α-bisabolol (**A**) and (**B**) relative proportions of different classes of terpenes isolated from *S. stenophylla* summarized from Kamatou (2006).

The presence and quantities of these compounds in the essential oil determine its commercial value and it is imperative to maintain a benchmark profile as a standard. Nonetheless, Kamatou et al. (2008b) and Viljoen et al. (2006) illustrated seasonal

and regional variations of secondary compounds found in South African sages, thus presenting the essential oil industry with a quality control challenge as consistency is paramount in natural product processing. This pattern is also explain the grand difference in the essential oil content documented in the Brunke and Hammerschmidt (1985) and Jequier et al. (1980) reports. For commercial processing, this means that there will be great variability of the oil content and composition leading to high price fluctuations. Propagation methods and breeding efforts must ensure that the variation is reduced or eliminated to ensure consistency of the essential oil.

2.3.2 Pharmacology of polar fraction

The sage water soluble fraction comprises of polyphenols, flavonoids and alkaloids. In *S. stenophylla,* phenolic acids are most important. They are dominated by caffeic acid and its derivatives, with rosmarinic acid being the most copious of these. Caffeic acid is a phenolic phytochemical associated with anti-cancer, anti-oxidant, anti-inflammatory, anti-depressant and anti-carcinogenic activities (Gülçin 2008, Kang et al. 2009, Tsai et al. 2011). Rosmarinic acid, a compound synthesized from the amino acids L-phenylalanine and L-tyrosine, is a cure for sore throats, wounds, fever and is anti-inflammatory as well as anti-viral chemical (Petersen and Simmonds 2002). It is also implicated in the treatment of Alzheimer's diseases because it is able to inhibit activity of the enzyme acetylcholinesterase (Falé et al. 2011). Other important constituents of the hydrophilic fraction are ursolic acid, carnosol, carnosic acid, lithospermic acid B and coumarins. Carnosol and its derivatives are strong antibacterial agents with proven antioxidant activity and thus add value to the methanol: chloroform solvent extract. Flavonoids are even more widespread in sages with quercetin, kaempferol, apigenin, luteolin and cirsmaritin being most prevalent. These compounds contribute towards the bioactivity of sages (Lu and Foo 2001, Kamatou 2006) and there is a rise in scientific literature reporting on their efficacy.

2.4 *Salvia* biotechnology

The importance of medicinal plants as a source of essential oils, food supplements and pharmaceuticals cannot be over-emphasized. It is no doubt that today that natural products are some of the best templates for drug design and synthesis (Baldi et al. 2007). To meet the fast increasing demand for natural products, biotechnology is one of the tools that are currently being exploited to enhance their production and availability in different plant species. Such biotechnological interventions are usually in the form of tissue culture and transgenesis (encompassing also metabolite engineering) of plants of interest. *Salvia* species have been cultivated for culinary, pharmaceutical and ritual purposes and *S. officinalis* (common sage) and *S. miltiorrhiza* being the commonly grown. Typical tissue culture investigations focus on developing efficient micropropagation systems (Frett 1987, Huang and van Staden 2002, Makunga and van Staden 2008), optimizing both growth and culture conditions for microplants and also to elevate biomass and subsequent metabolite levels using transgenic roots, cell and organ cultures (Frett 1987).

In the 1980's, the autumn sage (*S. greggii*) was one of the first sages to be micropropagated when Frett (1986, 1987) evaluated the influence of MS salts and phytohormones on its growth *in-vitro*. By the beginning of the decade that followed, the potential of hairy roots in tanshinone and ferruginol production became a key focus for scientific and industrial research (Zhi and Alfermann 1993). The production of phenolic acids and other caffeic acid derivatives fluctuates with alterations in the plant hormone concentration in *S. officinalis* shoots grown on different MS media (Santos-Gomes et al. 2003), suggesting that the yield of these metabolites can be regulated to fit market needs. In South Africa, sages only started to be micropropagated in 2002 when Huang and Van Staden induced callus formation from *S. chameleagnea* explants and from the callus they were able to generate shoots. Both callus and shoots were able to synthesize rosmarinic acid. This showed

the potential of tissue culture as an option for generating South African sages. Makunga and van Staden (2008) regenerated plantlets from cotyledons and hypocotyls of *S. africana-lutea* and designed a micropropagation protocol capable of multiplying one shoot to two-three thousand plantlets after a period of 12 weeks with four week sub-culturing in between. Ramogola et al. (2009) proceeded and employed different strains of *Agrobacterium rhizogenes* to induce hairy root growth from *S. africana-lutea.* The extracts from the transgenic roots proved to have superior antimicrobial activity compared with whole plantlet extracts.

Salvia hairy roots are currently being widely used for the production of secondary compounds due their prolific growth resulting in high productivity (Zhi and Alfermann 1993, Wu et al. 2008) and potential to make new compounds Ramogola (2009). Hairy roots can be an effective source of important compounds from the Chinese sage, Danseng. Of late, overexpression of the 3-hydroxy-3-methylglutaryl CoA reductase (*HMGR*), 1-deoxy-D-xylulose-5-phosphate synthase (*DXS*) and geranylgeranyl diphosphate synthase (*GGPPS*) genes improved tanshinone biosynthesis in *Salvia miltiorrhiza* root cultures (Kai et al. 2011). Unfortunately, South African research has not yet explored the option of recombinant DNA technology and yet it is evident from elsewhere that this is a potent tool for improving metabolite production. Hoelscher et al. (2003) have isolated and characterized the gene responsible for the synthesis of δ-3-carene in *S. stenophylla* thus paving the way for further work of such nature on this species.

2.5 Medicinal plant farming

In the sage genus, only *S. officinalis* and *S. miltiorrhiza* are the most widely grown as commercial field crops. *Salvia officinalis* is widely cultivated world-over for culinary and medicinal purposes (Santos-Gomes et al. 2003, Mirajalili et al. 2006). *Salvia miltiorrhiza* is widely grown all over China for the extraction of phenolic compounds

and in particular, tanshinones (Sun et al. 2007). Field culture forms the main source *S. miltiorrhiza* root in China and on average the country consumes in excess of 20 million kilograms of the plant (Liu et al. 2011). However, it was also noted that field cultivation of medicinal plants tends to increase the value of wild-harvested plants and thus even puts them at a greater risk of extinction (Canter 2005) as the case with the American ginseng (Mooney and McGraw 2007). Adoption of medicinal plants as commercial crops is also hindered by uncertainties of consumer interests in natural products, trade policies governing such products as well as weather and other climate events that affect yield and quality (Craker 2008) which are difficult to predict and control. For example; farming of *Harpagophytum procumbens* DC (commonly known as the devil's claw) has many challenges in taking off because potential farmers are either unable to finance such ventures or the tenure system adopted by the government is not economically favourable with harsh environmental conditions further reducing the viability for commercial cultivation (Stewart and Cole 2005, Cole and Bustan, 2009). Seed physiology and male sterility were cited by Frabetti et al. (2009) as some of the reasons for the poor success rate when attempting to propagate *Teucrium fruticans* L. (commonly known as tree or shrubby germander) as a field crop.

Tissue culture based on either differentiated (roots and shoots cultures) or undifferentiated (callus and suspension) cultures (Guilietti and Ertola 1999), has been explored as a variant form of medicinal sage production for the extraction of economically important metabolites (Tisserat and Vaughn 2008). It is a rapid method for propagation of plantlets that are true-to-type (George et al. 2008) without need for seeds, and the uniformity of the yield is almost guaranteed. Micropropagation also results in a constant supply of secondary metabolites irrespective of season and since it is an aseptic technique, resultant plantlets are free from diseases and pest attacks (Razdan 2003, Arikat et al. 2004, Debnath et al. 2006). In some cases, *in-vitro* nurturing leads to creation of plant lines with higher secondary compounds compared to field grown plants (Arikat et al. 2004), consequently providing a stable

continuous supply of secondary compounds (Debnath et al. 2006). *Salvia* species worldwide have successfully been tissue cultured for their secondary metabolites (**Table 2.1**) and in some cases micropropagation was coupled with mechanization to improve both growth rate and efficiency of metabolite harvesting.

Automation of tissue culture processes further increases the productivity of micropropagation. Mechanization of tissue culture protocols can further increase the plant's potential to boost biomass production which will subsequently translate to improved secondary metabolism. Nonetheless, this is limited by the genetic potential of the specific plant. Karam and co-workers in 2003 reported that essential oils and rosmarinic acid in *Salvia fruiticosa* synthesised more than double the amount of essential oils (0.7 %) when grown *in-vitro* as compared to greenhouse grown plants (0.34 %).

Selection and breeding of the best phenotypes in such population has been used in agriculture to produce superior hybrids. Artificial selection formed the basis of conventional plant breeding and is based on the understanding of Mendelian and Darwinian principles (Lužny 1978), resulting in phytochemically heterogenous populations. Reducing the chemical heterogeneity resulting from the genetic crosses is time consuming and expensive. Usually, it takes several back crosses to eliminate unwanted traits and achieve a stable variety (Tadmor and Lewinsohn 2007). In most perennial plants, this can easily translate to at least seven years of hybrid development. This technique of plant improvement often yields desired results, but since it is based on horizontal gene transfer, it can only allow recombination of a limited set of genes within a species' genome. On the other hand, metabolite engineering provides a faster and more specific approach to improve secondary compound biosynthesis.

Table 2.1 Micropropagation of medicinal *Salvia* species for secondary compound extraction

Species	Explant	Tissue Culture	Chemical /Tissue Culture System	Reference
Salvia africana-lutea (L).	hypocotyls	plantlet	efficient micropropagation protocol	Makunga and van Staden (2008)
S. blacoana marieolensis (Webb and Heldr.) *S. valentina* (Vahl)	shoots tips, nodal segments	plantlet	tissue culture protocol	Cuenca and Amo-Marco (1999, 2000)
S. brachyodon (Vandas)	shoot tips; nodal segments	plantlet	tissue culture protocol	Mišić et al. (2006)
S. canriensis (L.)	axillary buds	plantlet	16-acetoxycarnosol, carnosic acid, carnosol	Luis et al. (1992)
S. chamelaeagnea (P.J. Bergius)	nodal explants, shoots, callus, leaf	plantlet, cell suspension cultures	rosmarinic acid	Huang and van Staden (2002)
S. fruticosa (Mill.)	callus, shoots, nodal segment	plantlet	essential oils (α-pinene, 1,8-cineole, camphor, borneol)	Arikat et al. (2004)
	callus, leaf	callus, suspension cultures	rosmarinic acid	Kintzios et al. (1999)
S. leucantha (Cav.)	apical shoots	plantlet	tissue culture protocol	Hosoki and Tahara (1993)

S. miltiorrhiza (Bunge)	shoots	hairy roots, cell suspension cultures	lithospermic acid B, rosmarinic acid, cryptotanshinone I	Chen et al. (1999a)
	shoots	cell suspension cultures	tanshinone I, tanshinone II A, lithospermic acid B, rosmarinic acid, cryptotanshinone I	Chen et al. (1999b)
	shoots	cell suspension cultures	tanshinone I, tanshinone II A, cryptotashinone	Chen et al. (1997)
	shoots	cell suspension cultures	tanshinone I, tanshinone II A, cryptotanshinone I	Chen and Chen (1999)
	shoots	cell suspension cultures	cryptotanshinone I, ferruginol	Miyasaka et al. (1986a)
	cell cultures	cell suspension cultures	cryptotanshinone I, ferruginol	Miyasaka et al. (1986b)
	shoots	cell suspension cultures, hairy roots	ferruginol, lithospermic acid B, rosmarinic acid,	Chen and Chen (2000)
	shoots	callus and cell suspension cultures	cryptotanshinone I, rosmarinic acid	Yan et al. (2006)
	shoots	hairy roots	rosmarinic acid	Yan et al. (2006)
	shoots	hairy roots	tanshinone I, tanshinone II A, cryptotanshinone I	Chen et al. (2001)

	shoots	hairy roots	tanshinone I, tanshinone II A lithospermic acid B, rosmarinic acid, methylenetanshiquinone, tanshinone II B, dihydroxytanshinone I	Chen and Chen (2000b)
	shoots	cell suspension cultures	cryptotanshinone I	Zhi and Alfermann(1993)
	shoots	hairy roots	cryptotanshinone I	Hu and Alferman (1993)
	shoots	hairy roots	tanshinone I, tanshinone II A, tanshinone II B, tanshinone V, cryptotanshinone I, dihydroxytanshinone I, tanshinone VI; ferruginol	Ge and Wu (2005a; 2005b)
S. nemorosa (L.)	shoots	plantlet	tissue culture technique	Skala and Wysokińska (2004)
S. sclarea (L.)	zygotic embryo, callus, cotyledon	plantlet, hairy roots	tissue culture protocol	Liu et al. (2000)
	shoots	hairy roots	salvipsone, aethiopinone, 1-oxoaethiopinone, ferruginol	Kuźma et al. (2006)
	shoots, internodes	hairy roots,	Pygenic acid A 2,24-Dihydroxyursolicacid	Kuźma et al. (2007)
	shoots, internodes	callus, hairy roots, cell suspension cultures	salvipsone, aethiopinone, 1-oxoaethiopinone, ferruginol, sclareol	Banthorpe (1990)

S. valentina (Vahl)	shoots, nodal segments	plantlet	tissue culture protocol	Cuena and AmoMarco (2000)
S. officinalis (L.)	shoots, callus, nodal segments	plantlet	tissue culture protocol	Tawfik and Mohamed (2006; 2007)
	apical and axillary buds	plantlet	improved essential oil quality	Avato et al. (2005)
	shoots	callus, cell suspension cultures	ursolic acid	Bolta et al. (2000)
	leaf, callus, embryo	callus, embryonic cultures	rosmarinic acid	Kintzios (1999)
	internodes	callus, cell suspension cultures	gallic acid, tanshinone I, tanshinone II A, tanshinone II B, tanshinone V, cryptotanshinone I, dihydroxytanshinone I, tanshinone VI; ferruginol, 3-*O*-tanshinone I	Zhi and Alfermann (1993)
	shoots	plantlet, callus, cell suspension	carnosic acid, carnosol	Grzegorczyk et al. (2007)

2.6 Metabolic engineering

According to Çakar (2009), metabolic engineering was first defined by Bailey (1991) as the improvement of cellular activities and functions by manipulating enzymatic, regulatory and transport functions of the cell with the use of recombinant DNA technology. Thus, it is the purposeful and directed modification of intracellular metabolism and properties. The main goals of metabolite engineering are to facilitate and/or prevent accumulation of specific compounds, by promoting biosynthesis, storage and influx. In metabolite engineering, goals can be achieved by either increasing the number of producing cells, raising the rate of enzyme activity by using sense gene expression (genes coding for the biosynthesis of enzymes, proteins or nucleotides) or by reducing catabolism using anti-sense genes (Verpoorte et al. 2000). This will consequently lead to either accumulation of secondary compounds, over-expression of metabolic genes, anti-sense mediated down regulation of anabolic activity or in some cases a combination of any of these processes (Frick et al. 2007). Metabolic engineering has been used to improve the biosynthesis of existing metabolites, facilitate synthesis of new metabolites, instruct new metabolic activities and improve cell performance under suboptimal conditions (Cameron and Chaplen 1997).

Gains from metabolite engineering frequently surpass conventional breeding benefits (**Table 2.2**). The present increase in scientific evidence supporting that plant remedies are efficient as alternative medicines implies that there is need to enhance their production. Unlike conventional breeding, use of biotechnological techniques can help save time and surpass the hereditary potential of an organism without risking the genetic diversity of the species by over-expressing both homologous and heterologous genes.

Table 2.2 Metabolite engineering of some popular medicinal plants

Species	Modification	Result	Reference
Salvia miltiorrhiza (Bunge)	over- and coexpression of *SmGGPPS, SmHMGR* and *SmDXS*	increased production of pharmaceutically important active diterpenes tanshinones	Kai et al. (2011)
	HMGR cloning and overexpression	increased enzyme activity and production of tanshinones	Dai et al. (2011)
	AtPAP1 upregulation	elevated phlenolics (10 fold increase in Salvianolic acid B), total flavonoids and anthocyanin accumulation	Zhang et al. (2010)
	GUS	an efficient protocol for transformants regeneration	Yan and Wang (2007)
	AtHSP101 and OsHSP101 overexpression	developed a heat tolerant *S. miltiorrhiza* lines for propagation in hot climates	Hung (2011)
S. stenophylla (Burch *ex.* Benth)	3-carene synthase cloning	cDNA clone; synthesis of functional protein in *E. coli*	Hoelscher et al. (2003)
S. officinalis (L.)	hairy root induction with ATTCC15834 and A4	high yielding hairy roots producing more rosmarinicacid than untransformed roots	Grzegorczyk et al. (2006)
Catharanthus roseus (L.)	*CjMDR1* expression	improved accumulation of endogenous	Pomahačová et. al (2009)

		ajamline and tetrahydroalstonine	
	transcription factor ORCA2	catharanthine and vindoline accumulation was elevated	Liu et al. (2010)
	transcription factor *ORCA3*	up-regulated biosynthesis of catharanthine	Wang et al. (2010)
	TDC overexpression	increased tryptamine levels	Goddijn (1994)
Papaver somniferum (L.)	*PsCor1.1*overexpression	15-30 % rise in morphine content; 10-fold increase in *Cor* transcript	Larkin et al. (2006)
Mentha X piperita (L.)	*DXR* overexpression	increased essential oil accumulation	Mahmoud and Croteau (2001)
	Antisense-*MFS* and *DXR*	increased essential oil content; reduced (+)-menthofuran and (+)-pulegone	Lange et al. (2011)
	MFS and *PR*	improved essemtial oil quality (lowered (+)-menthofuran and (+)-pulegone)	Mahmoud and Croteau (2003)
	LS	altered essential oil composition; increased limonene yield	Krasnyanski et al. (1999)

Pathway engineering in medicinal plants naturally focuses on enzymes and processes involved with the synthesis and accumulation of secondary metabolites. Simultaneous modulation of different steps is another proven option for yield enhancement in metabolite engineering. Lange et al. (2011) successfully improved essential oil composition in mint by modifying geranyl diphosphate synthesis and isopentenyl diphosphate production. This enzyme is involved in one of the most important pathways in secondary metabolism, the terpenoid biosynthetic pathway. This pathway is responsible for the manufacture of most of the compounds that are involved in plant-environment interactions and therefore confers taste, odour and sometimes colour to plants. The essential oil component of the sage family is largely a function of terpene presence.

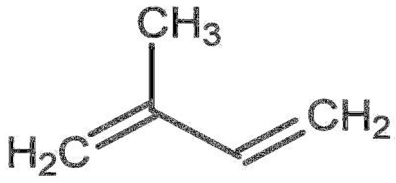
2.7 Terpenoid biosynthesis

The terpene group of secondary compounds are synthesized from five carbon (C5) isoprene units joined together in a head to tail arrangement (**Figure 2.4A**). The group comprises of monoterpenes, diterpenes, sesquiterpenes, tetraterpenes and polyterpenes depending on the number of isoprene units possessed by the compound (McGarvey and Croteau 1995, Dubey et al. 2003). Synthesis of terpenoids either follows the cytosolic mevalonate path or the plastid mevalonate independent pathway (Trapp and Croteau 2001). Monoterpenes usually follow the plastid mevalonate independent pathway and sesquiterpenes are synthesized in the mevalonate pathway (Dubey et al. 2003).

(-)-α-Bisabolol, is synthesized via the mevalonate pathway from the isoprenoid intermediate farnesyl diphosphate (Mercke et al. 2000). The pathway derived its name from mevalonate, a six carbon intermediate compound formed from the sequential condensation of three acetyl coenzyme A (Acetyl CoA) units. Isopentenyl diphosphate can also be synthesized through the methylerythritol phosphate pathway, which takes place in the plant cell plastid (Schmidt and Gershenzon 2007).

Sesquiterpene production starts with the polymerization of two diphosphorylated isoprene (a 5-carbon molecule) molecules to generate geranyl diphosphate (GPP) (Chappell 1995a, Chappell 1995b, Chappell 2002). Geranyl diphosphate is a ten-carbon intermediate that can be converted to cyclic or linear products representing monoterpenes (Trapp and Croteau 2001) and addition of a third isoprene unit to GPP will lead to the formation of farnesyl diphosphate (FPP). Farnesyl diphosphate can either be converted to cyclic or linear products of the sesquiterpene group, in which α-bisabolol is found.

A



B

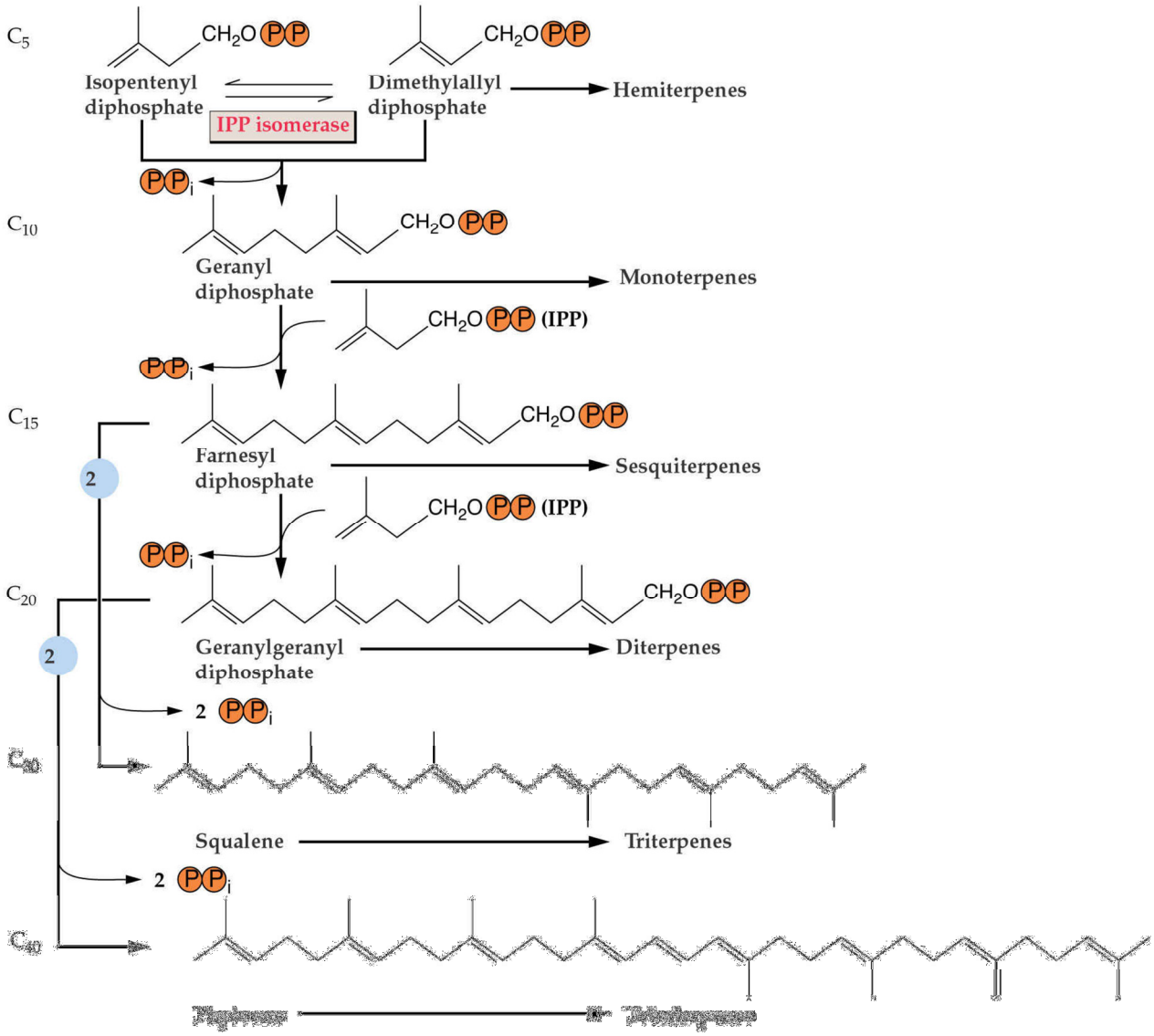


Figure 2.4 (**A**) An isoprene unit, the building block for the back of all terpenes and terpene derived compounds. (**B**) The diagrammatic representation of terpene biosynthesis (**http://www.biology.lsu.edu webfac/jmoroney/BIOL7063/**)

Products of terpene metabolism are usually volatile and may be detected using chemical profiling methods. More often, gas chromatography coupled with mass spectrometry (GC-MS) is the favoured method (Saito and Matsuda 2010). Polar portions of the metabolome are sometimes derivatized to increase volatility prior to GC-MS analysis. However, other methods such as nuclear magnetic resonance (NMR) and liquid chromatography coupled to mass spectrometry (LC-MS) are useful when analysing phenolics, terpenes, sugars and other polar compounds. The science dealing with the use of these methods is what is now known as metabolomics (Choi et al. 2010, Allwood et al. 2011)

2.8 Metabolomics

In the natural products industry, central to commercial and biological importance is the plant phenotype which is essentially a cocktail of chemicals they accumulate, manifesting as scent, colour and flavours, comprising the metabolome. Thus the metabolome is the sum of all compounds found in an individual, organ, tissue or cell system forming the basis of metabolomics. Allwood et al. (2011) defined the metabolome as the ultimate end product of gene expression and modulation of protein function and environmental cues, making metabolomics the functional component of the –omics technologies (Sumner et al. 2003). Accordingly metabolomics can be viewed as a comprehensive, non-biased high throughput chemical and statistical analysis of complex mixtures of metabolites that are characteristic of plant chemistry (Hall et al. 2002). It is a progressive extension of the –omics technologies to the larger scale analysis of the small molecular weight component of plants (Sumner et al. 2003). Metabolite accumulation is sensitive to both temporal (seasonal and developmental) and spatial variation (Allwood et al. 2011).

Results from metabolomics are essential in explaining gene function (in both space and time) and effects of environmental perturbation. It is thus a useful tool to characterize products of metabolism. Molecular breeding introduces a number of transgenes into a plant and metabolomics is the most efficient tool for assessing the

overall effect such introductions simultaneously (Saito and Matsuda 2010). Metabolomics was conceived in the biomedical industry and it is still used effectively as a tool for diagnosis in where it is known as metabonomics (Choi et al. 2007, Verpoorte et al. 2007). Chemotaxonomy is another field in which comprehensive metabolite compound analysis is increasingly becoming important as the classification of botanicals is now placing emphasis on chemical composition.

A truly comprehensive analysis of the metabolome is still unrealistic, mainly because analytical and biological variances are difficult to control and quantify. However, use of metabolomic tools such as NMR, GC-MS, LC-MS and a combination of these yields a near inclusive report of the metabolite profile.

2.7.1 Gas chromatography mass spectrometry (GC-MS)

For detection and identification of metabolites, GC-MS seems to be the best method since it is the most widely used technique in plant metabolomics (Kopka 2006, Saito and Matsuda 2010). Even though it works best when analyzing volatile compounds, it is equally useful in the quantification of non-volatiles provided that a derivatization step is added to sample preparation prior to GC-MS.

Derivatization is the transformation of non-volatile metabolites into volatile analytes amenable to GC-MS. The process reduces polarity of functional groups and thus making it easier for the analytes to separate on the gas chromatograph column (Halket et al. 2005). It involves either silylation, alkalylation, alkoxyamination or acetylation. The most common method is silylation. Here the silylation reagents, namely trimethyl-silyl (TMS) and dimethyl-(*tert*-butyl)-silyl (TBS), are introduced and potentially replace all exchangeable acidic protons (Kopka et al. 2006, Wittmann 2010). This method has the broadest range of derivatization (Kopka et al. 2006).

Equally useful when volatilizing sugars is the method known as alkylation. It results in the production of analytes that are more volatile than silylation derivatives. Alkylation is generally methylation of test samples with activated methyl groups and is most reactive with carboxylic acids, phenols and alcohols (Kopka et al. 2007, Wittmann 2010). The reaction results in the formation of esters, thioesters, ethers, n-alkylamines and n-alklyamides. The polarity of the sample is reduced because the added alkyl group replaces a hydrogen atom in the functional group, rendering the compound less hydrophilic. This method is now unpopular because of its carcinogenic and explosive nature, irrespective of its high analyte yield (Tønes and Johnsen 1989). Nonetheless, fatty acids are still esterified by methylation prior to GC-MS analysis. For weakly acidic group like alcohols, alkylation is best achieved by using basic catalysts such as sodium or potassium methoxide. Boron trifluoride and hydrogen chloride are ideal for the more acidic OH groups namely phenols and carboxylic acids. The most common reagents for alkylation are dimethylformamide dialkyl acetyls, diazoalkales and pentafluorobenzylbromide. The choice of reagent is dependent on the compound to be derivatized, for instance diazomethane cannot be used for esterifying phenols because it also methylates the OH of the phenolics leading to complex of partially methylated products but it efficiently esterifies fats, producing very little side reactions. It is also toxic, carcinogenic and explosive (Maurya et al. 2011).

Acylation reactions lead to the formation of amides, esters and thioesters as a result of conversion of metabolites containing –NH, –OH and –SH groups by action of carboxylic acid and its derivatives. The resultant analyte is stable and the reaction can easily confer volatility to carbohydrates and amino acids. N-methyl-bis(trifluoroacetamide) (MBTFA) is the most common reagent used for acylation. It trifluoroacylates amines, hydroxyl and thiol groups, resulting in the formation of N-methylfluoroacetamide. This is a stable analyte and does not interfere with chromatography. Other reagents include perfluoro acid anhydrides and perfluoroacylimidazoles. Addition of perfluoro acid anhydrites to amino and hydroxyl groups results in the formation of perfluoroacyl derivatives with reduced polarity, high stability and volatility. Compared to perfluoro acid anhydrites, perfluoroacylimidazoles

yield smoother reactions producing no acid by products (SUPELCO 1997) and as a result are more preferred by some scientist.

2.7.2 Liquid chromatography mass spectrometry (LC-MS)

When HPLC is coupled to mass spectrometry, it is commonly referred to as LC-MS. It is a fundamental separation technique with the ability to separate and quantify non-volatile compounds without prior derivatization and as a result can analyze a wide range of compounds. This is essential when analyzing polar non-volatile heat-sensitive molecules. Phenolics, alkaloids, flavonoids, saponins, poly amines and glucosinolates are usually most efficiently separated using LC-MS (Allwood et al. 2011). It is suited for analysis of unknown compounds of greater polarity and is far cheaper when compared to GC-MS.

2.7.3 Nuclear magnetic resonance (NMR)

Nuclear magnetic resonance can either be mono- or multidimensional. The 1D ^{1}H NMR been the method of choice for metabolic analysis but recently heteronuclear single-quantum coherence spectroscopy (HSQC) and 2D ^{1}H J-resolved (2D JRES) spectroscopy are increasingly being used since they are more powerful tools in metabolomics and can achieve absolute or relative quantification of metabolites (Allwood et al. 2011). Nuclear magnetic resonance is non-descriptive and requires minimal sample preparation and quickly gives metabolite profiles but, spectra obtained are composed of the signals of a lot of compounds and are difficult to separate (Koek et al. 2011). It is most effective when screening families of compounds rather than the individuals themselves.

2.9 Metabolomics of *Salvia* species

Research on *Salvia* in southern Africa has so far focused on elucidation and the validation of phytochemical activities of plant extracts (Kamatou et al. 2008b). Such studies were reported on investigations into antimicrobial (Kamtaou 2006, Ramogola 2009), anti-tumor and anti-inflammatory activities and reported on the near comprehensive analysis of the metabolome. Ramogola (2009) used GC-MS to characterize the secondary compounds found in *S. africana-lutea* foliage and hairy roots and revealed changes in the metabolome as a result of *Agrobacterium*-mediated transformation. Kamatou (2006) screened a number of southern African sages with the aid of GC-MS and LC-MS and confirmed that the metabolite compositions of most South African sages are dependent on season and geographical location. These studies did well in explaining the volatile component and anti-microbial activity of the *Salvia* metabolome but identity of the majority of non-volatile compounds still remains to be elucidated. Further research is crucial because plant metabolomics provides a platform on which drug development and design, and natural products formulation can be based on. Technologies such as metabolite engineering and tissue culture are also strengthened by information generated from metabolite profiles.

2.10 *Agrobacterium* transformation

The *Agrobacterium* genus has the ability to transfer and integrate portions of the plasmid DNA to plants. This leads to production of nutrients that specifically favour its growth by the host plant (Escobar and Dandekar 2003). Members of the *Agrobacterium* genus are commonly found in the soil and the majority of them are saprophytic. Nonetheless, other species are capable of causing neoplastic growth in dicotyledonous plant and in few monocotyledons.

For biotechnology applications, two species of *Agrobacterium* have been used to facilitate gene transfer; *Agrobacterium tumefaciens* and *A. rhizogenes.* These are

pathogens causing crown gall disease and hairy root disease, respectively. Other species in this genus with same ability to cause neoplastic diseases in plants are *A. vitis* which cause crown gall in grape and *A. rubi* which cause cane gall (Escobar and Dandekar 2003). *Agrobacterium* infects wounded plants and is attracted to the plant via chemotaxis in response to compounds released by the plant as part of its defence mechanism. These compounds include sugars and phenolics.

2.9.1 *Agrobacterium tumefaciens*

Agrobacterium tumefaciens is a Gram-negative, rod shaped and motile soil phytopathogen that induces crown gall disease in plants by specifically transfering a portion of its tumor inducing (T_i) plasmid DNA which is subsequently into the genome of the host plant (Lacroix et al. 2006). It is found in the rhizosphere and normally survives on nutrients released by plant roots. Upon arrival at the wound, *A. tumefaciens* induces cell division of the host and production of opines, which are the sole carbon source for *A. tumefaciens* (Slater et al. 2003). The increased cell division will then lead to the tumorous growth, referred to as the crown gall.

Growth of the crown gall depends on the presence of the T_i plasmid (Lacroix et al. 2006). A portion of the T_i plasmid known as the transfer DNA (T-DNA) is transferred to the host plant. The T-DNA carries genes that encode proteins involved in both phytohormone production and biosynthesis of opines and agropines. Genes on the T_i plasmid that do not encode production of hormone or opine production are responsible for proteins involved in the uptake (Slater et al. 2003). On the T_i plasmid, the T-DNA is bordered by two small nearly perfect direct repeats. These are the border sequence. Any gene between the borders is transferred to the plant genome and can be expressed as part of the plant's genotype at the point of infection (Escobar and Dandekar 2003).

2.9.2 *Agrobacterium rhizogenes*

Like *A. tumefaciens, A. rhizogenes* is Gram negative and is attracted to a wounded plant by signal molecules exuded from the plant resulting in the development of hairy roots at the site of infection (Guillon et al. 2006b). *Agrobacterium rhizogenes* infection is characterized by a high hormone independent growth rate resulting in genetically stable hairy roots with short doubling time when cultured (Guillon et al. 2006a, Giri and Narasu 2000). These traits make hairy roots a vital option for producing secondary metabolites comparable to intact plants since they can be easily industrialized.

2.9.3 T-DNA transfer

The process of T-DNA transfer begins with the recognition of signals from a wounded plant. The *A. tumefaciens* recognizes substances that are normally part of the plant's defence mechanism and their presence indicates the competence of the plant to be transformed (Lacroix et al. 2006). The recognition of the competent cell is then followed by the attachment of the bacteria to the plant cells. The attachment is via a polysaccharide transcribed from the *att*R locus (Slater et al. 2003). Chromosomal virulence genes (*chv*A and *chv*B*)* are also involved in the attachment. This is followed by gene induction. VirA senses phenolics and autophosphorylates and phosphorylate and activate VirG. VirG induces expression of all *vir* genes (Escobar and Dandekar 2003). The Induction of *vir* genes is enhanced by sugars (galactose, glucose, and xylose).

Production of the T-DNA strand follows the induction. The VirD1 and VirD2 complex recognizes the left and right border sequences. VirD2 produces a single stranded nick in the DNA and becomes attached to the 5` end of the displaced single strand of the T-DNA. VirC1 aids the repair synthesis to replace the displaced T-DNA. The T-DNA/VirD2 complex is exported out of bacterium by a T-pilus and VirD4, VirE2 and VirF are also exported with the complex. Inside the plant cell cytoplasm, the T-DNA is covered with proteins that are thought to; 1) protect it from nucleases, 2) facilitate nuclear localization and 3) give the complex the conformation the allow passage through the nuclear complex. VirD2 and VirE2 interact with plant proteins to ensure

nuclear localization. VirD2 have nuclear localization signals which allow it to interact with plant proteins called importins (Slater et al. 2003). Importins are plant proteins involved in recognition and transport via the nuclear pore complex and possess as nuclear localization signal. Once inside the plant nucleus the bacterium's T-DNA is incorporated into the plant's genome. The T-DNA strand is integrated into the plant's genome through a process that Bundock and Hooykaas (1996) referred to as 'illegitimate recombination'. It does not require extensive regions of similarity as in homologous recombination (Slater et al. 2003). Once stably integrated, genes between theT-DNA are then sxpressed. Thus when introducing foreign genes in a plant, DNA s inserted on sites within the T-DNA.

2.11 Aims and objectives

Plant metabolic pathways responsible for synthesis of compounds of industrial importance are under control of genes, the environment and the interaction of the two. The expression of secondary metabolism genes is regulated by the changes in ambient conditions, making secondary compounds available only when required and in the needed amount. Industrial processing of natural products is dependent on the presence or absence of these compounds which more often accumulate in minute quantities. Cultivation of medicinal plants for manufacturing purposes is still minimal and yet the demand of phytochemicals is on the rise. *Salvia stenophylla* is increasingly becoming important in the cosmetics and pharmaceutical industries as a result of the essential oils it accumulates and different wild populations exhibit diverse chemotypic variation. This makes quality control and formulation of uniform products difficult. However, metabolite engineering can be used to create plant lines with a potentially increased constant output of essential oils. Thus, this study was designed to provide an alternative and efficient source of bioactives synthesized by *S. stenophylla,* particularly terpenes and their derivatives. It attempted to achieve this through tissue culture and over-expression of *AgGPPS2* in microplants. As such the following objectives were set:

1. To develop a tissue culture system for *S. stenophylla*;

2. To determine effects of water, nitrogen, potassium and hormone stress on volatile metabolite accumulation;
3. To sub-clone the gene encoding for the production of geranyl diphosphate synthase in *E. coli* prior to genetic transformation;
4. To over-express the geranyl diphosphate synthase in *S. stenophylla* using *Agrobacterium* gene transfer technology;
5. To carry out molecular characterization of transgenic clones using Southern blot; and
6. To compare the effects of over-expressing geranyl diphosphate synthase on terpenoid production.

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Chapter Three

In-vitro seed germination and cultivation of the aromatic medicinal *Salvia stenophylla* (Burch. *ex* Benth.)[1]

3.1 Introduction

Salvia stenophylla (Burch. ex Benth.) (family Lamiaceae) is a South African sage found in almost all biomes in the country (Germishuizen et al. 2006, Jequier et al. 1980) showing that it is tolerant to a number of different environments. *S. stenophylla*, commonly known as the blue mountain sage is an upright perennial growing up to 40 cm with narrow linear oblong to oblong lanceolate aromatic leaves (Viljoen et al. 2006). Its square stem is covered with sparse hairs and brightly coloured pinkish-blue axial flowers. Traditionally, *S. stenophylla* is used as a disinfectant for rural homes or it is taken as an herbal remedy in the form of a tea. It produces an essential oil of cosmetic importance which has anti-microbial, anti-inflammatory, anti-malarial and anti-cancer activity (Darra et al. 2008, Kamatou et al. 2008). Compounds of note are α-bisabolol, δ-3-carene and limonene. α-Bisabolol is an oxygenated sesquiterpene that is often found at the highest concentration in the essential oil fraction of many wild populations of *S. stenophylla* (Viljoen et al. 2006). Out of the four known stereoisomers of α-bisabolol, (+)-epi-α-bisabolol has wound healing properties (termed cicatrizant activity) and (-)-α-bisabolol is popular in skin care formulations due to its anti-inflammatory activity, a particularly desired effect for sensitive skins (Viljoen et al. 2006). In *S. stenophylla* and *S. runcinata*, (-)-α-bisabolol may constitute a large fraction of the essential oil compounds (Kamatou and Viljoen 2010).

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Despite the demand for *S. stenophylla* as an herbal remedy for both traditional medicinal use and commercial dermalogical (aromatherapeutic) products, few scientific studies have focused solely on the plant. As a result the knowledge on its propagation and phenology is still scanty. We report on both the conditions that are important for the germination of *S. stenophylla in-vitro* and those that facilitated organogenesis during tissue culture.

Several published micropropagation protocols aim to encourage multiple plantlet production with minimal plant material as starter material (Cuenca and Amo-Marco 1999) for the sages. Many of these also aim to increase metabolite synthesis of key compounds (Ruffoni et al. 2000, Santos-Gomes and Fernandes-Ferreira 2003, Arikat et al. 2004, Avato et al. 2005). A positive impact on the essential oil quality and quantity of compounds is possible with tissue culture (Arikat et al. 2004, Avato et al. 2005) where synthesis of interesting compounds is then upregulated. Different species have different *in-vitro* requirements and this is exemplified by the variety of techniques used for successful propagation for *S. miltiorrhiza* (Morimoto et al. 1994); *S. fruticosa* (Arikat et al. 2004); *S. officinalis* (Tawfik et al. 1992, Santos-Gomes and Fernandes-Ferreira 2003, Avato et al. 2005) and *S. fruticosa* (Mišić et al. 2006). With the variation in reported protocols for sage species, it is thus important that for each species, specific plant growth regulator (PGR) requirements be investigated.

As the essential oil of *S. stenophylla* is becoming increasingly important in the natural products sector, determination of the chemical composition of volatiles released by *in-vitro* shoot cultures in comparison with *ex-vitro* plants is of paramount importance. This is best achieved by using headspace solid phase microextraction linked to gas chromatography–mass spectrometry (HS–SPME–GC–MS).

3.2 Materials and methods

3.2.1 Seed material

Salvia stenophylla seeds were purchased from B and T World Seeds (Paguignan, France) in August 2008, stored in the dark at room temperature and within the same month, germination experiments were set up.

3.2.2 Germination experiment

The study incorporated three experimental variables (treatments): scarification, smoke-water plus a combination of scarification and smoke-water. Acid scarification was achieved by soaking seeds in 70 % H_2SO_4 (5 min), followed by thorough washing with sterile distilled water before placing the seeds on a one-tenth (1/10) Murashige and Skoog (MS) (Murashige and Skoog 1962) medium with 0.8 % agar (w/v) (germination medium, henceforth). For all media, the pH was adjusted to 5.8 with 1 M KOH prior to autoclaving at 121 ℃ and 1.1 kg cm^{-2} for 20 min. Once the medium had cooled, 25 mL was poured under laminar flow into each Petri dish (10 cm). For the smoke treatment, concentrated filter-sterilized (0.2 µm) smoke solution [generated according to Baxter et al. (1994) from *Themeda triandra* Forssk. was added to a concentration of 10^{-5} (v/v)]. This medium was denoted as smoke-medium and seeds on this medium were referred to as smoked. Alternatively, scarified seeds were grown on the smoke-medium to examine smoke- and scarification-induced germination. Controls were left untreated. All seeds were surface-decontaminated in 3.5 % (w/v) sodium hypochloride (20 min) (August 2008); washed three times with sterile distilled water (5 min) and then transferred aseptically to respective germination medium and smoke medium. For each treatment 10 Petri dishes, containing 10 seeds were sealed with laboratory film (Parafilm ‘‘M’’ American National CanTM, USA); and incubated at 23 ± 2 ℃ in either a 16/8 h light regime or in total darkness. Light provided by cool-white fluorescent lamps (L75 W/20X Osram, USA; Code F96T12) gave out a photosynthetically active radiation (PAR) of 50 µmol m^{-2} s^{-1} (at shelf level). Cumulative percentage germination (here germination was defined as the emergence of at least 2 mm of the radicle) was recorded at 3-day intervals for 60 days. Observations in the dark were conducted under a green safe

light (0.5 µmol m^{-2} s^{-1}). When no further germination had taken place for several days, the final germination was calculated.

3.2.3 Culture induction and shoot multiplication

Salvia stenophylla seedlings (70-day-old) were used for culture induction (October 2008). Shoot tips, nodal explants (0.5 cm) (contains a pair of axillary buds) and basal explants (0.5–2.5 cm from the shoot base) were placed on solid MS medium with 0.1 g L^{-1} myo-inositol, 30 g L^{-1} sucrose and 8 g L^{-1} agar (Merck, Germany) (w/v) (pH of 5.8, adjusted with 1 M KOH). Auxin and cytokinin combinations (µM): 2,4-D: BA (4.5: 8.9), NAA: BA (2.7: 4.4 or 8.4: 8.9), and IAA: BA (5.7: 4.4 or 5.7: 8.9) were tested for *in-vitro* regeneration. Cultures were initiated in glass culture vessels (110 ^{x} 55 mm) with each culture vessel containing three explants on solid medium (25 mL). Cultures were incubated under a 16/8 h light regime (50 µmol m^{-2} s^{-1}) at a temperature of 23 ± 1 ℃. The number of shoots growing from each explant was counted, the length was measured and each shoot was weighed. Callus (in grams) was noted and all plantlets with roots were recorded. Experiments were repeated three times. Fifteen culture vessels were used per experiment. Each plantlet represented a single replicate.

3.2.4 Acclimatization

Plant cultures kept for 8 months with 6 weekly subcultures were acclimatized by transferring them into pots (110 ^{x} 90 mm) with a 1:1 (v/v) mix of vermiculite and soil (Double Grow Potting Soil, Durbanville, South Africa; www.stodels.com). Prior to transplanting, plantlets were washed thoroughly with a 1 % (w/v) Dithane® WG-45 solution (mancozeb 750 g kg^{-1} active ingredient; Efekto, South Africa) and then unrooted shoot bases were dipped into an IAA solution (570 mM) for 2 min to facilitate rooting. Rooted plantlets were left untreated. The soil mix was drenched with 2.5 % (w/v) Kelpak [a seaweed extract prepared from *Ecklonia maxima*; active ingredients: 2.2 mg L^{-1} auxins and 0.0062 mg L^{-1} cytokinins; Kelp Products Starke Aryes (South Africa)] to assist *ex-vitro* growth. Potted plants were covered with plastic bags to maintain a high relative humidity (about 90 %) and then opened over a period of 2 weeks to reduce the relative humidity to approximately 70 %. During

this time, the plants were watered every 3 days by hand whilst they were growing in a thermostatically controlled glasshouse at Stellenbosch University (South Africa). In the glasshouse, the temperature settings were adjusted to a minimum of 15 ℃ and a maximum of 25 ℃. The natural day-length increases from 10 h 30 min to 12 h 23 min and outdoor temperatures fluctuate between 11 and 25 ℃ during the day. Plants were exposed to natural sunlight with photosynthetically active radiation (PAR) ranging on a daily basis between 540 to 810 µmol m^{-2} s^{-1} (midday irradiance).

3.2.5 Chemical profiling for volatile compounds

Tissue cultures were maintained in continuous culture on MS medium containing IAA and BA at a ratio of 5.7: 8.9 µM for 12 months. On the other hand, non-propagated plants of *S. stenophylla* were maintained as potted plants from February 2009 until August 2009. To compare the volatile compounds produced by *in-vitro* plantlets to potted plants, HS-SPME of the leaflets was performed with Supelco SPME fibres [DVB/Carboxen/PDMS, StableFlex (Supelco)]. Leaves were placed directly into a 20 mL headspace vial, sealed with an aluminium-coated silicone rubber septum which is easily pierced with the needle of the SPME device. Volatiles were extracted at 80 ℃ for 15 min. The gas chromatography was performed with a Waters GCT Premier AS 2000 instrument coupled to a mass spectrometer, equipped with a HP5 column (30 m, 0.25 mm ID, 0.25 µm film thickness). Temperatures were set at 250 ℃ for both the injection (split injection ratio of 1:5) and the ion source temperature. Helium was used as the carrier gas (1 mL min^{-1}). The temperature ramp regime was initiated by heating at 40 ℃ for 5 min, followed by an oven ramp to150 ℃ at 5 ℃ min^{-1}; and a second ramp of 10 ℃ min^{-1} up until 280 ℃. A mass scanning range of 40–550 m/z (perfluorotri-N-butylamine as mass reference) was employed and mass spectrawere recorded at 2 scans s^{-1}. The Xcalibur™ software bundle version 1.2 (Finnigan Corporation 1998) was used for tentative compound identification and where possible, authentic standards [camphene, (1R)-(+)-camphor, b-caryophyllene, (1R)-(+)-α-pinene, (-)-α-bisabolol (Sigma–Aldrich; Steinheim Germany) and (+)-3-carene; R-(+)-limonene (Fluka, Sigma–Aldrich)] were used to confirm the identified compounds.

3.2.6 Data analysis

Percentage data were arcsine transformed prior to analysis of variance (ANOVA). For the seed germination data (**Figure 3.1**) after the repeated-measures ANOVA, factorial ANOVA followed (**Table 3.1**). To assess the effect of different explants (shoot tips, nodal and basal explants) a one-way ANOVA (**Table 3.2**) was used when the data were normally distributed and when the data were nonparametric the Kruskal–Wallis ANOVA was utilized. A Tukey's unequal N HSD *post-hoc* test followed after oneway ANOVA (**Table 3.3**; shoot length). Otherwise, the results were subjected to Kruskal–Wallis ANOVA, *post-hoc* multiple comparisons of mean ranks was carried out (**Table 3.3**; shoot number and root length). Kruskal–Wallis analysis was again utilized for data presented in **Table 3.4**. Statistica version 8 (StatSoft 2007) was used as the software package for all statistical procedures.

3.3 Results and discussion

3.3.1 *Salvia stenophylla* germination

The large medicinal flora and ethnobotanical knowledge presents many opportunities for continued development of a vibrant commercial medicinal plant industry in South Africa. Knowledge of the agronomic requirements of medicinal plants is still limited even though in-depth understanding of propagation steps would greatly facilitate commercialization. Makunga and van Staden (2008) reported on non-synchronous germination *in-vitro* in *S. africana-lutea*. To establish synchronized germination, *S. stenophylla* seeds were exposed to either light, smoke solution, or scarified chemically and as expected, the germination of *S. stenophylla* seeds followed a sigmoid pattern over 60 days (**Figure 3.1**). As a general trend, the first signs of germination were noted in a few seeds growing in smoke medium under dark or light conditions approximately 7 days after culturing. Germination slowed down after day 40 and from day 50, no new germinations occurred with smoke. Factorial ANOVA revealed that the type of treatment influenced the day in which the first lot of seeds started to germinate (**Table 3.1**). Germination was reliant on the removal of the seed coat (facilitated by chemical scarification) or smoke exposure but was independent of light as seeds incubated in the light or dark exhibited a similar germination rate.

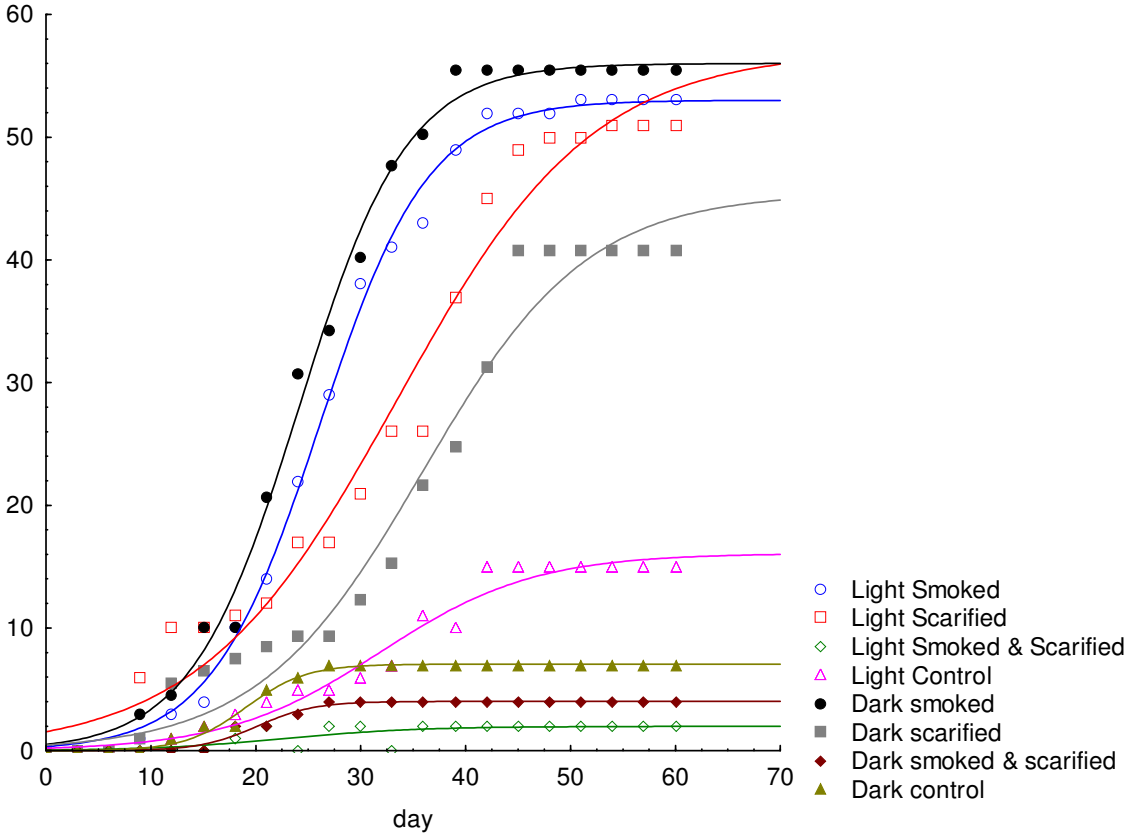

Figure 3.1 Germination frequency (%) of *S. stenophylla* seeds incubated under light or dark conditions for a period of sixty day. Seeds were treated with smoke and/or chemically scarified. Data were analysed using the repeated-measures ANOVA at the 95 % confidence limit.

Table 3.1 Summary of the results from the Repeated measures ANOVA on the effects of seed treatment (smoke and scarification), light regime, day and the interactions on the germination percentage of *S. stenophylla* seeds

Effect	Degrees of Freedom	F-test	*P*-value
day	20	222.4187	0.000000*
light regime	1	0.3734	0.543105
treatment	3	50.8238	0.000000*
day x light regime	20	1.2744	0.185737
day x treatment	60	48.6370	0.000000*
light regime x treatment	3	1.3975	0.250544
day x light regime x treatment	60	1.0477	0.378731

The asterik (*) indicates those treatments (interactions) that had a significant impact on the germination percentage at the 95 % confidence limit.

Table 3.2 Direct organogenesis (shoot and root regeneration) from nodal explants of *S. stenophylla* on MS media with auxins and cytokinins under a 16h light/8 dark regime.

PGR combination	Mean no. of shoots	Mean rooting percentage	Mean shoot length
NAA:BA			
2.7: 4.4 µM	1.07 ± 0.30ab	68.89 ± 17.36a	4.53 ± 1.50a
8.4: 8.9 µM	1.39 ± 0.26b	88.89 ± 7.03a	3.55 ± 0.24a
IAA:BA			
5.7: 4.4 µM	0.67 ± 0.21a	75.00 ± 17.08a	3.24 ± 0.74a
5.7: 8.9 µM	1.83 ± 0.68b	66.67 ± 16.67a	2.34 ± 0.82a

Different letters in the same column indicate values that are statistically different at 95 % confidence level. The asterisk (*) in the heading row indicate a variable whose means were compared using a one-way ANOVA whilst the rested of the data were inferred using a Kruskal-Wallis ANOVA.

The statistics summarized in **Table 3.1** provide strong unequivocal support for the stimulatory role of smoke-triggered germination and coat imposed dormancy in *S. stenophylla*.

Germination in most *Salvia* species (except for *S. pomifera*) readily occurs in the dark (Karamanos 2000). There is no consensus regarding the requirement for light to stimulate germination in this genus as *S. mellifera* requires light for germination (Keeley 1986). Our data show that light in *S. stenophylla* is not crucial for germination. It may be possible that because of its wide geographic distribution, this species has the capacity to germinate under a range of light conditions. These plants are thus exposed to different environments (Jäger and van Staden 2000, Germishuizen et al. 2006) with varying soil depths, soil composition and surface cover, consequently leading to different levels of light radiation reaching the seeds. For instance, germination in *S. mellifera* is highly dependent on light such that it remains dormant below the light penetration depth (Keeley 1986).

Figure 3.2 shows the final mean percentage germination after 60 days, irrespective of whether the seed lot was grown with or without light as light had no significant effect on germination (refer back to **Table 3.1**). In this study, overall germination was best on medium with smoke solution as an additive (**Figures 3.2, 3.3A**). This was recorded as a threefold increase in germination in comparison to controls. Seeds that were scarified and grown on smoke medium, referred here as 'smoked and scarified', germinated poorly with percentage germination being lower than controls. Smoke-treated seeds had the highest germination frequency (**Figure 3.2**). The influence of smoke on germination is well-documented and recently the main trigger for smoke-dependent germination was assigned to butenolide (3-methyl 2H-furo(2,3-c) pyran-2-one; (Flematti et al. 2004, van Staden et al. 2004). This compound enhances percentage germination as well as the germination rate (Jain et al. 2008). The biochemical and genetic mechanisms of smoke (or butenolide) as a germination cue are still unclear but research is ongoing in order to clarify the molecular signaling mechanisms responsible for smoke-derived germination in plants (Soós et al. 2009). A positive response to scarification also suggests exogenous seed coat-imposed dormancy in *S. stenophylla* as this process removes or damages the seed coat. According to Taiz and Zeiger (2002), dormancy is due to the seed coat preventing

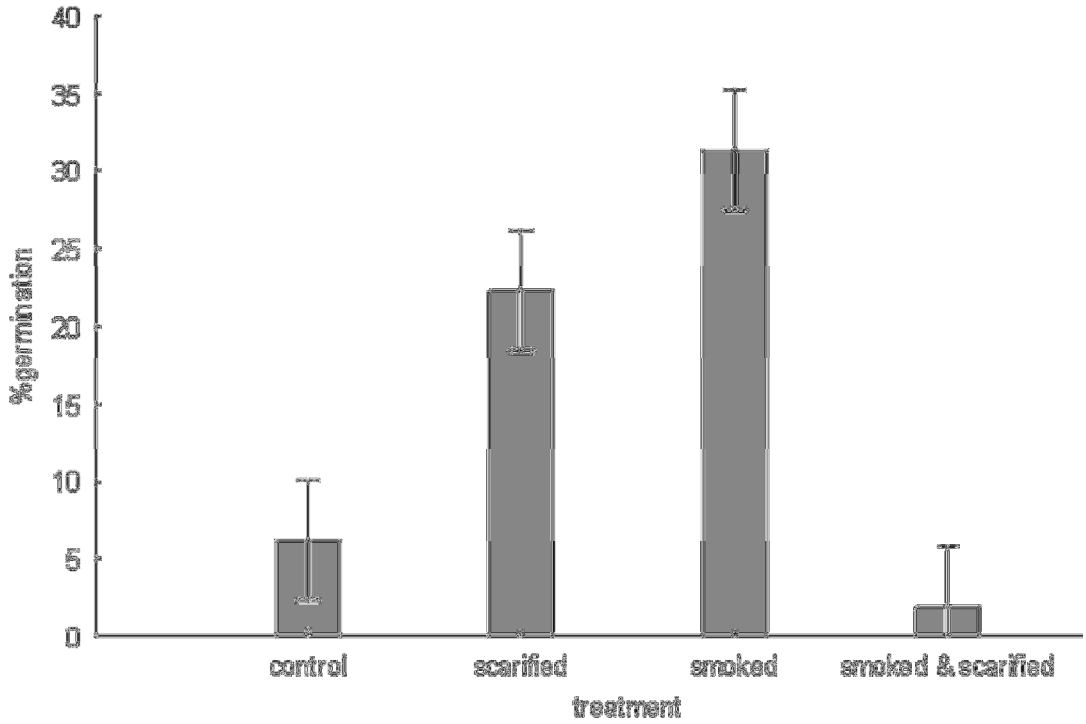

Figure 3.2 Effects of smoke and scarification on *in-vitro S. stenophylla* seed germination on 1/10 MS medium. Grey bars indicate the total number of seeds that germinated after 60 days irrespective whether seeds were grown in the light or dark. Bars with different letters above them are statistically different and vertical bars denote standard error at the 95 % confidence interval

Table 3.3 Micropropagation of *S. stenophylla* on Murashige and Skoog (1962) medium supplemented with different combinations of auxins and cytokinins

PGR combination (µM)	Number of shoots	Shoot length (cm)	Rooting frequency (%)
NAA:BA			
2.7:4.4	1.07ab	4.5a	68.9a
8.4:8.9	1.39b	3.55a	88.9a
IAA:BA			
5.7:4.4	0.67a	3.24a	75.0a
5.7:8.9	1.83a	2.34a	66.67a

Different letters in the same column indicate values that are statistically different at a 95 % confidence level. The data for shoot length were compared using a one-way ANOVA whilst for the rest of the data statistical significance was inferred using a Kruskal–Wallis ANOVA

water imbibition and gaseous exchange or due to internal embryo inactivity. Primary (physiological) dormancy is assigned to the phytohormonal balance with gibberellins (promoter) acting antagonistically to abscisic acid (inhibitor), thus inducing germination (Rehman and Park 2000). Poor germination on smoke medium of scarified seeds was detrimental for germination. It is possible that scarification which removes the testa enabled higher concentrations of smoke compounds to reach the embryo. Smoke has a negative influence on germination (Daws et al. 2007) when the concentrations used to treat seeds are high as it becomes inhibitory. Daws et al. (2008) showed that concentrations of butenolide beyond 1×10^{-7} significantly reduced germination in *Striga hermonthica*. For this study, the smoke solution was included at a concentration of 10^{-5} (v/v). However, seeds were in contact with smoke solution for prolonged periods (60 days). A study which involves the addition of the smoke-water extract at varying concentrations may prove useful in the future as this will serve to determine the optimal concentration of smoke solution that is important for germination in *S. stenophylla*. To break pronounced seed dormancy, a broader range of methods should be tested, e.g., temperature, hormonal and/or light treatments. Stratification which mimics moist chilling is a standard procedure utilized to enhance and speed up dormancy (Rehman and Park 2000). Exogenously applied GAs as a substitute for stratification successfully aid in alleviating dormancy in seeds. Results derived from such a study designed to optimize these factors would also be useful for those interested in the domestication of this particular species for commercial cultivation for the essential oil constituent epi-α-bisabolol or the ornamental horticultural industry.

3.3.2 Culture induction and shoot multiplication

Seedlings (shoot tip and nodal sections) proved a highly regenerative explant source for the induction of *S. stenophylla* cultures (**Figure 3.3A**). Shoot tips had a tendency to elongate prior to shoot regeneration. Only one shoot was produced from shoot tips as compared to nodal explants that had a capacity to produce two shoots per nodal section but this difference was not statistically significant (**Table 3.2**). As there were no statistical differences with regards to the explant type used, data were combined for statistical analysis and the pooled data are presented in **Table 3.3.**

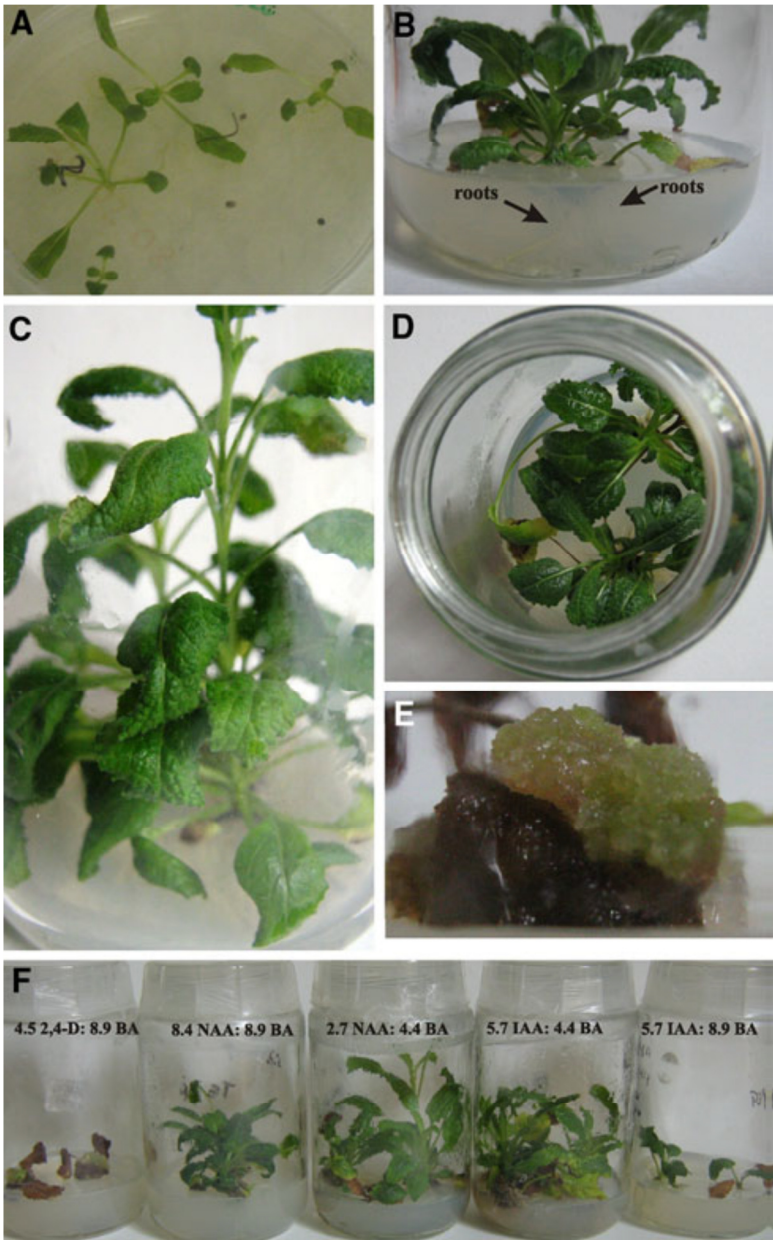

Figure 3.3 *In-vitro* culture of *S. stenophylla*. (**A)** Scarified and smoke-treated seeds germinating seedlings; (**B**) Multiple plantlet production on 5.7 µM IAA and 8.9 µM BA MS medium. Rooting occurred spontaneously (arrows indicate adventitious roots); (**C**) Plantlet elongation on 2.7 µM NAA: 4.4 µM BA MS medium; (**D**) Healthy green shoots, true-to-type with a waxy cuticle growing on 2.7 µM IAA and 4.4 µM BA MS medium; (**E**) Callus production on 4.5 µM 2,4-D: 8.9 µM BA MS medium; (**F**) Morphological differentiation on different media: 4.5 µM 2,4-D: 8.9 µM BA medium encouraged callus production; 8.4 NAA: 8.9 µM BA medium resulted in shoots with roots; 2.7 µM NAA: BA medium promoted shoot elongation; 5.7 µM IAA: 4.4 µM BA medium produced multiple rooted shoots associated with callus; 5.7 µM IAA: 8.9 µM BA medium produced shoots that were short.

Data were derived from all explants used in the study. The first signs of micropropagation were noted 16 days after culture initiation. The highest shoot regeneration was achieved on a combination of 5.7 µM IAA and 8.9 µM BA (**Table 3.3**). This combination produced an average of 1.83 shoots per explant. Even so, statistical analysis revealed that this effect was similar to all NAA: BA combinations tested [2.7:4.4 and 8.4:8.9 (µM)] where 1.07 or 1.39 shoots, respectively, were recorded. The medium with 5.7 µM IAA and 4.4 µM BA produced the least number of shoots. Plantlets produced on the different media appeared to be true-to-type (**Figures 3B–D**). None of the plants were hyperhydric. Shoots were healthy without obvious abnormalities. Phenotypic abnormalities associated with *in-vitro* culture often indicate suboptimal microenvironmental conditions. Plantlets elongated rapidly reaching a length of 3.55 cm (**Table 3.3**) on medium with 8.4 µM NAA and 8.9 µM BA. Several PGR combinations were tested during this study but none of these had a statistically significant effect on shoot elongation. The shoot length varied from 2.34 to 4.53 cm.

Rooting *in-vitro* is largely genotype-dependent (de Dorlodot et al. 2007, Ioio et al. 2008). All four combinations of auxins and cytokinins had a similar effect on adventitious rooting despite the length of the shoot. A 8.4 µM NAA and 8.9 µM BA medium encouraged extensive rhizogenesis (**Table 3.3**) displaying a high rooting rate of 88.89 % although it was not significantly different from the other combinations (P>0.05). Rooting was spontaneous and the adventitious roots produced at the shoot base were strong, elongating rapidly after root induction (**Figure 3.3B**). As a consequence, we did not seek to investigate alternative rooting protocols. Even though rooting was spontaneous for most media tested (**Table 3.3**), it was sometimes accompanied by extensive callus proliferation. Naphthalene acetic acid alone is often used as a rooting agent in tissue culture and this study showed that increasing its concentration in MS medium led to both increased shoot and root development. Subjecting plants of *S. brachyodon* to increasing IAA concentrations (Mišić et al. 2006) positively influenced rooting to a point beyond which this hormone inhibited root formation (1.1 µM). Makunga and van Staden (2008) showed a similar pattern when they cultured *S. africana-lutea* hypocotyl-derived plants on MS medium. In that study, increasing concentrations of NAA (from 2.7 to 5.7 µM) improved rooting. Here IAA-supplemented MS medium had a lower rooting percentage as

compared to NAA. Nonetheless, the difference was insignificant at the 95 % confidence level. Over ten to twenty roots were initiated on each plantlet (**Figure 3.3B**).

Combining 2,4-D with BA produced only callus from nodal explants (100 %; mass of 6.16 g; **Table 3.4**). The rapid conversion of all nodal explants to callus was particularly prevalent with 2.4-D (**Figure 3.3E**) but was not limited to this medium. Callus formation was also extensive with NAA and BA at a ratio of 8.4: 8.9 (µM) (**Table 3.4**). It was surprising that none of the nodal explants exposed to IAA formed callus. Production of callus is often undesired as it increases the likelihood of somaclonal variation and subsequent expression of epigenetic and genetic instability (Cassells and Curry 2001). With regards to medicinal plants, there are cases when callus is desirable. This is exemplified by the production of the strong anti-oxidant rosmarinic acid in *S. chamelaeagnea* callus (Huang and van Staden 2002). In this study transfer of callus onto respective media for subsequent culture cycles did not exhibit an organogenic response but at times, some shoots produced callus at the base after subculture. Different morphological effects of using auxins and cytokinins in combination are shown in **Figure 3.3F**. The prolific nature of the current protocol for plantlet production warranted 6 weekly subculture cycles; effected by transferring nodal explants onto fresh medium. For a continuous culture system, we now maintain plantlets of *S. stenophylla* on a medium with 2.7 µM NAA and 4.4 µM BA. Shoots produced on this medium may reach a length of over 4.53 cm. On average, three to five nodal explants may be excised from each shoot with two shoots being regenerated per axillary bud. Therefore, each shoot has the potential to produce over 1000 new shoots during a 3-month cycle. Even though rooting *in-vitro* is not a prerequisite for acclimatization, rooted plants establish faster out of culture as they have a fully functional rooting system (George and Sherrington 1984). Both rooted and unrooted plantlets (**Figure 3.4**) were easily transferred to soil and upon potting in the glasshouse, 75 % of the plantlets (data not shown) continued to develop normally.

Table 3.4 Callus production from explants of *S. stenophylla* on Murashige and Skoog (1962) medium supplemented with different combinations of auxins and cytokinins

PGR combination (µM)	Callus production	Mass of regenerating tissue
2,4D:BA		
4.5:8.9	100a	6.2a
NAA:BA		
2.7:4.4	44.5ab	3.8ab
8.4:8.9	88.9ab	5.1a
IAA:BA		
5.7:4.4	0b	1.4b
5.7:8.9	0b	2.5ab

Different letters in the same column indicate values that are statistically different at a 95 % confidence level. Statistical significance was inferred using a Kruskal–Wallis ANOVA as the data were nonparametric

Figure 3.4 Example of acclimated *S. stenophylla* plantlets

3.3.3 Headspace analysis

Different chemotypes of *S. stenophylla* were identified by Viljoen et al. (2006) according to their essential oil data and plants containing α-bisabolol, δ-carene and limonene were found in Biesiesvlei, Dordrecht, Molteno, Sannieshof and Steynsrus. Monoterpenes such as α- and β-pinene, p-cymene, ɣ-terpinene, δ-3-carene and limonene vary significantly in *S. stenophylla*. The chemical composition was analysed using SPME for *in-vitro* derived *S. stenophylla* plants as it is reported by Gonçalves et al. (2008) that it requires smaller sample sizes, minimizes the formation of artifacts and is less time-consuming. In the tissue cultured sample, α- and β-pinene (2.2 %; 8.6 % respectively), camphene (2.0 %), δ-3-carene (14 %) and limonene (7.9 %) were represented in the chemical complex of volatiles (**Table 3.5**). Qualitative and quantitative variation in the chemical constituents of the essential oil of *S. stenophylla* plants is closely related to the genetic and phenotypic characteristics as well as being largely influenced by climatic conditions and water availability in different habitats where these plants are naturally found. Plants found at different localities accumulated different levels of major compounds with the concentration of a-bisabolol ranging from 1.3 % (Springfontein) to 46.5 % (Dordrecht) (Viljoen et al. 2006). (-)-α-Bisabolol was still the most dominant compound in *in-vitro*-derived plantlets as the relative abundance of this compound was estimated at 21 % (**Table 3.5**) with levels being comparable to unpropagated samples. The volatile fraction of both propagated and nonpropagated plants was extremely complex with over 160 compounds occurring in these plants. Chemical similarity of *in-vitro* plants and *ex-vitro* plants indicated that tissue culture propagation did not have a significant impact on the volatile compounds. This is highly desirable if this biotechnological strategy is to be adopted for commercial purposes. Environmental conditions are controlled and optimized *in-vitro* to facilitate rapid vegetative growth and mass multiplication. This important pre-requisite for successful organogenesis has the potential to lead to altered secondary metabolism with plants that have been produced in this way being vastly different from those grown out of culture. The impact of environmental conditions on secondary metabolism is well-supported (Palić et al. 1982, Perry et al. 1999, Skoula et al. 2000) and great plasticity can be expected within a taxon (Viljoen et al. 2006).

Table 3.5 Major volatile organic components (relative abundance, %) released from *in-vitro* and *ex-vitro* plants *S. stenophylla* plants identified by HS-SPME-GC/MS

retention time (min)[a]	Component	*Ex-vitro* plants (%)	*In-vitro* plants (%)
9.45	α-Pinene	1.7	2.2
9.97	Camphene	1.4	2.0
11.79	β-pinene	7.5	8.6
12.36	δ-3-Carene	17.4	14.0
12.96	D-Limonene	6.4	7.9
24.42	β-caryophyllene*	-	2.34
25.25	cis-α-Bisabolene*	-	1.03
30.12	(-)-α-Bisabolol	20.1	21.0

([a]) Compounds are listed in order of their elution time from a HP5 column
(*) Detected only in *in-vitro* shoot-cultures

β-Caryophyllene (2.34 %) and cis-α-bisabolene (1.04 %) were present in *in-vitro* propagules whereas these compounds were not detected in the foliage of *ex-vitro* material tested in this study. This is not unusual as these compounds are generally found in wild populations of *S. stenophylla*. Jequier et al. (1980) quantified β-caryophyllene at 2.5 %. This particular volatile was represented in 20 populations out of 25 from different habitats examined by Viljoen et al. (2006). When present, variation in the quantity of β-caryophyllene in plants from different locations was evident, ranging from 31.9 to 1.3 %. Here, we can thus conclude that *in-vitro* culture did not result in the production of unwanted compounds that are not generally present in *S. stenophylla* species. *De novo* or upregulated synthesis of both β-caryophyllene and cis-α-bisabolene (attributed to the microenvironmental conditions established for this species) is thus regarded as an added advantage, providing further supporting evidence for use of such a biotechnological strategy. This approach was thus beneficial as chemical profiles of propagated plants closely matched those of plants found in nature. Our continued interest in the *in-vitro* cultivation of *S. stenophylla* will now lead to genetic engineering and for this particular purpose sufficient plantlet material is available as a target for genetic transformation using *Agrobacterium* as a vector.

3.4 Conclusion

Using an *in-vitro* system, germination of *S. stenophylla* could be improved with smoke treatment or chemical scarification suggesting that the seeds exhibit dual dormancy (both exogenous and endogenous). The protocol established in this paper has several advantages as it was simple with *in-vitro* shoots rooting naturally, negating the inclusion of a separate rooting stage. This makes it highly attractive as it fulfils the criteria for the production of *S. stenophylla* as a commercial crop. Micropropagation is preferred for molecular genetic engineering but more importantly, clonal propagation may possibly provide an alternative source of (-)-α-bisabolol in the future.

3.5 References

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Chapter Four

Effect of potassium, nitrogen and water on *Salvia stenophylla* phytochemistry[2]

4.1 Introduction

Plant secondary metabolism is biochemically and genetically complex. It is a function of spatial, temporal, and phenological factors, with seasonal changes and species growth patterns impacting both metabolite synthesis and subsequent accumulation. Therefore, metabolite heterogeneity within species is often reliant on biotic and abiotic factors. *Salvia stenophylla* Burch. *ex* Benth. (Lamiaceae) (**Figure 4.1A**), or blue mountain sage, is one of 26 indigenous South African species (Kamatou et al. 2005, Germishuizen 2006). This small, bushy perennial herb (average height = 40 cm), with flowers varying from blue to purple and which bloom from August to February (Jequier et al. 1980), has a wide geographical distribution in South Africa (Germishuizen 2006).

Similar to other sages, *S. stenophylla* produces a diverse range of volatile compounds of commercial significance (Kamatou et al. 2008) consisting primarily of monoterpenes and sesquiterpenes along with their oxygenated derivatives, which

[2] This chapter is currently available as a peer-reviewed original scientific paper. It was published in the June 2012 edition of the Journal of Plant Growth Regulation. One of the pictures became the cover-page for that issue. The full reference is as follows:

Musaruwa HT, Koegelenberg, Makunga NP (2011) Chemical variation in essential oil profiles detected using headspace solid phase microextraction gas chromatography spectrometry in response to potassium, nitrogen and water available to micropropagated plants of *Salvia stenophylla* (Burch. ex Benth). *J Plant Growth Regul* 31: 206-220

could be alcohols, aldehydes, esters, ethers, ketones, phenols, or oxides (Longaray Delamare et al. 2007), rendering the oil aromatic.

Figure 4.1 *In-vitro* propagation of *Salvia stenophylla*. (**A**) Wild plant grown outdoors as potted garden plant. (**B**) Continuous stock plant culture grown on agar-solidified MS medium with 2.9 µM IAA and 4.4 µM BA. (**C**) Plantlets grown for experimental use in test tubes on MS medium. (**D**) Plantlets grown on medium with reduced nitrogen levels (½ N) became chlorotic with increased production of callus. (**E**) Plantlets grown on medium with increased nitrogen levels (2N medium) maintained prolific shoot regeneration and rhizogenesis. (**F)** Roots produced on the 2N medium were strong, thick and fibrous. **(G**) An example of rooted plants growing on ½ N medium with roots which were thin and fragile. (**H**) Plants grown on the 2 K medium produced well-formed *in-vitro* flowers. (**I**) Inclusion of sorbitol reduced plantlet productivity, increasing the incidence of callus at shoot bases. (**J**) *In-vitro*-generated *Salvia stenophylla* plants acclimated and growing in the greenhouse.

Apart from (-)-α-bisabolol, an important component of dermaceutical and cosmeceutical products due to its anti-inflammatory, anti-irritant, antimicrobial, and cicatrizant activity (Kamatou et al. 2010), major compounds of industrial importance include manool, limonene, δ-3-carene, α- and β-pinene, camphor, and camphene, among many others. These biochemicals play a major role in the quality of essential oils and are thus monitored for quality assurance in commercial preparations. The essential oil of *S. stenophylla* shows chemical polymorphism, a phenomenon that has been linked to changing ecological microclimates (Viljoen et al. 2006) as populations of these plants are found in a diverse range of habitats (ranging from the Eastern Cape, KwaZulu-Natal, and the Highveld of Gauteng Province).

Field cultivation has been applied broadly to aromatic plants for essential oil production worldwide [for example, *Pelargonium graveolens* L'Heritier (Saxena 2008), *Lippia javanica* (Burm. f.) Spreng, *Anthemis nobilis* L., and *Matricaria recutita* L. (Nikolova et al. 1997)], ensuring adequate supply to meet commercial demands. Even though in its infancy, domestication of aromatic and medicinal plants (both exotic and indigenous species) is becoming increasingly important for poverty alleviation schemes (Makunga et al. 2008, Horak et al. 2009) in South Africa as the essential oils industry is part of a wider government initiative to stimulate rural socioeconomic development. Currently, both cultivated and wild-crafted plant material is distilled for the production of ''African indigenous'' essential oils to serve local and export markets, but *S. stenophylla* cultivation is limited to a few agricultural commercial producers that are mainly resident in the Western Cape Province. In view of this, a better understanding of the nutritional and other cultivation requirements would assist with the domestication of key aromatic indigenous crops such as *S. stenophylla*.

Like most medicinal and aromatic plants, genetic and abiotic factors such as water availability, temperature, photoperiod, and herbivory affect the essential oil production of *S. stenophylla* populations (Viljoen et al. 2006). The need for the cultivation of medicinal plants in an economy with a fast-growing phyto-pharmaceutical industry cannot be overemphasized as access to many aromatic

plants in South Africa is largely through wild harvesting, increasing concerns regarding the loss of biodiversity. Tissue culture is also a well-used means of conservation (Kamatou et al. 2008, Makunga and Van Staden 2008) for those medicinal plants that are accessible only through wild harvesting. *In-vitro* culture also allows for rapid plant propagation (Nalawade et al. 2003). The generation of disease-free, clonal propagules is highly attractive for commercial use (Kintzios 2000) as the production of clonal plants minimizes qualitative and quantitative chemical variation, ensuring a reliable source of bioactive ingredients. Apart from the studies that focused on the *in-vitro* propagation of *S. chamelaeagnea* (Huang and Van Staden 2002), *S. africana-lutea* (Makunga and Van Staden 2008), and *S. stenophylla* (Musarurwa et al. 2010), investigations have centered mostly on the phytochemical characterization and pharmacological efficacy of local endemic *Salvia* species which are used as traditional medicines (Kamatou et al. 2005, 2008, 2010, Viljoen et al. 2006).

Here, the *in-vitro* system was ideal for generating plants that are genetically similar, facilitating the aim of studying the effect of changing nitrogen, potassium, water supply, and plant growth regulator (PGR) combinations on the volatile oil profiles of *S stenophylla*. Such manipulations cause great shifts in secondary metabolic flux leading to significant changes in the chemical constituents of essential oils (Rout et al. 2000). We chose to vary the concentrations of nitrogen and potassium supplied to the plants as they greatly influence plant growth and vigor (Van Iersel et al. 1998, Kadota et al. 2001). Volatile constituents were monitored easily through headspace solidphase microextraction gas chromatography spectrometry (HS-SPME-GC-MS). Plants derived from the *in-vitro* system may serve as an *ex-situ* stock for commercial agricultural cultivation.

4.2 Materials and methods

4.2.1 General procedures and *In-vitro* plantlet culture

A continuous microplant culture of *S. stenophylla* Burch. Ex Benth. (**Figure 4.1B, C**), generated from seedling explants in 2008 according to the protocol described by Musarurwa et al. (2010), was used in this investigation. Eighteen month-old microplants were maintained in culture vessels (110 × 55 mm) on a basal medium (30 mL) of Murashige and Skoog (1962) (MS), with 2.9 µM NAA, 4.4 µM BA, 0.1 g L^{-1} myo-inositol, 30 g L^{-1} sucrose, and 10 g L^{-1} agar (Merck, Germany) (pH 5.8; 1 M NaOH) (unless otherwise stated). All media were autoclaved at 121 ℃ and 103 kPa for 20 min. *In-vitro* plantlets were grown under cool white fluorescent lights (L75 W/20 9 Osram, USA; code F96T12), supplying light for 16 h (50 µmol m^{-2} s^{-1}) at shelf-level in the growth room (25 ± 2 ℃). Nodal segments (2–5 cm), each containing an axillary bud pair that had the capacity for producing adventitious shoots, were subcultured to generate plantlet stocks every 6 weeks. Plants were acclimatized similarly to the methods described by Musarurwa et al. (2010). Throughout the study, a set of *ex-vitro* plants derived from tissue culture was maintained as potted glasshouse plants. The plants were acclimated by reducing the relative humidity over a 2-week period from about 90 to 70 %. Plants were watered regularly every 3 days by hand. The conditions in the glasshouse were maintained using a thermostat that regulated the temperature between 15 ℃ (minimum) and 25 ℃ (maximum). In Stellenbosch, the natural sunlight ranged daily between 540 and 810 µmol m^{-2} s^{-1} at midday during the time of acclimatization. We also kept a stock of plants, donated by Mrs. Jenny Ferreira (Wellington, Western Cape, South Africa), under garden conditions. The plants were watered daily using a sprinkler system but otherwise they were exposed to normal environmental conditions prevailing in Stellenbosch during the study period from February 2009 until December 2010.

The control medium (referred to here as MS) had NH_4NO_3 at 25.76 M and KNO_3 at 18.79 M as the nitrogen source, and all other macro- and micronutrients were kept as stipulated by Murashige and Skoog (1962). For the nitrogen experiment, the concentration of NH_4NO_3 and KNO_3 was either doubled (2 N), tripled (3 N), or increased by a factor of one and a half (1 ½ N), or two and a half (2 ½ N); all other

nutritional elements were kept similar to the control. The potassium source, KH_2PO, which was added to MS medium at a concentration of 1.25 M (Murashige and Skoog 1962), was varied in a similar manner as described above and treatments henceforth are denoted as 1 ½ K, 2 K, 2 ½ K, and 3 K. Furthermore, plants were also grown on a medium that had half the concentration of all MS salts and vitamins (½ MS). For this treatment, the sucrose and myo-inositol were added at 15 and 0.05 g L^{-1}, respectively. Otherwise, for all other media, sucrose and myo-inositol were added at a rate of 30 and 0.1 g L^{-1}, respectively. The PGRs were omitted from all media, including the control. The experiment was conducted in glass vessels (110 × 55 mm) with one plantlet representing a replicate. Each treatment had at least ten replicates (unless otherwise stated), and three samples from each treatment (sampled at random) were used for metabolite profiling (**Tables 2, 3**). All experiments were conducted twice. Data were collected at the end of 6 weeks by recording the number of regenerated shoots, shoot length (cm), plantlet mass (g), and root number from each explant. The number of leaves from each regenerating shoot was also counted (**Table 4.1**), and in this particular instance, each regenerating shoot was regarded as a replicate for statistical purposes

4.2.2 Effect of osmotica

Two dehydrating compounds were added independently as osmotica [sorbitol and polyethylene glycol (PEG)] to the MS medium (**Table 4.4**). One group of plantlets was treated with 110 µM sorbitol, similar to the study of Kadota (2001), as plants are still able to maintain normal shoot proliferation at this concentration. In parallel, 5 % (w/v) PEG-6000 (Merck, Germany) was added to the basal medium of the second group of plantlets. Plant growth regulator supplementation was achieved by adding a combination of NAA and BA at 2.9 and 4.4 (µM), respectively (Musarurwa et al. 2010). The controls had no sorbitol or PEG added to the basal MS medium. *S. stenophylla* internodal sections (1–3 cm in length) were individually transferred to glass test tubes (7 cm × 2 cm) containing 10 mL growth medium (**Figure 4.1C**). Plastic caps were used as a seal and secured with a Parafilm ‘‘M’’ strip (American National Can, USA). Each plant in a test tube was regarded as a replicate (for examples refer to **Figure 4.1C**). The experiment was terminated after 4 weeks. The

number of leaves per plantlet was counted at day 0 and day 30. The shoot mass, the callus mass, and the total biomass produced from each plantlet were recorded. The number of roots formed was also noted. Thereafter, these plantlets were immediately sent for GC-MS analysis (**Table 4.5**).

4.2.3 Effect of plant growth regulator combinations

Because plant metabolite synthesis is also affected by the presence and concentration of PGRs, plantlets were grown on MS media with varying concentrations of cytokinins and auxins. The PGR combinations were 2.9 µM IAA and 4.4 µM BA, 2.9 µM IAA and 8.9 µM BA, and 1.1 µM IAA and 8.9 µM BA. Data were collected at the end of 6 weeks.

4.2.4 Routine HS-SPME-GC-MS protocol

The essential oils were assessed through HS-SPME-GC-MS, in a manner similar to the report of Musarurwa et al. (2010) but oven ramping temperatures and injection ratios were modified. Briefly, directly after harvesting, all plant material (0.4 g) was placed inside 20 mL headspace vials. Each container was sealed with an aluminum-coated silicone rubber septum. The extraction of the volatiles was facilitated by heating samples for 15 min at 80 ℃. Released shoot volatiles were adsorbed by the SPME fiber [DVB/Carboxen/PDMS, StableFlex (Supelco)]. With the exception of the osmotica-treated material and those experiments that examined the effect of PGRs, a 1:20 split injection ratio was used to introduce compounds into the Waters GCT Premier instrument fitted with a HP5 column (30 m, 0.25 mm i.d., 0.25 lm film thickness). Otherwise, a 1:5 split injection ratio was used. An initial temperature of 40 ℃ with a 5 min hold was used. Thereafter, the temperature was raised to 150 ℃ at a rate of 5 ℃ min^{-1} before the rate was further adjusted to 10 ℃ min^{-1} until 280 ℃. For mass spectrometry, the chemical standards [namely, (-)-α-bisabolol, camphene, (1R)-(+)-camphor, (+)-3-carene, b-caryophyllene, (1R)-(+)-α-pinene, R-(+)-limonene] (Sigma-Aldrich, Germany) used for analysis were kept the same as in the report by Musarurwa et al. (2010). An additional chemical standard, azulene, also purchased from Sigma-Aldrich (Germany), was included in this set used in the present study.

The NIST Mass Spectral Search Program Library ver. 2.0 d (2005; Standard Reference Data Program of the National Institute of Standards and Technology, USA), in conjunction with previous work of Viljoen et al. (2006), was used for compound identification. The plantlets for each experiment were pooled together for analysis as we had determined that the differences between the chemical constituents of three individual tissue culture samples were negligible.

4.2.5 Data collection and statistical analysis

Each treatment had at least ten replicates and three samples from each treatment (sampled at random) were used for metabolite profiling. All experiments were conducted twice. The means were compared using the analysis of variance (ANOVA; $P \leq 0.05$) followed by the Tukey's HSD *post-hoc* analysis. For the data that were not normally distributed, Kruskal-Wallis *post-hoc* analysis was used to separate means. All percentage data were first arcsine-transformed prior to ANOVA. A principal component analysis (PCA) was also carried out to show differences between *in-vitro* propagules and *ex-vitro* propagated plants and those exposed to varying potassium and nitrogen medium components. All statistical procedures were performed using Statistica ver. 9 (2007; StatSoft Inc, Tulsa, USA).

Table 4.1 Effect of potassium and nitrogen concentration on *in-vitro* growth and organogenesis of *S. stenophylla* on Murashige and Skoog (1962) medium

Nutrient	Nutrient Level	Shoot Number	Shoot Weight (g)	Shoot Length (cm)	Leaf Number	Root Number
control	½	1.63 ± 0.25 abcd	0.21 ± 0.04 ab	**4.89 ± 0.59 b**	10.9 ± 1.71 a	**16.8 ± 2.65 b**
	1	1.17 ± 0.28 ab	0.16 ± 0.05 ab	1.83 ± 0.68 a	7 ± 1.97 ab	7.50 ± 3.06 ab
nitrogen	1½	1.00 ± 0.28 a	0.16 ± 0.04 ab	1.85 ± 0.68 a	7 ± 1.97 ab	2.67 ± 1.71 ab
	2	1.78 ± 0.23 bcd	0.17 ± 0.03 ab	1.58 ± 0.55 a	8.11 ± 1.61 ab	0.67 ± 0.55 a
	2½	**2.22 ± 0.23 d**	0.35 ± 0.11 ab	1.68 ± 0.55 a	7.22 ± 1.61 ab	1.00 ± 0.57 a
	3	2.00 ± 0.28 cd	0.30 ± 0.04 ab	1.68 ± 0.68 a	6 ± 1.97 ab	0.00 ± 0.00 a
potassium	1½	1.86 ± 0.26 bcd	**0.68 ± 0.34 b**	2.16 ± 0.63 a	**11.3 ± 1.83 a**	5.86 ± 2.99 ab
	2	1.14 ± 0.26 ab	0.17 ± 0.06 ab	1.53 ± 0.63 a	9.57 ± 1.83 ab	5.00 ± 3.92 ab
	2½	1.02 ± 0.22 a	0.09 ± 0.02 a	1.30 ± 0.68 a	4.83 ± 1.97 b	2.17 ± 2.17 ab
	3	1.40 ± 0.31 abc	0.20 ± 0.05 ab	2.34 ± 0.74 a	8.6 ± 2.16 ab	8.40 ± 5.88 ab

Letters that are different within the same column indicate statistical differences ($P < 0.05$). Values in bold font type are the highest recorded means for that variable

4.3 Results and Discussion

4.3.1 Effect of nitrogen and potassium on plantlet growth

The impacts of mineral nutrition on both microplant productivity and volatile leaf constituents of *S. stenophylla in-vitro* were determined. Most treatments were able to induce the development of two microplants per explant, with the exception of the 1 N and 2 ½ K treatments. Plants growing on 1 ½ K medium were able to maintain prolific shoot mass production (0.68 g) (**Table 4.1**) which was paralleled by adventitious leaflet regeneration. Although this was statistically insignificant, the MS medium strongly promoted rhizogenesis. Roots produced in culture were typically well developed and appeared to be thick and strong (**Figure 4.1C**). The number of leaves produced per shoot was not correlated to increasing concentrations of nitrogen. However, explants on ½ MS and 1 ½ K media produced the highest number of leaves per individual regenerated shoot. Those plants grown on ½ MS elongated best (4.89 cm), while all other treatments were statistically similar in terms of their ability to promote shoot extension. However, nutrient manipulation strongly influenced overall growth patterns and organogenesis. Increased levels of supplied potassium salts corresponded to a higher rooting frequency and the number of adventitious roots formed in culture.

It is evident from **Table 4.1** that total shoot mass correlated with supplied nitrogen, but this effect was also accompanied by a drastic reduction of root formation. Plants in nutrient limiting environments invest in mass proliferation of roots so as to increase the surface area for maximum nutrient absorption. The root network thus becomes much thicker with increased production of lateral root branches (Pellny et al. 2008). Our data corroborate with these well-established effects as reduction of nutrients (½ MS) dramatically stimulated rooting. Although unrooted microplants are able to survive *ex-vitro* transplantation, acclimatization is often better when roots are present. Furthermore, plants with roots associated with callus often have a lower hydraulic activity due to the disruption of the vasculature, potentially lowering their *ex-vitro* acclimatization frequencies (Makunga and Van Staden 2008). The *in-vitro*

propagation system for *S. stenophylla* is generally highly prolific, with adventitious shoot regeneration from axillary nodes.

Stitt (1999) reported that high nitrate concentrations may inhibit root growth. This may explain the loss of the rooting potential. Nitrates are important in nucleic acid and protein production, acting as a stimulant for gene expression while playing a crucial role in resource allocation (Hermans et al. 2006). Moderate nitrogen deficiency reduces shoot growth but it may have a stimulatory effect on root growth resulting in a smaller shoot: root ratio (Scheible et al. 1997). Plantlets often became chlorotic on lower nitrogen medium (**Figure 4.1D**), indicating inefficient production of chlorophyll pigments. Production of roots under high levels of nitrogen (2 N) coupled with inefficient reallocation of carbohydrates due to lowered potassium resulted in strong, thick roots that were more fibrous (**Figure 4.1E and F**), but those on lower nitrogen levels appeared more fragile, breaking off easily when plants were extracted from culture tubes (**Figure 4.1G**).

Salvia stenophylla shoot regeneration was inversely proportional to changing potassium concentrations (**Table 4.1**), suggesting that a shortage of this mineral may favour shoot growth (Zhang et al. 1999, Lahti et al. 2005). A similar effect was noted by Benlloch-González et al. (2010) using sunflower plants deprived of potassium. Potassium plays a crucial role in osmoregulation, photosynthesis, enzyme activation, and transport of assimilates in plants and, thus, it is possibly responsible for a biomass production shift. To improve their uptake in low-potassium environments (Li et al. 2006), plants activate more potassium transporters to counter low levels of this mineral. All these factors affect *in-vitro* plantlet regeneration and metabolite synthesis. Interestingly, doubling the potassium in the medium precipitated *in-vitro* flowering (**Figure 4.1H**), and those plantlets that flowered had floral parts that were well developed and typical of wild *S. stenophylla* plants in flower.

Effect of nitrogen and potassium on essential oil components

A metabolomic approach was used in this study to differentiate the effects of changing the micro-environmental medium components. Compounds that had a NIST library match of 85–100 % were regarded as likely hits (**Tables 4.2 and 4.3**).

The true identity of nine chemical compounds was confirmed through the use of standards and the molecular ion peaks, fragmentation patterns, and retention times.

Table 4.2 Volatile secondary metabolite accumulation in response to changes in potassium concentrations in MS media

Compound	Retention time	Kovats index	Relative abundance (%)					
			MS		Potassium			
			0.5	1	1.5	2	2.5	3
α-Pinene	8.74	937	0.23 ± 0.08 ab	-	0.18 ± 0.06 ab	0.18 ± 0.01 ab	0.23 ± 0.15 ab	0.13 ± 0.02 b
Camphene	9.24	947	0.19 ± 0.05 a	-	-	0.10 ± 0.04 a	0.18 ± 0.14 a	0.10 ± 0.03 a
α-Phellandrene	11.34	1000	0.97 ± 0.22a	0.34 ± 0.04 bc	0.20 ± 0.02 b	0.90 ± 0.31 a	0.66 ± 0.18 abc	0.62 ± 0.16 ac
δ-3-Carene	11.52	1009	12.60 ± 0.05 a	-	9.42 ± 1.35 ab	12.18 ± 2.99 a	9.57 ± 2..52 ab	9.51 ± 2.09 ab
D-limonene	12.1	1018	5.12 ± 1.05 a	4.05 ± 1.08 ab	3.62 ± 0.64 ab	5.08 ± 1.49 a	3.63 ± 1.68 ab	3.53 ± 0.86 ab
D-sylvestrene	12.19	987	1.81 ± 0.40 ab	1.36 ± 0.35 ab	1.33 ± 0.30 ab	1.87 ± 0.53 ab	1.31 ± 0.46 ab	1.23 ± 0.30 ab
Terpinolene	14.15	1052	0.97 ± 0.19 bc	0.55 ± 0.13 ab	0.47 ± 0.01 ab	0.92 ± 0.31 bc	0.21 ± 0.11 b	0.68 ± 0.17 abc
Camphor	15.95	1144	0.29 ± 0.06 a	0.38 ± 0.21 ab	0.63 ± 0.22 ab	0.19 ± 0.05 a	0.14 ± 0.09 a	0.19 ± 0.10 a
Borneol	16.67	1156	1.25 ± 0.30 a	1.73 ± 1.38 ab	1.22 ± 0.22 a	1.22 ± 0.32 a	1.39 ± 1.28 ab	1.22 ± 0.28 a
β-Caryophyllene	23.62	1415	2.95 ± 0.22 a	1.39 ± 0.28 a	-	2.84 ± 0.47 a	3.13 ± 0.60 a	1.69 ± 0.34 a
trans-ß-Bergamotene	24.07	1674	2.98 ± 0.23 abc	2.47 ± 0.46 a	2.76 ± 0.46 ab	3.89 ± 0.58 c	3.05 ± 0.71 abc	2.48 ± 0.19 a
α-caryophellene	24.47	1579	0.69 ± 0.17 b	-	1.31 ± 0.38 b	0.65 ± 0.11 a	0.74 ± 0.05 a	0.42 ± 0.08 a
Z-ß-Farnesene	24.62	1438	2.46 ± 0.45 abc	1.51 ± 0.32 ad	1.86 ± 0.26 abd	2.44 ± 0.35 abc	1.72 ± 1.09 abd	1.24 ± 0.25 d
β-Bisabolene	25.87	1483	2.16 ±0.44 a	1.73 ± 0.21 a	2.60 ± 0.09 a	2.28 ± .036 a	1.87 ± 0.56 a	1.62 ± 0.06 a
τ-Muurolene	25.98	1486	0.64 ± 0.12 a	0.70 ± 0.13 a	0.36 ± 0.09 a	0.56 ± 0.06 a	0.24 ± 0.10 bc	.059 ± 0.04 b
Cedrene	26.23	1398	0.60 ± 0.10 abcd	-	0.49 ± 0.63 acd	-	0.41 ± 0.15 a	0.44 ± 0.08 a
α-Patchoulene	26.43	1464	0.21 ± 0.05 a	-	0.25 ± 0.03 a	-	0.30 ± 0.09 a	0.27 ± 0.02 a
α-Muurolene	28.7	1440	0.82 ± 0.17 a	-	0.71 ± 0.10 a	0.55 ± 0.16 a	-	0.83 ± 0.14 a
Bisabolol oxide B	29.02	1707	0.21 ± 0.05	-	0.32 ± 0.04	0.31 ± 0.14	-	0.36 ± 0.14
(-)-α-Bisabolol	29.52	1625	47.58 ± 3.75 ac	51.87 ± 4.57 ab	50.25 ± 7.25 abd	56.98 ± 4.19 ac	55.04 ± 6.25 abd	61.01 ± 3.70 d
cis-Lanceol	30.61	1737	1.48 ± 0.32 ae	1.21 ± 0.24 e	2.48 ± 0.41 abcd	1.60 ± 0.53 abe	1.03 ± 0.39 e	2.51 ± 0.62 abcd
Verticiol	33.29	1290	2.57 ± 1.38 ab	2.04 ± 0.38 ad	2.85 ± 0.49 abc	2.85 ± 1.27 abc	1.35 ± 1.01 d	2.66 ± 0.48 ab
Trachylobane	33.56	1698	0.09 ± 0.01 b	-	-	-	0.25 ± 0.23 ab	0.28 ± 0.03 ab

Different letters in the same row indicate values ()that are statistically different at 95% confidence level. Values were compared using one-way ANOVA

Table 4.3 Volatile secondary metabolite accumulation in response to changes in nitrogen concentrations in MS media

Compound	Retention time	Kovats index	Relative abundance (%)					
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Camphene	9.24	947	0.38 ± 0.03 b	0.24 ± 0.02 ab	0.16 ± 0.02 a	-	0.16 ± 0.04 a	0.35 ± 0.06 b
α-Phellandrene	11.34	1000	0.77 ± 0.02 ac	0.87 ± 0.04 a	0.82 ± 0.12 a	0.81 ± 0.15 a	0.35 ± 0.02 bc	0.61 ± 0.13 abc
δ-3-Carene	11.52	1009	8.33 ±0.34 ab	10.26 ± 0.09 ab	9.90 ± 0.87 ab	10.27 ± 1.56 ab	5.60 ± 0.24 b	10.05 ± 2.56 ab
D-limonene	12.1	1018	3.25 ± 0.16 ab	4.06 ± 0.09 ab	4.04 ± 0.43 ab	3.90 ± 0.77 ab	1.96 ± 0.12 b	3.55 ± 0.97ab
D-sylvestrene	12.19	987	1.19 ± 0.05 ab	1.42 ± 0.05 ab	1.42 ± 0.14 ab	154 ± 0.34 ab	0.85 ± 0.09 a	2.05 ± 0.67 b
Terpinolene	14.15	1052	-	1.07 ± 0.05 c	-	-	-	0.69 ± 0.14 abc
Camphor	15.95	1144	0.62 ±0.06 ab	0.61 ± 0.12 ab	0.44 ± 0.09 ab	0.97 ± 0.65 b	0.20 ± 0.03 a	0.48 ± 0.05ab
Borneol	16.67	1156	3.13 ± 0.44 b	1.24 ± 0.24 a	0.62 ± 0.06 a	0.72 ± 0.28 a	1.24 ± 0.57 a	1.66 ± 0.37 ab
β-Caryophyllene	23.62	1415	10.59 ± 2.05 b	3.15 ± 0.24 a	3.08 ± 0.48 a	1.10 ± 0.14 a	2.03 ± 0.38 a	1.76 ± 0.21 a
trans-ß-Bergamotene	24.07	1674	1.76 ± 0.45 ad	2.71 ± 0.55 ab	3.56 ± 0.51 bc	1.97 ± 0.47 a	2.36 ± 0.27 a	2.82 ± 0.28 ab
α-caryophellene	24.47	1579	2.70 ± 0.50 c	0.82 ± 0.04 a	0.75 ± 0.01 a	-	0.53 ± 0.10 a	0.45 ± 0.06 a
Z-ß-Farnesene	24.62	1438	2.61 ± 0.13 bc	2.42 ± 0.12 abc	2.98 ± 0.37 c	-	2.02 ± 0.38 abcd	2.07 ± 0.12 abcd
β-Bisabolene	25.87	1483	7.54 ± 0.54 b	2.25 ± 0.09 a	2.57 ± 0.17 a	1.77 ± 0.29 a	2.09 ± 0.08 a	2.01 ± 0.13 a
τ-Muurolene	25.98	1486	0.07 ± 0.02 a	0.63 ± 0.06 a	0.65 ± 0.01 a	0.50 ± 0.08 ac	0.24 ± 0.03 bc	0.53 ± 0.06 a
Cedrene	26.23	1398	0.77 ± 0.05 b	0.71 ± 0.04 bcd	0.77 ± 0.08 b	0.49 ± 0.11 ac	0.74 ± 0.14 bd	0.61 ± 0.02 abcd
α-Patchoulene	26.43	1464	0.24 ± 0.00 a	0.28 ± 0.02 a	0.23 ± 0.01 a	0.22 ± 0.03 a	0.25 ± 0.01 a	-
α-Muurolene	28.7	1440	-	0.57 ± 0.04 a	0.56 ± 0.05 a	-	0.92 ± 0.14 a	0.64 ± 0.06 a
Bisabolol oxide B	29.02	1707						
(-)-α-Bisabolol	29.52	1625	40.12 ± 2.78 c	48.08 ± 0.53 ac	48.58 ± 1.91 ac	54.82 ± 3.67 abd	52.01 ± 0.77 bd	61.14 ± 0.10 ab
cis-Lanceol	30.61	1737	2.75 ± 0.43 bcd	2.20 ± 0.12 abcde	2.48 ± 0.29 abce	2.01 ± 0.42 abce	3.37 ± 0.14 d	2.85 ± 0.69 bc
Verticiol	33.29	1290	1.76 ± 0.45 ad	2.71 ± 0.55 ab	4.22 ± 1.21 bc	3.02 ± 0.77 abc	4.87 ± 0.97 c	3.80 ± 0.56 abc
Trachylobane	33.56	1698	0.22 ± 0.06 ab	0.29 ± 0.05 ab	0.45 ± 0.15 ab	0.27 ± 0.08 b	0.45 ± 0.05 a	0.33 ± 0.04 ab

Different letters in the same row indicate values (mean ± se) that are statistically different at a 95% confidence level. Values were compared using one-way ANOVA

These concluded a positive match, providing us with greater confidence in the mass spectral analysis conducted for putative compound identification in this study. Over 100 volatile compounds were detected in propagated plants of *S. stenophylla*, and the profiles of the propagated plants were similar to those occurring in the wild (**Figure 4.2**). These compounds accounted for 85–95 % of the headspace volatiles (**Tables 4.2 and 4.3**). As expected, the major compounds were monoterpenes and sesquiterpenes and overall profiles were similar to those previously reported by Viljoen et al. (2006). Altering supplied levels of both macronutrients had little effect on many secondary volatiles with the exception of (-)-α-bisabolol, which responded positively to an increase in potassium and nitrogen levels (**Tables 4.2 and 4.3; Figure 4.3**). The MS- and MS-grown plants were the least complex in terms of chemical composition (**Figure 4.3**). Furthermore, individual plantlets extracted from these media did not show significant essential oil intraspecific variation upon carrying out the PCA (**Figure 4.4**), implying an almost uniform accumulation of metabolites reflected by 27 major constituents (**Figure 4.3**). All replicates for ½ MS and MS, denoted as A and B in **Figure 4.4**, loaded on the same component. We regarded this as being good and beneficial when considering commercial production because uniformity can be used as a measure of quality.

Overall, changing the composition of potassium and nitrates did not significantly alter the major volatile chemical constituents *produced by S. stenophylla* plants *in-vitro* as few to no effects were detectable regarding the essential oil quality. There was no direct relationship (or statistically valid trend) linking increasing concentrations of these nutrients with elevated accumulation of the major essential oil metabolites, with the exception of a few chemicals (**Table 4.2**). Only a few metabolites responded positively to higher amounts of tested nutrients. For instance, (-)-α-bisabolol (**Figure 4.2**), which makes up the greatest fraction of headspace volatiles irrespective of treatment, was positively influenced by changing concentrations of supplied potassium and nitrogen (**Table 4.2; Figure 4.3**). (-)-α-Bisabolol accumulation showed an interesting trend in response to higher potassium supply, with its relative abundance rising from a mean of 48.58 % (½ MS) to 61.01 % when potassium was increased threefold in the growth medium (3K) (**Table 4.2**). Abundance of the monoterpene D-limonene was reduced when potassium was increased, but varying

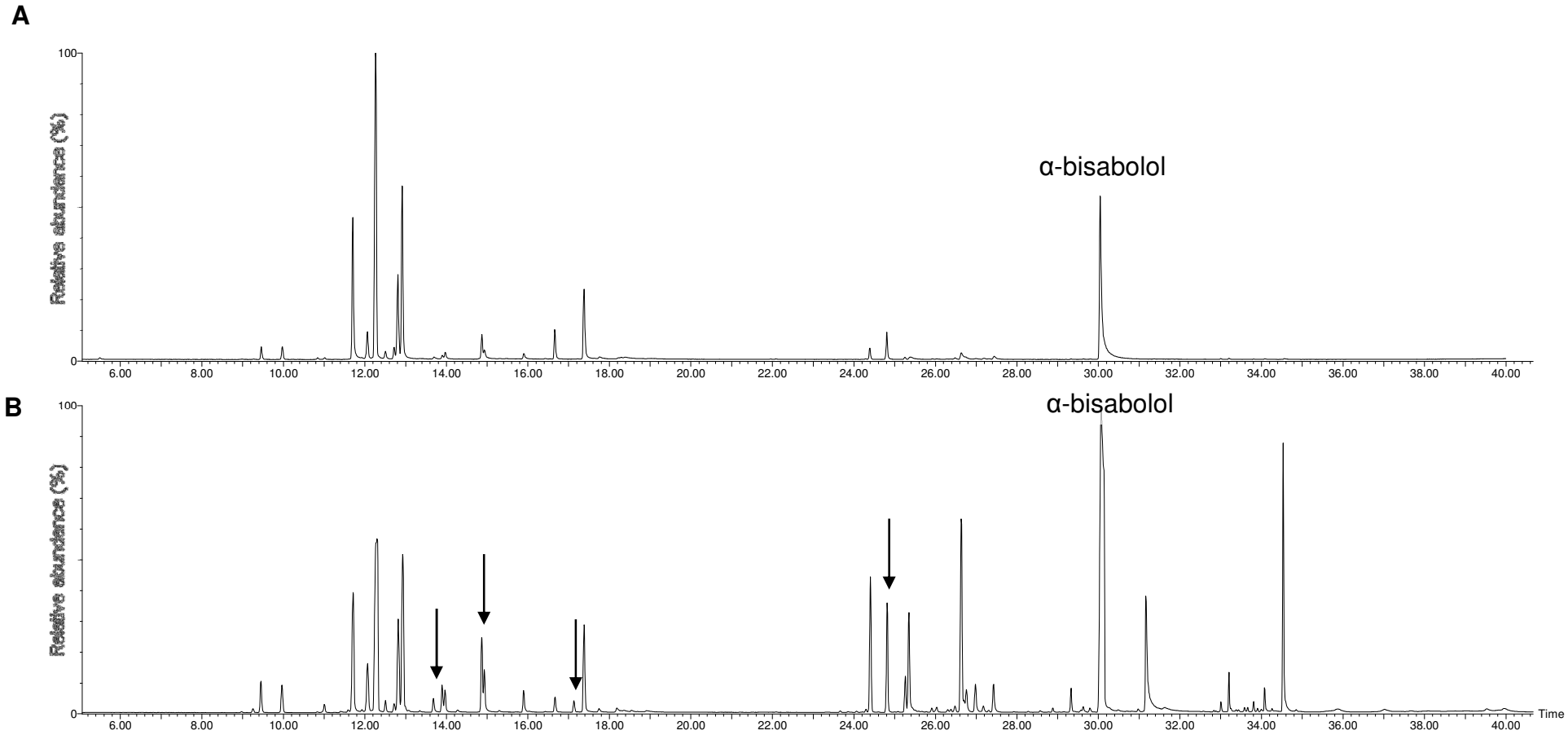

Figure 4.2 HS-SPME-GC-MS analysis of (**A**) wild plants maintained in the garden and (**B**) *in-vitro*-derived microplants of *S. stenophylla*. Overall, metabolite profiles were similar, with tissue-cultured samples at times having higher levels of specific compounds (indicated by arrows) and (-)-a-bisabolol (eluting at 30 min) was one of the major constituents. Arrows indicate peaks that were more prominent in the tissue-cultured material

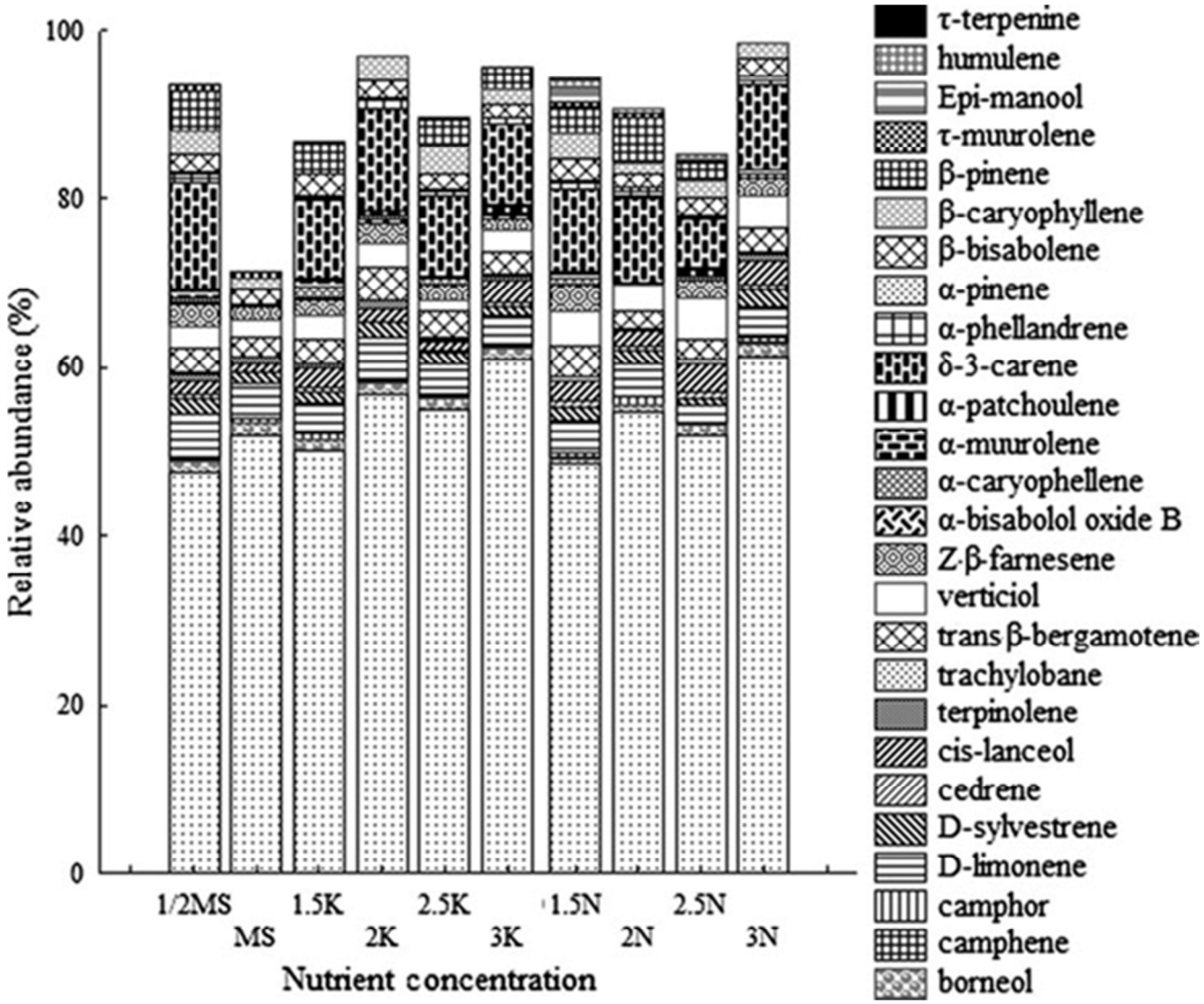

Figure 4.3 Relative abundance (%) of *Salvia stenophylla* metabolites in response to different nutrient concentrations *in-vitro* after a six week growth period on solid agar MS medium. Stacked columns represent major metabolites identified for each treatment using the NIST05 library.

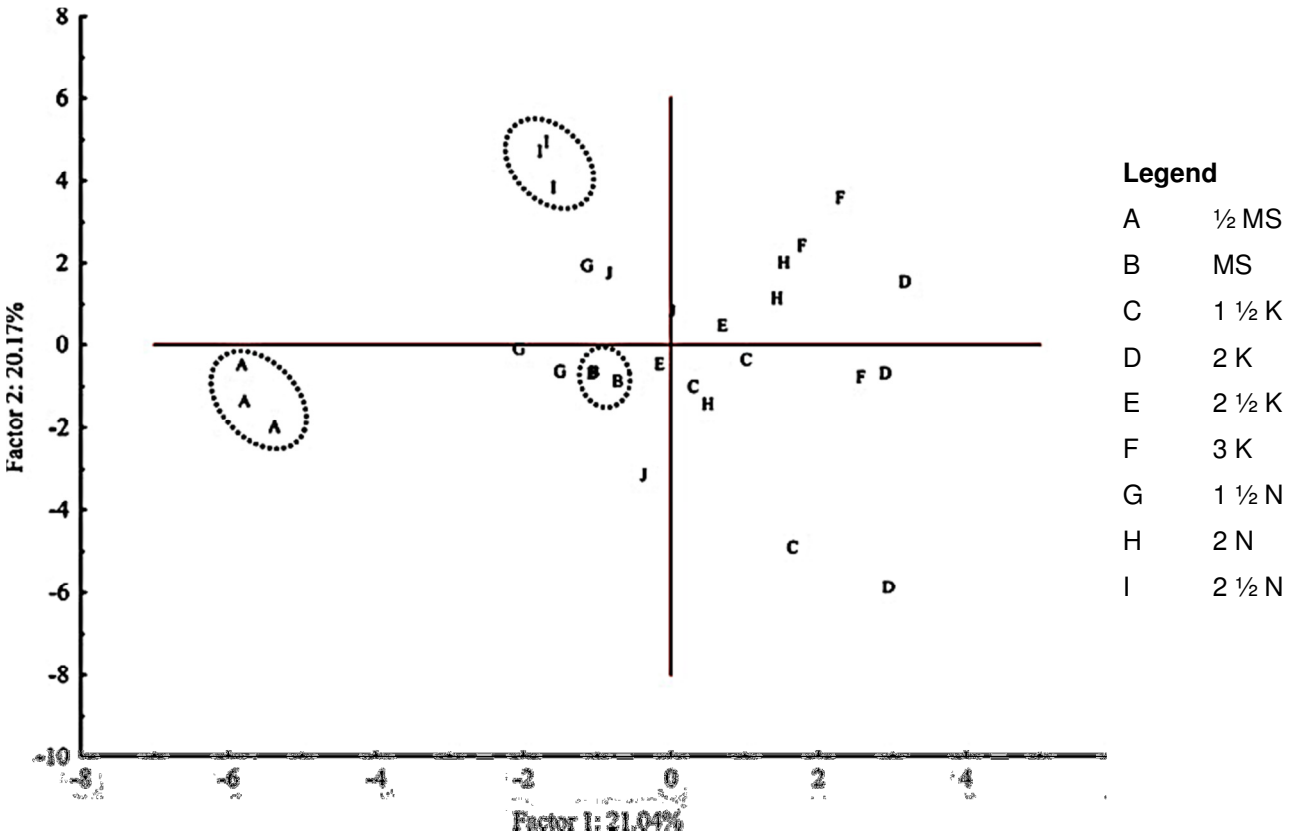

Figure 4.4 A score plot obtained from PCA showing aggregation of HS-SPME-GC-MS profiles based on ½MS, MS, nitrogen and potassium treatments. Factor 1 captured 21.04 % of the variance whilst factor 2 captured 20.17 % of the variance.

the nitrogen provided to microshoots had an insignificant effect on this particular metabolite. However, for those plants exposed to the 2 ½ N medium, a decrease was also noted. Among these, cis-lanceol and verticiol are likely to be some of the metabolites that distinguish this treatment as a separate cluster (**Figure 4.4**). The growth of plantlets on media with different nitrogen or potassium content did not affect the accumulation of a-muurolene (a sesquiterpene). Its relative abundance ranged from 0.56 to 0.92 % and was not significantly different at the 95 % confidence level (**Table 4.3**).

There is no congruent idea on the effect of genetic regulation and/or environmental impact on essential oil biochemistry in plants, so several research groups (Nikolova et al. 1997, Emongor and Chweya 1992, Van den Heever et al. 2008) have monitored the effects of nitrogen and potassium nutrition on the essential oil yield in plants. Low essential oil heterogeneity in response to alterations in nutrient supply may indicate that genetic control of secondary metabolism has a larger role in controlling the synthesis of compounds rather than the environment in *S. stenophylla*, as was also noted by Ma and Gang (2008) in micropropagated *Phlegmariurus squarrosus*. Nikolova et al. (1997) reported that essential oil synthesis in different lines of chamomile was not affected by different combinations of nitrogen, potassium, and phosphorus. Nitrogen is often associated with enhanced vegetative growth and differentiation, which in aromatic plants may possibly result in increased peltate glandular trichome development. Trichomes are vital for oil accumulation and storage, leading to elevated monoterpene production (Yamaura et al. 1989). (-)-α-Bisabolol and bisabolol oxides B and A remained within their normal range. On the other hand, Emongor and Chweya (1992) noted higher (-)-α-bisabolol and chamazulene levels. The increased essential oil yield due to higher nitrogen supplementation led to lowered antifungal activity in *Tulbaghia violacea*, thus implying a decrease in the accumulation of secondary bioactives responsible for pharmacological action (Ma and Gang 2008). In this study we were unable to detect any azulene in the plant samples, even though analysis of the cocktail containing the pure chemical standards showed positive identification of this compound. Differences in the response of plants to minerals suggest that perceived metabolomics changes are species-specific.

Plants that grow in the Dordrecht region in South Africa have the highest (-)-α-bisabolol content of 46 % (Viljoen et al. 2006). The region is characterized by low rainfall, plus *S. stenophylla* and other broad-leafed plants occur along highly nutritious drainage lines. Through *in-vitro* manipulation, we could supersede recorded levels of (-)-α-bisabolol in naturally occurring plants, making our strategy a powerful tool to increase production of this commercial metabolite. Similarly, nitrogen supplementation was correlated to the amount of (-)-α-bisabolol produced *in-vitro*, with superior production recorded at 61.14 % when plants were grown on medium with three times the normal levels of nitrogen (**Table 4.3**).

(-)-α-Bisabolol in wild *S. stenophylla* plants may range from 1.8 to 46.50 % as the primary compound, depending on the locality of the plant population (Viljoen et al. 2006). We therefore cannot emphasize enough the importance of being able to easily produce plants with a superior, more predictable (-)-α-bisabolol content. Such treatments could thus be directly adopted for agricultural production because (-)-α-bisabolol synthesis responds readily to microenvironmental alterations. This is of great benefit to the pharmaceutical and cosmeceutical industries as this chemical product is highly sought after. Although ½ MS was beneficial for microplant production and organogenesis (**Table 4.1**), (-)-α-bisabolol production decreased (**Table 4.2**). Effects of this medium could be clearly discriminated from the other treatments, resulting in both fewer and lower metabolite concentrations. This was confirmed through its distinct grouping upon carrying out the PCA (**Figure 4.4**). Interestingly, Green et al. (2009) recently concluded that potassium is required for the synthesis of a terpene synthase, known as a-farnesene synthase, isolated from apple. This enzyme is paramount in the formation of sesquiterpenes, and its response to potassium is similar to other terpene synthases from unrelated gymnosperms (Degenhardt et al. 2009). Production of (-)-α-bisabolol, a sesquiterpene, is linked to prenyl transferases which increase the amount of available precursors that ultimately lead to greater a-farnesene synthase activity, and subsequent synthesis of the sesquiterpene intermediate, farnesene, is of vital importance in bisabolol production. It is thus possible that potassium functions in a similar fashion in *S. stenophylla*, that is, increasing farnesene production. Some compounds occur as trace elements and are thus not easily detectable via HS-

SPME-GC-MS. Nonetheless, adjustment to the GC-MS method helps with the detection of these minor chemical constituents. In this study, those compounds that generally accumulated at a proportion of 2 % or less were regarded as trace chemicals and modifications to them were also obvious (**Tables 4.2 and 4.3**) with changing available nutrients. Some compounds (bisabolol B oxide and terpinolene) were not always quantifiable in nitrogen-treated samples (**Table 4.3**), just as trachylobane was not readily detected in potassium treatments (**Table 4.2**), accumulating at undetectable levels which suggests production at extremely low to negligible concentrations.

4.3.2 Effect of osmotica on plantlet growth

Inclusion of osmotic agents in the growth medium adversely influenced morphogenetic responses, and plantlets treated with 5 % (w/v) PEG were unable to maintain normal *in-vitro* organogenesis (leaf mass = 0.07 g) during the culture period. Instead, the frequency of callus accumulation became higher (18 %) (**Table 4.4**). Intense callugenesis (**Figure 4.1l**), which is undesirable as it increases the incidence of somaclonal variation, lowers the capacity for *in-vitro* microplants to produce volatile compounds. Dedifferentiation negatively affects the development of glandular trichomes which are crucial as essential oil storage organs (Serrato-Valenti et al. 1997, Rout et al. 2000, Arikat et al. 2004, Kamatou et al. 2006). Plantlets subjected to the dehydrating treatments became chlorotic compared to controls. Generally, dehydration influences osmotic potential, lowering the capacity for water uptake and absorption of macro- and micronutrients from the surroundings.

Table 4.4 Effect of sorbitol and PEG as osmotica on *in-vitro* growth and development of *S. stenophylla* plantlets

Treatment	No. of new leaves[a]	Leaf weight (g)	Callus weight (g)	Total biomass	Root formation
Sorbitol	4.70 ± 0.54 a	0.08 a	00.18 ± 0.03 a	0.25 a	0.00 ± 0.00 a
PEG	6.00 ± 0.71 a	0.07 a	00.18 ± 0.03 a	0.24 a	0.00 ± 0.00 a
Control	4.22 ± 1.22 a	0.05 a	0.34 ± 0.14 a	0.39 a	0.00 ± 0.00 a

Means within columns noted with different letters are significantly different according to Kruskal–Wallis ANOVA at P ≤ 0.05. ([a]) The amount of new leaves was calculated as number of leaves on day 30—number of leaves on day 0 associated

In turn, one of the effects of water deprivation is reduced plantlet growth and photosynthetic ability (Pasternak 1987). Apart from this, the probability for ion toxicity in plants becomes elevated (Levitt 1980) and this may lead to many genetic and biochemical changes related to the stress responses (Cassells and Curry 2001). The negative impacts of water stress are known to be complicated and plants in tissue culture respond to stress in many ways. Among other effects (such as lowered nutrient translocation across membranes and enzyme malfunction that may lead to poor functioning of biochemical pathways), the incidence of callugenesis and somaclonal variation increases (Cassells and Curry 2001), leading to unpredictable and suboptimal plant growth and vigor.

4.3.3 Effect of osmotica on essential oil components

Differences in the quantity of headspace volatiles were evident from one culture cycle to another. For instance, control plants used in the nutrient experiment had more (-)-α-bisabolol (48.08–51.87 %) compared with those assayed to study the effects of water stress (19.95 %). Age differences might explain this increase in (-)-α-bisabolol content, which was more than twofold. Even so, within a population of plants utilized for experimentation, little to no intraspecific clonal differences were detectable with our analytical method. (-)-α-Bisabolol was used as an internal marker (standard) and the injection ratio was adjusted accordingly for each experiment. Emission of (-)-α-bisabolol (27.5 %), as a headspace volatile in PEG-supplemented medium, was more pronounced (**Table 4.5**) in comparison to controls and sorbitol-exposed microplants. Several publications provide supporting data. Positive upregulation of essential oil components produced by *S. officinalis* due to water deprivation (Bettaieb et al. 2009) and salt stress (Taarit et al. 2010) has been shown. In the latter situation, where NaCl was used to dehydrate plants, quantitative chemical differences were evident (Cassells and Curry 2001). Surprisingly, sorbitol treatments positively influenced production of α-guaiene, α-copaene, a-caryophyllene, muurolene, d-guaiene, and guaiol, eluting between 28.6 and 30.1 min; these were either in trace quantities or undetectable in control and wild aerial parts (**Table 4.6**). (-)-α-Bisabolol andazulene,1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)-,1S-

(1a`,4a`,7a`) (also known as α-guaiene) are commercially important in the cosmeceutical industry due to their anti-inflammatory and anti-irritant properties.

Table 4.5 Effect of phytohormone combinations (auxins and cytokinins) on major essential oil compounds accumulation in *in-vitro Salvia stenophylla* plants analyzed using HS-SPME-GC-MS

Major compounds	Retention time (min)	Kovats index	Tissue culture	Wild type	2.7:4.4 BA:NAA(µM)	2.9:4.4 BA:NAA(µM)	IAA:BA 2.9:8.9 (µM)	IAA:BA 1.1:8.9 (µM)	IAA:BA 2.9:4.4 (µM)
*α-Pinene	9.46	939	1.072	1.48	1.42	1.234	-	-	0.973
*Camphene	9.97	947	-	-	1.124	-	-	-	-
β-Pinene	11.80	965	3.709	8.04	4.727	4.572	3.364	3.592	4.081
Myrcene	12.14	958	1.504	3.68	1.425	2.686	1.458	1.738	1.992
*δ-3-Carene	12.38	1009	8.330	14.04	7.159	10.617	7.244	8.399	9.352
Sylvestrene	12.89	987	3.462	5.59	3.771	4.643	3.189	3.677	3.815
*D-Limonene	13.03	1018	4.458	6.54	4.214	5.328	4.072	4.604	5.053
γ- Terpinene	13.91	998	-	1.06	-	-	-	-	-
Isoterpinolene	14.89	1023	1.914	2.74	1.735	2.509	2.001	2.236	2.448
Terpinolene	14.95	1052	1.028	1.13	1.021	1.272	1.192	4.605	1.444
α-Terpinene	15.88	998	-	-	-	-	-	1.001	-
Borneol	17.40	1156	-	-	1.752	1.170	-	1.388	1.159
α-Terpineol	18.16	1143	-	2.06	-	-	1.184	1.086	-
β-Caryophyllene	24.41	1415	9.310	-	6.499	3.598	5.839	4.598	2.137

Major compounds	**Retention time (min)**	**Kovats index**	**Tissue culture**	**Wild type**	**2.7:4.4 BA:NAA(µM)**	**2.9:4.4 BA:NAA(µM)**	**IAA:BA 2.9:8.9 (µM)**	**IAA:BA 1.1:8.9 (µM)**	**IAA:BA 2.9:4.4 (µM)**
trans β-Bergamotene	24.82	1674	3.365	3.51	3.958	3.286	1.601	3.425	3.914
Aromaden drene	24.89	1386	-	-	-	-	2.546	-	-
α-Caryophellene	25.25	1579	-	-	2.016	1.009	-	1.578	-
Z-β-Farnesene	24.34	1438	2.087	2.50	2.772	2.469	1.058	3.532	2.971
Varidiflorene	26.30	1419	-	-	-	-	1.356	-	-
β-Bisabolene	26.63	1483	4.662	2.75	4.192	3.336	4.101	6.789	2.863
β-Sesquiphellandrene	26.96	1446	-	-	1.301	-	-	1.216	1.119
α-Guaiene	28.54	1490	-	-	-	-	9.368	-	-
δ-Guaiene	28.54	1490	-	-	-	-	4.262	-	-
Guaiol	29.64	1614	-	-	1.090	-	1.923	-	-
(-)-α-Bisabolol (levomenol)	30.10	1625	18.457	21.07	19.949	20.477	11.646	19.127	21.599
cis-Lanceol	31.15	1737	5.116	2.42	3.996	4.636	3.692	5.499	4.559
Epi-manool	33.19	2085	1.166		-	1.336	-	1.078	1.645
Trachylobane	34.06	1698	-	-	-	-	-	-	1.073
Manool	34052	2016	5.194	2.87	4.488	5.451	3.885	4.848	6.328

Table 4.6 Effect of PEG (5 %, w/v) and sorbitol (2 %, w/v) on major essential oil compounds of *in-vitro Salvia stenophylla* plants analyzed using HS-SPME-GC-MS

Major compounds	Retention time (min)	Kovats index	Tissue culture	PEG 5 % (w/v)	Sorbitol 2 % (w/v)
α-Pinene	9.46	937	1.07	-	-
β-Pinene	11.80	965	3.71	4.43	3.43
Myrcene	12.14	958	1.50	-	-
δ-3-Carene	12.38	1009	8.33	9.03	6.79
Sylvestrene	12.89	987	3.46	2.51	1.59
D-limonene	13.03	1018	4.46	4.40	3.38
Isoterpinolene	14.89	1023	1.91	-	-
Terpinolene	14.95	1052	1.03	-	-
Camphor	16.65	1144	-	1.04	-
β-Caryophyllene	24.41	1415	9.31	7.10	7.33
trans-β-Bergamotene	24.82	1674	3.37	2.81	1.31
α-Caryophyllene	25.25	1579	-		
Z-β-farnesene	25.34	1438	2.09	1.74	1.08
β-Bisabolene	26.63	1483	4.66	4.15	3.34
cis-α-Bisabolene	28.54	1518	1.04	-	-
α-Guaiene	29.30	1490	-	1.37	11.61
α-Copaene	28.80	1221	-	-	1.13
δ-Guaiene	29.54	1490	-	-	6.11
Guaiol	29.64	1614	-	-	1.57
(-)-α-Bisabolol	30.10	1625	18.46	27.50	1559
cis-Lanceol	31.15	1737	5.12	3.55	3.95
Epi-manool	33.29	2085	1.17	-	-
Trachylobane	34.06	1698	-	1.02	-
Anthracene	34.23	1782	-	1.04	-
Manool	34.52	2016	5.19	4.63	4.60

When plants of *S. stenophylla* were subjected to water stress, a-guaiene became favored over (-)-α-bisabolol as it accumulated at higher levels than previously detected (**Table 4.5**). Although not statistically significant, water stress was detrimental to (-)-α-bisabolol accumulation. Both (-)-α-bisabolol and a-guaiene are sesquiterpenes which are synthesized via terpenoid metabolism (Ganzera et al. 2006). Similar to (-)-α-bisabolol, which is used in the production of numerous products such as eye drops, body creams, sunscreens, and after-sun creams (Brown and Dattner 1998), guaiene derivatives are also useful as additives to skincare products. Furthermore, a-guaiene and related compounds are potent antiseptics.

4.3.4 Effect of plant growth regulator combinations

The ratio of PGRs is important as small changes in the concentrations of phytohormones have the ability to greatly affect *in-vitro* plantlet growth and development by affecting the multiplication, elongation, and rooting of plantlets (Emongor and Chweya 1992, Van den Heever and 614 others 2008). Extensive discussion on the effect of PGRs on *in-vitro* performance of *S. stenophylla* cultures has been conducted elsewhere (Musarurwa et al. 2010). We focused on analyzing the metabolomic profiles of propagated plants and their responses to different PGR combinations. Plants grown on the 2.9:4.4 BA: IAA medium had harder and more serrated leaves, which were often chlorotic, hardly elongating (data not shown). Plants grown on the 2.9:4.4 IAA: BA medium produced (-)-a-bisabolol at similar levels to wild plants. Although explant elongation was minimal, with this PGR combination callus production could be circumvented. However, callus regeneration was more abundant on the 2.7:4.4 NAA: BA medium. Plants also failed to root. a-Guaiene (and other guaiene derivatives) plus a-caryophyllene became more prominent in plants maintained on IAA: BA (2.9:8.9 µM) (**Table 5**). Otherwise, chemical profiles were generally similar in propagated tissues irrespective of PGR treatment. Interestingly, *S. stenophylla* plants maintained their ability to flower *in-vitro*, especially with a higher potassium supply. Flowers *in-vitro* appeared to have fully formed floral parts that were morphologically similar to those found in nature (**Figure 4.1l**). *In-vitro* flowering occurred at the same time as *ex-vitro* flowering in the greenhouse. Naturally, these plants flower from spring until the end of Southern Hemisphere summer (Jequier et al. 1980). Micropropagated

plants (**Figure 4.1J**) were similar in their morphology to traditionally propagated *S. stenophylla* plants and there were no distinguishing metabolomic features derived from tissue culturing (**Figure 4.2**), demonstrating that this propagation method can easily be adapted for massive agricultural multiplication of this commercially important medicinal crop. However, all tissue-cultured and glasshouse-acclimatized plants showed a slight difference in secondary compounds from the wild-growing plants, possibly due to different growth environments (**Table 4.5**). In conclusion, micropropagation of *S. stenophylla* is not only an efficient system of producing plant material for agricultural cultivation and industrial essential oil processing, ensuring the production of quality essential oils, it is also a unique platform to optimize growing conditions and for studying metabolism within a highly controlled microenvironment. Such practices may easily be adopted for field production of *S. stenophylla* plants. Further greenhouse and field trials are required to maximize ex- vitro performance of these plants.

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Chapter Five

Over-expression of the geranyl diphosphate synthase gene (*AgGPPS2*) in *Salvia stenophylla* via *Agrobacterium*-mediated transformation[3]

5.1 Introduction

Plant transgenic modification offers possibilities to change or redirect biosynthesis of pharmaceutical compounds and is viewed as an appropriate response bioeconomy demands in modern society. Medicinal plant biotechnology focuses on increasing the synthesis of pharmaceutical compounds. Gene over-expression is often used in metabolite engineering for improving the synthesis of plant biochemical pathways. This is achieved by either increasing the production potential of the plant systems or the storage capacity of the organs responsible for amassing compounds of interest by elevating the frequency of the regulatory genes (Chen et al. 2010). Engineering can either be done in the native host or in a different heterologous host. Native host engineering focuses mainly on raising the production levels of target compounds whilst on the other hand, heterologous host modification can also be done in hosts with undeveloped or no pathway for that metabolite (Withers and Keasling 2007).

Isoprenoids are the largest family of secondary compounds and all synthesized from sequential condensation of isoprene units. They play an important ecological role as they are involved in plant defense as toxins, repellents or attractants for natural enemies of potential predators such as herbivores (Kappers et al. 2005) or for allelopathic interactions and pollination (Bouvier et al. 2000). Biosynthesis of isoprenoids has been extensively reviewed by a number of authors (Newman and

[3] Chapters 5 and 6 are going to be published as a paper with the following title "Effects of over-expressing the AgGPPS2 gene in *Salvia stenophylla* on terpenoid biosynthesis". The paper will be submitted to Metabolic Engineering. The manuscript is currently in preparation.

Chappell 1999, Dubey et al. 2003, Cheng et al. 2007, Withers and Keasling 2007, Kirby and Keasling 2009) and was also summarized in **Chapter 2**.

Isoprenoid synthesis pathway has been considerably engineered to increase output of pharmaceutically essential terpenes. Lange and Croteau (1999) identified the gene coding for 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) and over-expressed it in mint, *Mentha x piperita.* Mahmoud and Croteau (2001) reported that the up-regulation of DXR gene expression resulted in a 50 % increase in essential oils in peppermint. The pacific yew, *Taxus brevifolia,* produces the diterpene taxol, used in cancer chemotherapy. Studies on *T. brevifolia* extracts showed significant variation in concentration between and within populations (Wheeler et al. 1992) with an average content of 130 kg per 1000 tonnes of bark (Liu and Khosla 2010). Engineering of the upstream reactions in the terpene biosynthetic pathway resulted in increased production of the first committed precursor, taxadiene, in taxol synthesis in *Escherichia coli* (Ajikumar et al. 2010). Similar experiments were carried out in yeast (DeJong et al. 2005) and *Arabidopsis* (Besumbes et al. 2004). These studies were all able to increase taxol production by the heterologous biosynthesis of precursor molecules. The sesquiterpene lactone artemisinin is naturally manufactured by *Artemisia annua* and is essential in the treatment of malaria. Committed precursors for lactone artemisinin biosynthesis have been synthesized by bacteria and yeast, thus providing raw materials for its synthesis (Withers and Keasling 2007).

Volatile chemicals in *S. stenophylla* are largely monoterpenes and sequiterpenes and geranyl diphosphate (GPP) is a crucial substrate in their synthesis (see **section 2.6**). An increase in the GPP synthase was postulated as being likely to lead to increased accumulation of GPP, which in turn would result in the elevation of terpenoid production since the enzyme activity is dependent on substrate availability. With this understanding, this study was set up to assess the effects of over-expression of the *GPPS* gene isolated from *Abies grandis* (*AgGPPS2*) (Burke and Croteau 2002a) on the yield and composition of essential oils produced by *S. stenophylla*. It was hypothesized that increasing the GPP accumulation in the cell cytoplasm would raise precursor availability for essential oil metabolite synthesis as it would enhance production of secondary compounds.

5.2 Materials and methods

5.2.1 Plant material

Salvia stenophylla seeds were germinated and seedlings were tissue cultured on Murashige and Skoog (1962) (MS) medium with six-week sub-culturing intervals (described earlier in **Chapter 3**)**.** Briefly, seeds were decontaminated by soaking them in 3.5 % (w/v) sodium hypochloride for 20 min. They were then rinsed three times with distilled water prior to acid scarification with 70 % sulphuric acid for 5 min. Seeds were again washed thoroughly with sterile distilled water and germinated on 1/10 MS medium solidified with 1 % agar (Merck, Germany). Some plantlets were transferred to MS medium supplemented with 2.7 µM NAA and 4.4 µM BA. For the first transformation attempt, hypocotyls were used for the transformation and three to four-week[4] old micro-plants were used.

5.2.2 Preparation of competent cells

Escherichia coli cells (DH5α) were prepared following the protocol described by Sambrook and Russell (2002) with minor modifications. Cells were plated out from the -80 ℃ freezer stock and were grown overnight on solid Luria-Bertani (LB) medium (5 g L^{-1} yeast extract; 10 g L^{-1} tryptone; 10 g L^{-1} NaCl; 15 g L^{-1} agar) at 37 ℃. Single colonies were picked and incubated overnight in sterile 50 mL polypropylene tubes with liquid LB medium with continuous shaking at 37 ℃. One milliliter of the overnight culture was transferred to fresh LB medium and was incubated for 3 h with shaking until the optical density (at 600 nm wavelength, OD_{600}) was between 0.3-0.4. Cell cultures were chilled on ice for 15 min prior to spinning them down at 5000 rotation per minute (rpm) for 10 min at 4 ℃. The supernatant was discarded and the pellet was resuspended in 30 mL of ice cold 0.1 M $CaCl_2$. Cells were incubated on ice for 30 min and were recovered by centrifugation as described above and were resuspended in 2

[4] The age of micropropagated plants was determined using the day of subculturing as day 0, thus making four-week old plantlets 28 days old.

mL of ice-cold 0.1 M $CaCl_2$. An equal volume of 15 % (w/v) glycerol solution was added and aliquots of 0.5 mL were placed in 1.5 mL micro-tubes prior to cells being frozen in liquid nitrogen. Tubes were stored at -80℃ until use. Eletrocompetent cells were prepared from overnight DH5α colonies. Single colonies were inoculated into 13 mL of LB broth and incubated at 37 ℃ for 3-4 h or until the OD_{600} reached 0.5. Cells were harvested by centrifuging the suspension for 10 min at 4 ℃ at 5000 rpm. The pellet was washed two times by adding 10 mL of ice-cold dH_2O. The cells were vortexed each time before being collected by centrifugation (15 min at 4 ℃ at 3000 rpm). The supernatant was poured off and the cells were resuspended in ice-cold 10 % (w/v) glycerol. The cells were frozen by immersing them in liquid nitrogen prior to being stored at -80 ℃ until use.

5.2.3 Plasmid construction and *Agrobacterium* transformation

The geranyl diphosphate synthase gene (*AgGPPS2*) from the grand fir, *Abies grandis,* was kindly donated by Prof. Rodney Croteau from the Washington State University. The *AgGPPS2* gene was harbored in the pBluescript SK- plasmid within the *Eco*RI and *Xho*I sites and was 1524 bp in size. The gene was excised from pBluescript SK- through a double restriction digestion reaction using *Eco*RI and *Xho*I restriction enzymes. The digestion was conducted at 37 ℃ overnightovernight in a 20 µL reaction with the following components: 0.2 µL (2 units) *Xho*I; 0.2 µL (2 units) *Eco*RI; 4 µL (2 X) of 10 X Tango™ buffer; 5 µL (100 ng) pBluescript (SK-)-*AgGPPS2* plasmid and 10.6 µL dH_2O. Unless otherwise stated, all restriction enzymes were supplied by Fermentas Inc (Maryland, USA). The digested DNA was then separated using gel electrophoresis on a 1 % (w/v) agarose-Tris-borate-EDTA (TBE) gel with 4 µL (10 mg mL^{-1}) ethidium bromide (EtBr) per 1 g of agarose gel (Fermentas) in 100 mL TBE buffer as the stain to visualize the DNA. The samples were run at a voltage of 80 V for 45-60 min using the PowerPac™ Basic (Bio-Rad Laboratories Inc.) with 0.5 X TBE as the running buffer. The DNA segment that migrated to the 1524 bp position was taken to be *AgGPPS2.* The DNA was extracted from the gel using the QIAquick® Gel Extraction Kit (Qiagen Inc.) following the manufacturer's protocol.

Briefly, the DNA fragment was excised from the gel under UV-light illumination and was weighed in a clean 2 mL micro-centrifuge tube and three volumes (w/v) of Buffer QG were added to the gel. To dissolve the gel completely, the tube was incubated at 50 ℃ for 10 min with vortexing at three minute intervals. One gel volume of isopropanol was added to the mixture and it was added to a QIAquick® column in a 2 mL micro-tube and was centrifuged for 1 min at 13 000 rpm. The flow through was discarded and the column was placed back into the same tube. A volume of 750 µL of Buffer PE was added to the QIAquick® spin column to wash and was centrifuged for 60 s at 13 000 rpm. The flow through was also disposed off and the tube was spun for a further minute to remove residual wash buffer. The elution of DNA from the QIAquick® column was done by adding 50 µL of Buffer EB. It was allowed to stand for 60 s and then the DNA was collected by centrifuging for 1 min at 13 000 rpm.

The pBluescript SK- was also used to transform competent DH5α cells using the heat shock method. The DH5α cells were thawed on ice for 10 min. Two microliters of pBluescript SK- were added to 50 µL of competent DH5α and mixed gently prior to incubation on ice for 30 min. Cells were then placed in a water bath at 42 ℃ for 45 s and 450 µL of liquid LB was added to the cells immediately thereafter. Cells were incubated, with vigorous shaking, at 37 ℃ for 1 h. Aseptically, 50 and 100 µL of DH5α cells were plated out on LB plate with 100 mg L^{-1} ampicillin. The remaining cells were centrifuged for 60 s and 450 µL of the supernatant was pipetted out and the pellet was resuspended in the remaining 50 µL. This was also plated out onto the LB plates with 100 mg L^{-1} ampicillin. A number of single colonies were inoculated into fresh LB medium with ampicillin (100 mg L^{-1}) and were incubated for 6 h prior to plasmid isolation.

5.2.3.1 Plasmid Isolation – alkaline lysis

Positive colonies growing in liquid LB medium with 100 mg L^{-1} ampicillin were grown overnight in preparation for plasmid isolation which was done using the alkaline lysis method as described by Sambrook and Russell (2002) with a few changes. Two milliliters of the overnight cultures were centrifuged and the pellet was resuspended in 200 µL Lysis Buffer I [50 mM glucose; 25 mM Tris-HCl [pH 8.0]; 10 mM EDTA [pH 8.0)].

Thereafter 200 µL of Lysis Buffer II (0.2 N NaOH; 1 % (w/v) SDS) was added and mixed gently by inverting the tubes repeatedly for 20 s. Cells were placed on ice for 2 min before 200 µL of the ice cold Lysis Buffer III (per 100 mL of buffer, 60 mL of 5 M potassium acetate; 11.5 mL of glacial acetic acid and 28.5 mL of dH_2O) was added and the bacterial lysate was returned to ice for another 3 min. The tubes were then centrifuged for 5 min at 13 000 rpm at 4 ℃. The supernatant was transferred to fresh tube and an equal volume of chloroform: phenol [1:1 (v/v)] was added. Tubes were vortexed for 30 s and this was followed by centrifugation at 13 000 rpm at 4 ℃ for 5 min. The upper aqueous phase was transferred into a clean eppendorf tube. The DNA was then precipitated by adding two volumes of absolute ethanol. The contents were mixed by vortexing and reaction was allowed to stand at room temperature for 2 min before centrifuging for 5 min at 13 000 rpm at 4℃ to recover the DNA. The supernatant was discarded and the tubes were air-dried for at least 60 min after which pellet was resuspended in TE buffer [100 mM Tris; 100 mM EDTA (pH 8.0)]. Two microliters (about 200-250 ng) of the DNA were used for PCR using full length *AgGPPS2* primers described in **Table 5.1**. The following PCR reaction conditions were used; initial denaturation at 95 ℃ for 3 min followed by 30 cycles of amplification (denaturation, 95 ℃ for 30 s; annealing, 56 ℃ for 30 s; elongation, 72 ℃ for 1 min 30 s) and 7 min final elongation at 72 ℃. This was done to ascertain the presence and identity of the *AgGPPS2* in the isolated pBluescript SK- plasmid.

The *AgGPPS2* PCR product was then ligated to a pGEM®-T Easy vector for sub-cloning and sequencing following the manufacturer's instructions. The reaction is indicated in **Table 5.2**. The reaction was mixed by pipetting and was incubated at room temperature for 1 h. The ligation product was then used to transform DH5α cells. Uncut pGEM®-T Easy vector was cloned in DH5α cells was used as a negative control whilst PCR product used for the ligation was used as a template for the positive control.

Table 5.1 Primers and annealing temperatures used for PCR reactions in *AgGPPS2* amplification. *Kpn*I and *Sal*I primers amplified the full length of the gene (1524 bp) whilst the truncated primers amplified the middle (1100 bp) of the 1524 bp gene

Primers	Sequence	Size of fragment amplified (bp)	Annealing temperature (ºC)
Full length			
*Kpn*I-forward	5′-GTACCGATTTTCCCT GCTACAAAAATCTA-3′	1524	56
*Sal*I-reverse	5′-GTCGACTGAGTATT GCTCCCTTGAATCAC-3′		
Truncated			
Forward	5′-CCACTTCGTTATC CCAGAA-3′	1100	58
Reverse	5′-AGTGAAGGCAGC ACCTCTGTT-3′		

Table 5.2 The ligation reaction joining *AgGPPS2* to the pGEM®-T cloning vector. The transgene was isolated from the gel extraction experiment described in section **5.2.3**

Reaction component	Volume (uL)	Final concentration
pGEM®-T Easy Vector	1	2.5 ng μL^{-1}
T4 DNA ligase	1	0.25 u μL^{-1}
PCR product (*AgGPPS2* segment)	3	0.3 ng μL^{-1}
2 X rapid ligation buffer	10	-
Nuclease free water	5	-

5.2.3.2 *Agrobacterium* transformation

The pGEM-T-*AgGPPS2* construct was transformed into *E. coli* DH5α using the heat-shock method as detailed by Sambrook and Russell (2002) with minor changes as described earlier or through electroporation using the BioRad Gene Pulser® following a preset program. Transformation was confirmed by carrying out colony PCRs of the putative transformed colonies growing on ampicillin (100 mg L^{-1}) selection medium as described in section **5.3.3.1**. Putative transformants were grown overnight in LB medium with 100 mg L^{-1} ampicillin prior to plasmid isolation using the Zyppy™ Plasmid Miniprep Kit. A *Kpn*I and *Sal*I double digestion in 20 µL reaction (0.4 µL (4 u) *Kpn*I; 0.4 µL (4 u) *Sal*I; 2 µL of 10 X *Bam*HI buffer; 15 µL plasmid DNA (2 ng); 2.2 µL dH_2O) was carried out at 37 ºC for 5 h to excise the *AgGPPS2* segment which was then ligated onto the pCAMBIA1301 plasmid with cauliflower mosaic virus 35S (*CaMV35S*) promoter in another 20 µL reaction (1 µL (2 ng) pCAMBIA1301; 3 µL (0.3 ng) *AgGPPS2*; 5 µL T_4 ligase buffer; 1 µL (0.25 u) T_4 ligase; 11 µL dH_2O). The reaction was incubated for 2 h at room temperature (22 ºC) and the ligation product (pCAMBIA1301-*AgGPPS2*) was electroporated into *E. coli* DH5α to generate multiple copies of the expression vector. Cells were plated out on kanamycin (100 mg L^{-1}) selection medium and transformation was confirmed by carrying out a colony PCR using the full length primers (**Table 5.2)**. A this stage the PCR product was sequence using these same primers and the sequence wasconfirmed using the nucleotide basic local alignment search tool (BLASTn) developed by Altschul et al. (1990). The pCAMBIA1301-*AgGPPS2* construct was isolated using the protocol described in section **5.2.3.1** and also using the Zyppy™ Plasmid Miniprep Kit and was transformed into *Agrobacterium tumefaciens* strain (EHA105) using the freeze thaw method (An et al. 1988). Two milliliters of the overnight EHA105 cells (OD_{600} between 0.5 and 1.0) were chilled on ice for 10 min and centrifuged at 5000 rpm for 5 min at 4 ºC to harvest the cells. The supernatant was discarded and cells were resuspended in 1 mL of ice cold 20 mM $CaCl_2$. Aliquots of 100 µL were dispensed into 1.5 mL micro-tubes. One microgram of pCAMBIA1301-*AgGPPS2* was added to each tube and the cells were frozen by immersing tubes in liquid nitrogen for 30 s prior to 5 min incubation at 37 ºC in a water bath. One milliliter of yeast-mannitol broth (YMB) (1 g L^{-1} yeast extract; 10 g L^{-1}

mannitol; 0.5 g L^{-1} di-potassium sulphate; 0.2 g L^{-1} magnesium sulphate, 1 g L^{-1} sodium chloride; 1 g L^{-1} calcium carbonate) was immediately added followed by 4 h incubation at 28 ºC with vigorous shaking. Cells were centrifuged for 30 s using the Hettich Mikro 120 (Hettich Instrument, Beverly, Massachusetts) bench top microcentrifuge. Selection was done on YMB medium supplemented with rifampicin (150 mg mL^{-1}), streptomycin (100 mg mL^{-1}) and kanamycin (100 mg mL^{-1}). Putative transformants were subjected to colony PCR using the conditions and reaction described in section **5.2.3.1**.

5.2.4 *Salvia stenophylla* transformation

Sterile *S. stenophylla* microplants were established and maintained as described in **Chapter 3**. Four to six-week old plants were used for the transformation procedure. To introduce the *AgGPPS2* gene into *S. stenophylla, A. tumefaciens* (EHA105) carrying the plant vector pCAMBIA1301-*AgGPPS2* was used. The transformed EHA105 colonies were cultured in YMB medium and incubated at 28 ℃ for 2 to 3 days in the dark. Sterile explants of about 1 cm x 1 cm were immersed in transgenic bacterial solution for 30 min. Explants were then blotted dry on decontaminated paper towels and were incubated on solid MS (Murashige and Skoog 1962) medium [30 g L^{-1} sucrose, 0.1 g L^{-1} myo-inositol and 10 g L^{-1} agar, (pH 5.8)] at 23 ℃ ± 2 ℃ in the dark for 2 days.

After the co-cultivation period, explants were washed three times with sterile water and blotted dry on sterile paper towels prior to placing them on MS medium with 100 mg L^{-1} kanamycin and 500 mg L^{-1} cefotaxime to select for putative transformants and to hinder bacterial growth, respectively. Surviving explants were transferred onto fresh MS medium with the same concentration of cefotaxime and kanamycin at four-week intervals. On the second transfer, plant growth regulators were added at a concentration of 2.7 µM NAA and 4.4 µM to the MS medium to facilitate fast shoot growth and multiplication (Musarurwa et al. 2010). The plants remained on antibiotics for about 20 weeks after which no residual bacteria grew when plantlets were sub-cultured on antibiotic free medium.

5.2.4.1 Nucleic acid extraction and PCR analysis

To verify transfer of *AgGPPS2* to the plant genome, a PCR was carried out on the genomic DNA of the putative transformants and non-transgenic plants were used as negative controls.

5.2.4.2 DNA isolation

Firstly, genomic plant DNA isolation was carried out using a standard cetyl trimethyl ammonium bromide (CTAB) protocol (Doyle and Dolyle, 1990) with minor alterations. Plant material, weighing 500 mg, was homogenized in liquid nitrogen and 1 mL of the CTAB buffer [2 % (w/v) CTAB; 2 % (w/v) Polyvinylpyrrolidone (PVPP) (Sigma-Aldrich, St Louis); 100 mM Tris-HCl, (pH 8.0); 25 mM ethylenediaminetetraacetic acid (EDTA); 2 M NaCl; 2 % (v/v) β-mercaptoethanol (added just before use)] was added before the mixture was incubated at 65 ℃ in a water bath for 60 min. The reaction was vortexed at 15 min intervals during incubation and centrifuged for 5 min at 13 000 rpm. The supernatant was subjected to chloroform: isoamyl 24:1 (v/v) extraction before the DNA was precipitated by adding 96 % (v/v) ethanol and the reaction was left overnight at -20 ℃. The reaction was then spun down for 5 min at 13 000 rpm and pellet was washed with 70 % (v/v) EtOH twice. The pellet was dried using the Genevac® EZ2 Personal Evaporator (Genevac LTD, Ipswich, England) and was resuspended in 50 µL of the TE buffer and stored at -20 ℃ until use.

Thereafter, three microliters (200 – 300 ng) of the resuspended DNA were used as a template for a 20 µL PCR reaction. The reaction mix had 0.4 µL of 10 mM truncated primer mix (**Table 5.1**), 0.4 µL of 10 mM dNTPs mix, 1.2 µL of 25 mM $MgCl_2$, 0.5 µL (5 u) of GoTaq® Flexi DNA polymerase (Promega), 5 µL 5X GoTaq® Flexi buffer and distilled water was added to make up to volume. A PCR mix without the template and another with *AgGPPS2* fragment were included in the PCR reaction to act as negative and positive controls for the PCR analysis respectively. The DNA was initially denatured at 95 ℃ for 5 min followed by 30 cycles of amplification (denaturation, 95 ℃ for 30 seconds; annealing, 58 ℃ for 30 s; elongation, 72 ℃ for 1 min 30 s) and final

elongation at 72 ℃ for 7 min. The PCR products were then separated on a 1 % (w/v) agarose gel by electrophoresis.

5.2.4.3 RNA isolation

Total RNA was extracted from leaves using the CTAB method modified by White et al. (2008). In short, 300-500 mg of leaf material was ground to a fine powder in liquid nitrogen and 1 mL of pre-heated (65 ℃) 2 % CTAB buffer (2 % (w/v) CTAB; 2 % (w/v) PVPP; 100 mM Tris-HCl, [pH 8.0]; 25 mM EDTA; 2 M NaCl; 0.5 g L^{-1} spermidine) was added and the mixture was incubated in a water bath for 60 min with intermittent shaking at 65 ℃. The reaction was spun down at 13 000 rpm for 10 min prior to the purification of the nucleic acids using a chloroform: isoamyl alcohol [24:1 (v/v) min and this was done twice. To the supernatant, 8 M of LiCl was added to the reaction to make a final concentration of 2 M prior to overnight incubation at 4 ℃. The RNA was harvested by centrifuging the sample for 60 min and the supernatant was discarded. The pellet was washed two times with 500 µL of 70 % EtOH and resuspended in 20 µL of dH_2O.

5.2.4.4 cDNA synthesis

To confirm transcription of the inserted gene, cDNA was synthesized from RNA using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas). The kit was stored at -20 ℃ and before use components of the kit were thawed on ice gently vortexed and spun down for 60 s using the Hettich Mikro 120 (Hettich Instruments, Beverly, Massachusetts) benchtop microcentrifuge. The reaction was set up on ice and initially 500 ng of mRNA and 0.1 mM of truncated *AgGPPS2* primers were added to nuclease free dH_2O in a sterile microfuge tube to make up a volume of 12 µL. The reaction was incubated at 65 ℃ for 5 min, chilled on ice, centrifuged and the tube was placed back on ice. Four microliters of 5 X reaction buffer, 1 µL of RiboLock™ RNase Inhibitor, 2 µL of 10 mM dNTPs mix and 1 µL (200 u) of RevertAid™H Minus M-MuLV Reverse Transcriptase were added to reaction bringing the reaction volume to 20 µL. The mixture was gently vortexed and then centrifuged. The reaction was then incubated for 60 min at 42 ℃ and it was terminated by heating it at 70 ℃ for 5 min. The first strand

cDNA synthesized was used as a template for PCR amplification as described earlier in section **5.2.4.2** to confirm expression of *AgGGPPS2* in *S. stenophylla.*

5.2.5 Southern blot

To determine the number of transgenes incorporated into the plant, a Southern blot was carried out as reported by Southern (1975) using four-week old microshoots. Wild type microplants were used as negative controls and the pCAMBIA1301-*AgGPPS2* plasmid as the positive control.

5.2.5.1 DNA isolation

Genomic DNA was isolated from four-week old micropropagated plants using Invisorb® Spin Plant Midi Kit (STRATEC Group, Birkenfeld, Germany) according to the manufacturer's protocol. For maximum DNA yield, 500 mg of plant material was homogenized by mortar and pestle in liquid nitrogen. The homogenate was transferred to a 15 mL tube and 2 mL of Lysis Buffer P with 80 µL of Proteinase K was added. The mixture was vortexed gently prior to 1 h incubation at 65 ℃ in a water bath. Tubes were vortexed every 15 min to enhance lysis. The lysis solution was transferred onto a prefilter membrane and this was centrifuged for 5 min at 4000 rpm and 1 mL of Binding Buffer P was added to the filtrate. The suspension was transferred to the spin filter and was incubated for 2 min at room temperature. The suspension was centrifuged at 4000 rpm for 1 min and the filtrate was discarded. To wash off the residual lysis buffer, 3 mL of Wash Buffer I was added and the spin filter was centrifuged for 5 min at 4000 rpm. The filtrate was discarded and the spin filter was returned to the same receiver tube and 3 mL of Wash Buffer II was added followed by 5 min centrifugation at 4000 rpm. The flow through was discarded and spin filter was centrifuged for 15 min to get rid of Wash Buffer II.

The spin filters were then placed in new clean tubes and 1 mL of Elution Buffer D was added. This was incubated at room temperature for 5 min before centrifuging for 5 min at 4000 rpm. Another 500 µL of **Elution Buffer D** was added and the above step was repeated. The DNA yield was quantified using the NanoDrop® ND-1000

spectrophotometer (Thermo-scientific, USA) and samples were stored at 4 ℃ or at -20 ℃ longer period.

5.2.5.2 Restriction digestion of genomic DNA

Twenty to thirty micrograms of DNA was digested with *Eco*RI (Fermentas) for 72 h in a 1 mL reaction at 37 ℃. Spermidine (100 µM of final volume) was added to enhance restriction digestion. The digestion results were concentrated using Genevac® EZ2 Personal Evaporator (Genevac LTD, Ipswich, England) until it was 50 uL in volume. Cut DNA was separated on a 1 % (w/v) agarose gel and was visualized using ethidium bromide.

5.2.5.3 DNA transfer and pre-hybridization

The gel was immersed in a denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 10 min. This was repeated twice. The reaction was neutralized by washing the gel for 15 min with gentle shaking twice with neutralizing buffer (1 M Tris [pH 7.8], 1.5 M NaCl) and was kept in 10 X saline-sodium citrate (SSC) (3 M NaCl, 0.3 M $Na_3C_6H_5O_7$) solution for at least 30 min before DNA was transfer to Hybond-N^+ membrane (Amersham, United Kingdom) by means of downward capillary transfer as illustrated in **Figure 5.1**. DNA was then cross-linked onto the membrane using the UV-Crosslinker (ULTRA.LUM, Scientific Associates; USA) for 2.5 min at 120 mJ cm^{-2}. ULTRAhyb™ hybridization buffer was used for 5 h pre-hybridization prior to addition of the probe. The membrane was placed in a hybridization tube with the side with the DNA facing up and 10 mL of the hybridization buffer was added. The tube with the membrane was then incubated in a hybridization oven at 50 ℃ for 5 h with gentle rotation at a constant speed.

5.2.6 Probe synthesis and DNA hybridization

The radio-labeled probe was synthesized using *AgGPPS2* fragment from the gel extraction described in section **5.2.3** as the template. Two microliters (200 ng) of the DNA fragment was added to the PCR reaction with truncated *AgGPPS2* primers

described in **Table 5.2.** The full PCR amplification reaction used is detailed in **Table 5.3** and the reaction conditions are summarized in **Table 5.4**. The [^{32}P]-dCTP (Perkin-Elmer, Inc. Massachusetts) was added to the dNTPs mix and the PCR was run in a radio-isotope lab. Twenty microliters of the PCR reaction were separated using gel electrophoresis as described in section **5.2.3** The PCR product was cleaned up using a GeneJet™ PCR Purification Kit (Fermentas) and this was used to generate the probe following the manufacturer's instructions. Twenty microliters of Binding Buffer was added to the PCR mixture and the reaction was mixed uniformly by inverting the tubes several times before they were transferred to a GeneJet™ purification column. The column was placed in a 1.5 mL micro-tube and was centrifuged for 1 min at 12 000 rpm and the flow through was discarded. To wash the column, 700 µL of wash buffer was added to the GeneJet™ purification column and this was washed twice and between each wash the flow through was centrifuged through the filter for 60 s.

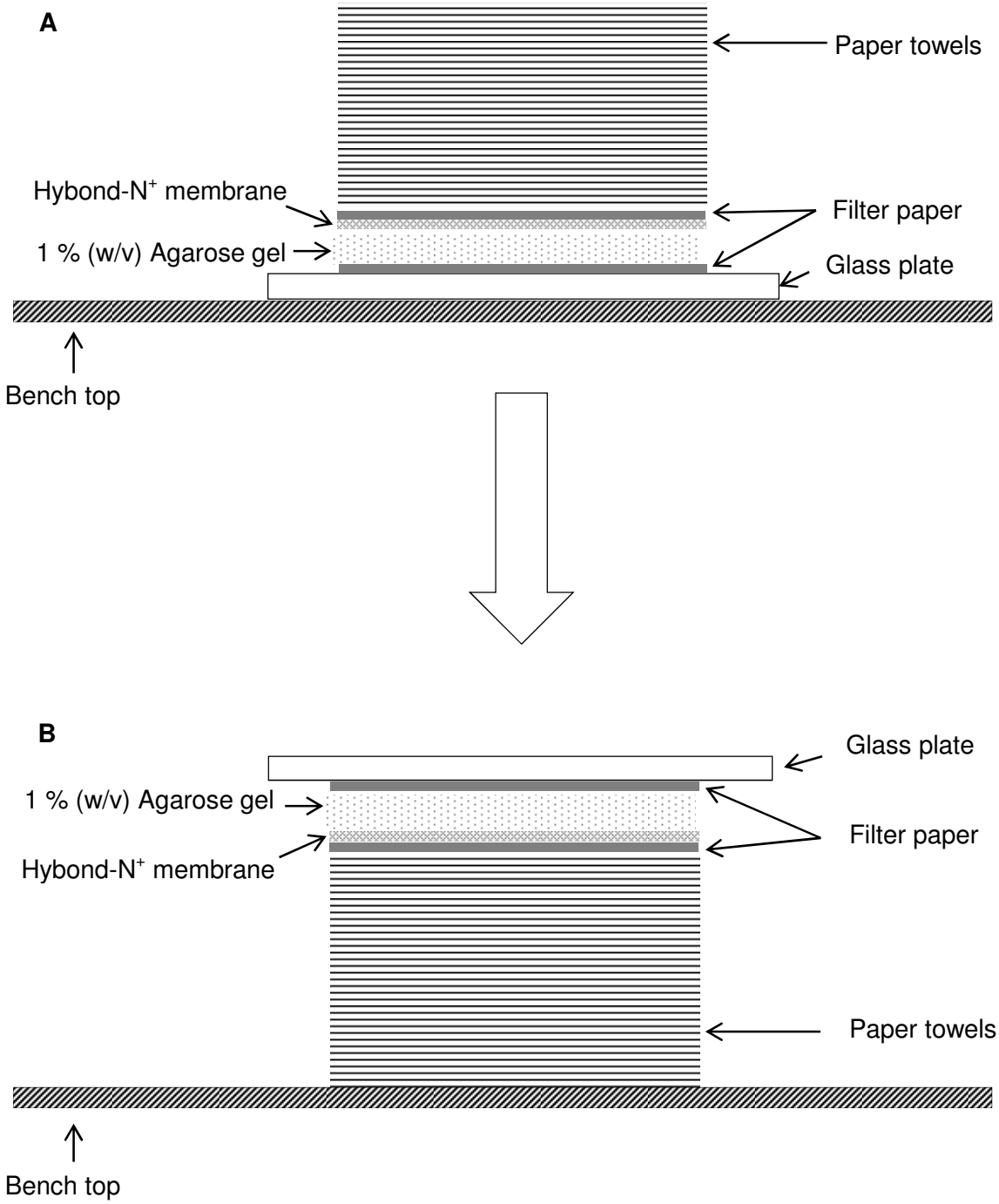

Figure 5.1 The downward capillary transfer of DNA from a 1 % (w/v) agarose gel on to a nylon membrane for Southern hybridization. The gel is set up as shown in (**A)** on and was turned upside-down (**B**). It was left over-night in this position to allow downward migration of DNA to the Hybond-N^+ membrane.

Table 5.3 The PCR reaction for *AgGPPS2* probe synthesis used in for Southern hybridization. Truncated primers were used in the amplification and [^{32}P]-dCTP was use for radio-labeling the probe

Component	**Volume (µL)**	**Final Concentration**
10 X *Taq* Buffer	2.0	1 X
25 mM $MgCl_2$	2.5	2.5 mM
10 mM dNTPs	0.5	0.2 mM
[^{32}P]-dCTP (PerkinElmer, Inc. Massachusetts)	1.25	3000 µCi
DNA (*AgGPPS2*)	2.0	20 ng
Taq polymerase	0.5	2.5 units
dH_2O	11.25	------
Reaction	**20 µL**	

Table 5. 4 PCR conditions for the synthesis of the radioactive *AgGPPS2* probe. All PCR reactions were carried out using the GeneAmp® PCR system 9700 (Applied Biosystems™, Carlsbad, California)

PCR Stage	**Temperature (°C)**	**Time**	**Cycles**
Initial denaturation	95	3 min	1
Denaturation	95	30 s	30*
Annealing	58	30 s	
elongation	72	1 min 30 s	
Final elongation	72	4 min	1

The asterisk (*) indicates the number of PCR amplification cycles for the reactions shown in the bracket

Fifty microliters of the elution buffer was applied on the center of the column and was left to stand for 1 min before it was centrifuged for 60 s to wash off the DNA probe from the membrane. To produce a single stranded DNA probe, the reaction was boiled for 5 min and this was snap cooled on ice. The probe was then added to the 10 mL of hybridization buffer to start hybridization of the Hybond-N+ membrane. The membrane was hybridized for at least 16 h at 50 ℃ with constant gentle rotation (section **5.2.5.3**). After hybridization, the membrane was washed with 50 mL 2 X SSC and 0.1 % (w/v) sodium dodecyl sulphate (SDS) buffer for 20 min at room temperature and for another 20 min at 50 ℃. The final wash was at 50 ℃ with 0.5 X SSC and 0.1 % (w/v) SDS solution. Excess buffer was removed from the membrane before it was sealed in plastic and was exposed using Cyclone Phosphorimager cassette in the dark for 16-72 h. The image was visualized using the Cyclone Phosphorimager (Packard, Meriden, CT, USA).

5.2.7 Greenhouse acclimatization

Six-week old *in-vitro* transgenic and non-transgenic plantlets were transplanted into 110 X 90 cm greenhouse pots with soil: vermiculite mixture of 1:1 (v/v) as was detailed in **Chapter 3**. After the first week of acclimation, plants were watered at least once a week until they senesced after they had flowered (**Figure 5.3 C and D**). In the fourth week, the number of surviving plants was noted. The plants were then allowed to grow to maturity and the seeds were harvested and stored at room temperature.

5.2.8 Metabolite fingerprinting using thin layer chromatography (TLC)

Both polar and non-polar metabolites were extracted from at least 100 mg of plant material with 5 mL of chloroform: methanol (1:1 v/v) as a solvent from both transgenic and non-transgenic six-week old explants (**Figure 5.3 B**). Twenty microliters of each solvent extract were loaded on a TLC silica gel 60 F_{254} (Merck, Darmstadt, Germany) aluminium plate with a 10 mm distance between samples. The samples were run in a vertical position in a chromatography tank with a toluene: ethyl acetate (97:3 v/v) solution. The solvent was allowed to run up the plate for 1 h and the plate was dried at room temperature for 1 min before it was sprayed with anisaldehyde: glacial acetic acid:

concentrated sulphuric acid; 1:20:10; (v/v/v). The plate was first visualized under 365 and 254 nm wavelengths of UV-light generated by the LF-206.LS (UVItec, Cambridge, UK) lamp in the dark, before it was baked for 10 min at 100 ℃ to observe the separated compounds.

5.3 Results and discussion

5.3.1 Plasmid construction and *Agrobacterium* transformation

Colony PCR was used to confirm transformation of *E. coli* (DH5α) and *Agrobacterium tumefaciens* (EHA105) as vectors. The plasmid bearing the transgene, pCAMBIA1301-*AgGPPS2,* was easily cloned into *E. coli* (DH5α) (**Figure 5.2 A**) and *Agrobacterium tumefaciens* EHA105 (**Figure 5.2 B**). More transformed colonies (more than 100 colonies per plate from 50 µL of suspension with a 95 % transformation rate) were observed with the DH5α as compared to the EHA105 (less than 50 colonies per plate from 50 µL of cell suspension with 92 % transformants). This was probably because *E. coli* is the less complex organism of the two and it can easily take up foreign DNA. A fragment of about 1524 bp was visualized via PCR, confirming transformation of both bacteria (**Figure 5.2**). The protocols for transformation were also developed with *E. coli* as the model organism and thus are optimized for its transformation.

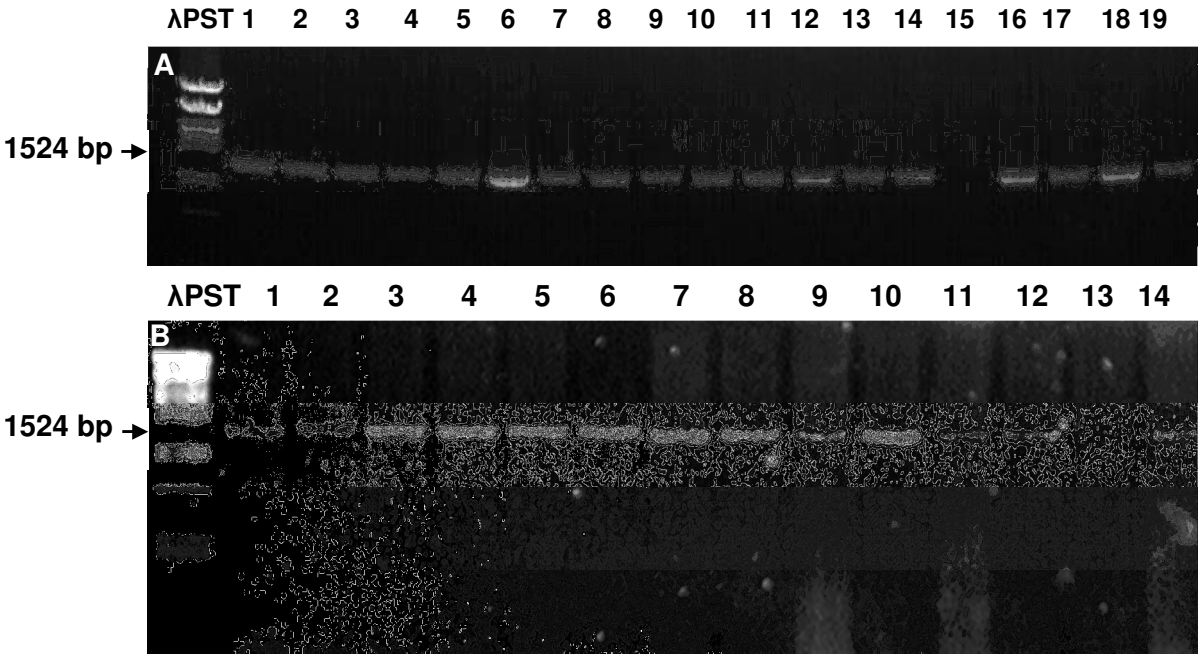

Figure 5.2 Colony PCR screening for *E. coli* (DH5α) (**A**) *Agrobacterium* (**B**) transformation with pCAMBIA1301-*AgGPPS2* construct Transformation was achieved by using the heat shock and electroporation methods. Each lane represents an individual clone picked from the plates after overnight incubation.

5.3.2 *Salvia stenophylla* transformation

This chapter reports on the first successful introduction of the geranyl diphosphate synthase from *Abies grandis* (*AgGPPS2*) gene responsible for the production of an enzyme catalysing synthesis of geranyl diphosphate into *S. stenophylla*. In total more than 2000 attempts were made to transform *S. stenophylla,* only 189 plantlets from explants survived on kanamycin selection (**Figure 5.3 A)** indicating that kanamycin reporter gene was being functionally expressed. Of these 5 were positive when tested for transgene integration using PCR with the truncated primers (**Table 5.1**) with a 1100 bp band being resolved (**Figure 5.4**). Success of transformation is largely a function of the strain of *Agrobacterium* used and the plant genotype (Anderson and Moore 1979). It is plausible that the low transformation efficiency of EHA105 on *S. stenophylla* is a result of reduced virulence of this *Agrobacterium* strain as Kaneyoshi et al. (2001) suggested that host specificity varies with the strain. Addition of acetosyringone is

aimed at enhancing the infective potential of *Agrobacterium* strains, but Godwin et al. (1990) concluded that in some strains and plants, it supresses virulence of the bacterium.

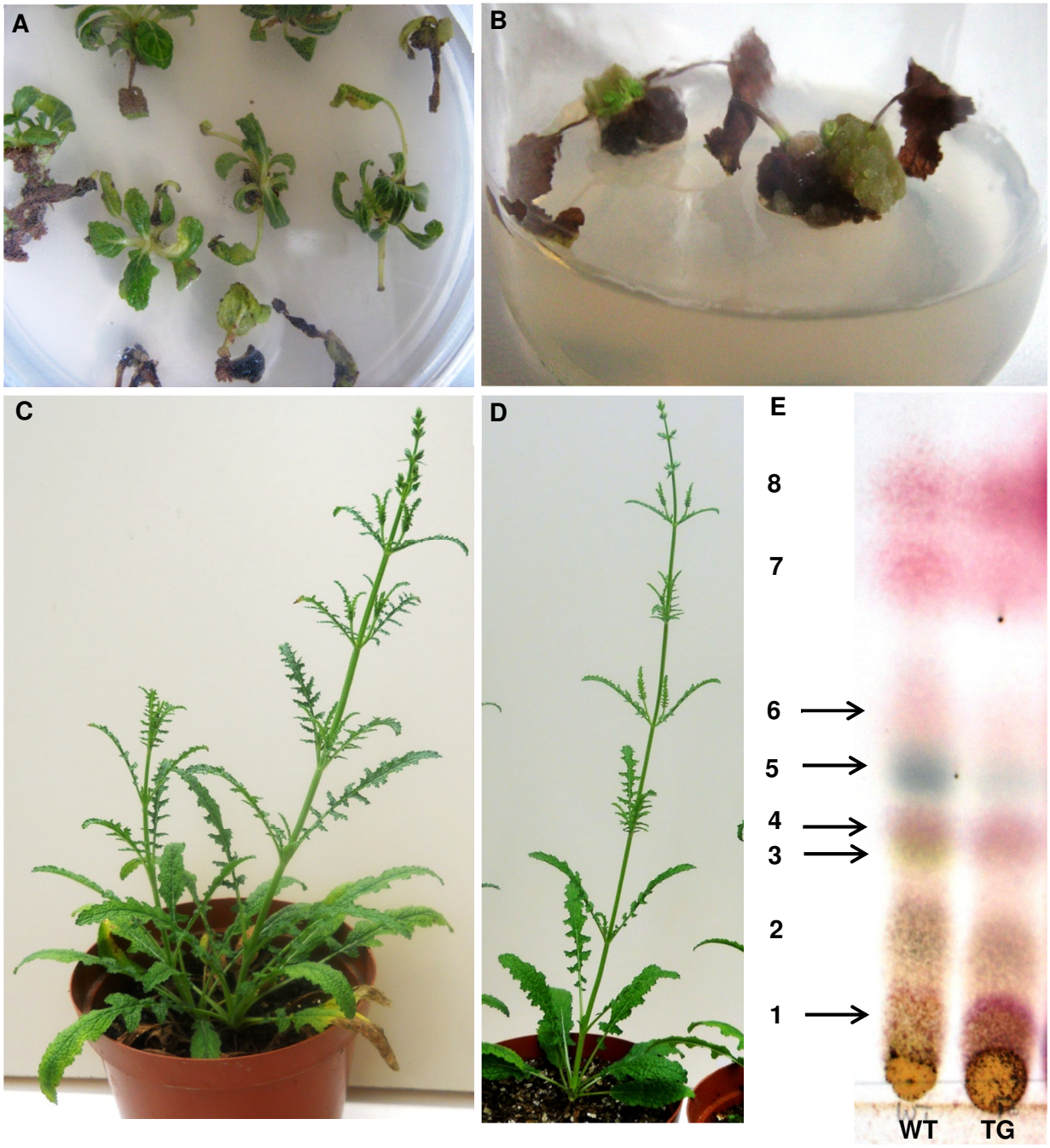

Figure 5.3 Four-week old putative transgenic *S. stenophylla* growing on hormone free kanamycin (100 mg L^{-1}) selection medium with cefotaxime (500 mg L^{-1}) (**A**). After the four-weeks on kanamycin plantlets were transferred to MS medium with 2.7 µM NAA and 4.4 µM BA. Putative transgenic plants callused before shoots sprouted (**B**). Both transgenic (**C**) and wild type (**D**) plants were visually morphologically similar when acclimatized in the greenhouse and flowered after an average of 8 weeks post acclimation. (**E**) Transgenic (right) and non-transgenic metabolite profiles extracted with chloroform: methanol (1:1 v/v) separated on a toluene: ethyl acetate (93:7 v/v) solvent TLC system and visualized through

anisaldehyde staining. Arrows indicate compounds showing significant variation in abundance between the two genotypes

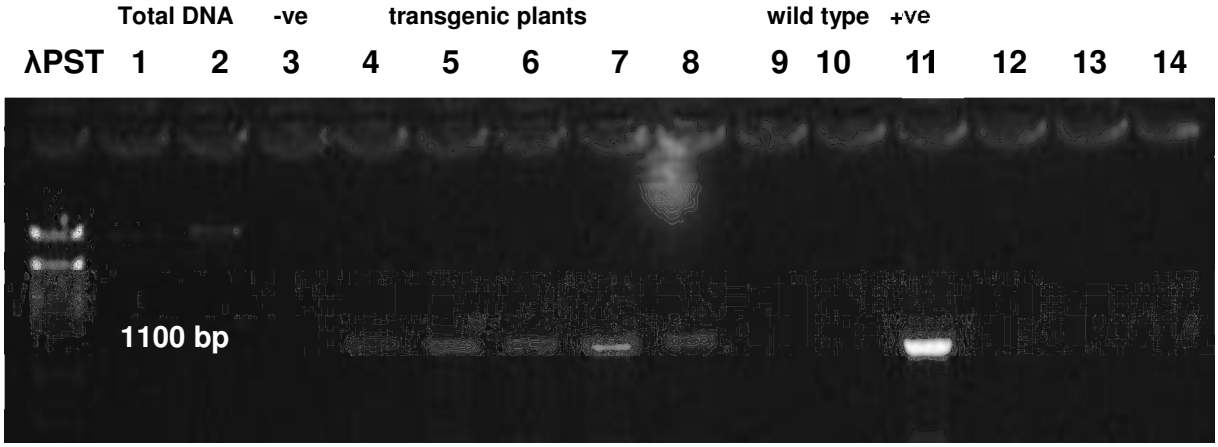

Figure 5.4 PCR confirmation of *AgGPPS2* integration in putative *S. stenophylla* transformants amplified using *AgGPPS2* truncated primers. Lanes 1 and 2 show total genomic DNA for the wild type and transgenic plants respectively. PCR results of transgenic individuals are shown from lane 4 to 8 and lane 9-10 are PCR amplicons for the wild type plants. Negative and positive controls denoted by –ve and +ve, in lane 3 and 11, respectively.

5.3.3 *AgGPPS2* expression

Only 20 % of the plants with the confirmed *AgGPPS2* gene (lane 4 - 8 in **Figure 5.4**) were positively amplified via RT-PCR (**Figure 5.5**). Methylation of the DNA sometimes interferes with gene expression and this might be a possible explanation (Razin and Cedar 1991) for the failure to express *AgGPPS2* in some of the putative kanamycin resistant lines. In tomato, DNA methylation in the promoter sequence silenced the *LeSPL-CNR* gene resulting in suppression of fruit ripening (Manning et al. 2006). The position of integration, gene orientation and mutation can explain the absence of gene expression in some of the plants bearing the transgenes (Gelvin 1998, Bae et al. 2008) since endogenous genes flanking transgenes can sometimes exert repressive pressure on them (Matzke and Matzke 1998). Southern hybridisation revealed that only one heterologous copy was incorporated into the transgenic plant (**Figure 5.6**). Also, the

neomycin phosphotransferase II (*nptII*) was not assessed for in this study. Nonetheless, the *nptII* gene encodes for kanamycin resistance and transgenes are not necessarily co-transformed or co-introduced, explaining kanamycin resistance but showing no presence of *AgGPPS2* gene in their genome. Single gene copy transfer makes *Agrobacterium* transformation more favourable since there are less chances of post-transcriptional homologous gene silencing, occasionally brought about by multiple gene insertions common in biolistic transformation (Tang et al. 2007). Clones of the positive transgenic plants were successfully acclimatized in the greenhouse and were able to produce seed. This demonstrated that the process did not tamper with the reproductive potential of the plant which is essential when considering future breeding success of the plant. The random nature of agrobacterial gene insertion does not guarantee stability of transgene expression and vertical transfer to subsequent offspring (Koprek et al. 2001) and thus there is need to also assess presence and expression of the heterologous *AgGPPS2* in the F_1 generation.

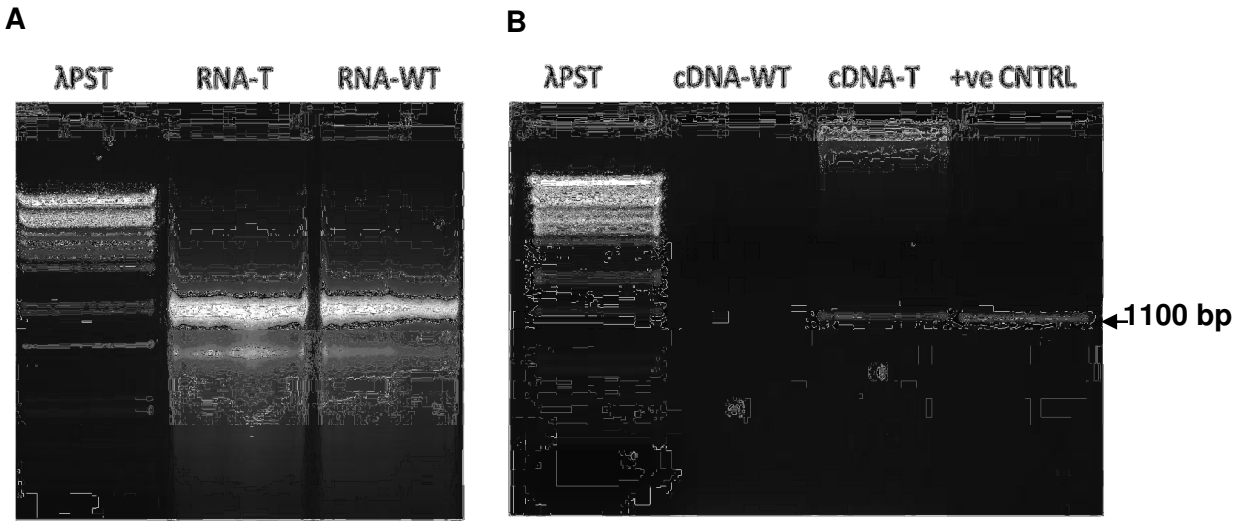

Figure 5.5 Total RNA (**A**) for transgenic (RNA-T) and wild type (RNA-WT), and (**B**) confirmation of *AgGPPS2* expression using RT-PCR from transgenic (cDNA-T) and wild type (cDNA-WT) of *S. stenophylla*. PCR was carried out using truncated *AgGPPS2* primers.

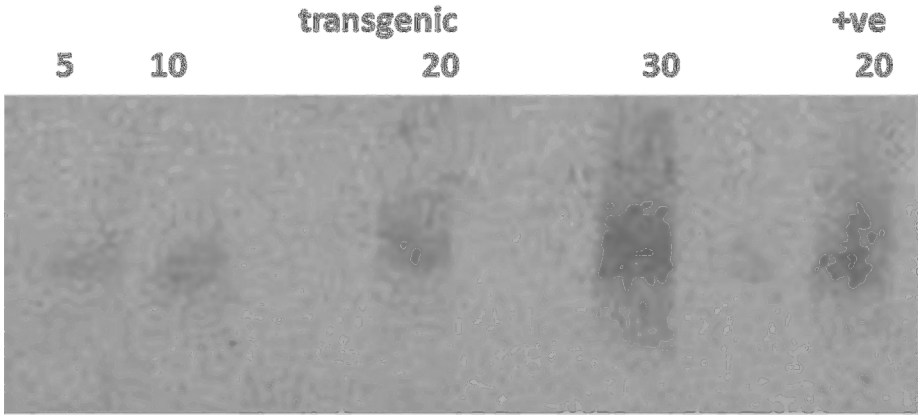

Figure 5.6 Southern hybridization analysis of putative transformed *S. stenophylla* plants. Genomic DNA was digested with *Eco*RI and probed using a [^{32}P]-dCTP labeled probe prepared from pCAMBIA1301-*AgGPPS2* construct using primers described in section **5.2.4.** Lanes marked 5, 10, 20 and 30 show amount transgenic DNA loaded in the respective lanes (µg). Twenty micrograms of the positive control (+ve) were loaded.

5.3.4 Effect of *AgGPPS2* on the *S. stenophylla* phenotype

Both transgenic and non-transgenic plants were similar in appearance and growth habit for both *in-vitro* and *ex-vitro* plants. However, there was evidence of changes in the metabolite profile based on the TLC analysis. TLC profiling is a quick and easy method which facilitated fast analysis of transgenic plants. Even though there were no noticeable differences when the plate was exposed to UV-light, there was, nonetheless, a marked deep colouration for some bands regarding the non-transgenic profile when compared to the transformed plants, suggesting that the compounds are more abundant in wild type plants than in transgenic plants (compound **3** and **5** in **Figure 5.3)** and *vice versa* (compound **1** in **Figure 5.3**). Some compounds were only present in wild type plants (compound **3** and **6** in **Figure 5.3**). Usually gene transformation may lead to changes in metabolite profile, thus it is no surprise that there seem to be variable inter-clonal metabolite accumulation.

However, the structural identity of these metabolites cannot easily be resolved with TLC and so other comprehensive metabolite analysis such as GC- and LC-MS (**Chapter 6**) to better understand the impacts of transgenesis. Changes in abundance of some metabolites in transgenics could possibly be a result of suppression of expression for

the biosynthetic gene by the introduction of a heterologous gene (van der Krol et al. 1990, Matzke and Marjori A Matzke 1998). In *Petunia* over-expression of genes coding for the biosynthesis of the floral pigment, dihydroflavonol-4-reductase or chalcone synthase genes, resulted in reduced pigmentation in flowers of 25 % of the transformants (van der Krol et al. 1990). The objective of this study was to assess the effect heterologous gene expression on the metabolite profile of *S. stenophylla* and the TLC profiles seem to imply that significant alterations in chemical accumulation did occur. Nevertheless, there was still need for further characterization of both genotypes to fully understand the effect of the foreign gene expression in the plant as TLC profile alone is not conclusive.

Transformation of *S. stenophylla* resulted in viable plants with similar growth patterns but metabolite profiles showed some differences in abundance between the two genotypes. However, it is also of paramount importance that further analysis of the first filial generation of the transgenic line be carried out. Transgenes sometimes deviate from the classic Mendelian inheritance sexual reproduction might bring about allelic rearrangements which might alter the transgene position subsequent expression as a result of ectopic homologous pairing (Yin et al. 2004).

5.4 Conclusion

The results presented in this chapter forms the basis for manipulation of South African sages for increased synthesis of isoprenoids of industrial importance. With time, the protocol can be optimized for transformation of sages and thus improve the transformation efficiency of *S. stenophylla* when using *Agrobacterium*. However, it would be worthwhile to test several other agrobacterial strains to determine whether the best strain to use when transforming *S. stenophylla* and other South African sage species. The terpene biosynthetic pathway is governed by more than one enzyme and it will be valuable to introduce several genes when engineering for increased metabolites. **Chapter 6** attempts to characterize the metabolic profiles of the two genotypes.

5.5 References

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Chapter Six

A comparison of transgenic and wild type *Salvia stenophylla* metabolomics[5]

6.1 Introduction

The presence of functional secondary compounds in large quantities has made sage species important since the beginning of civilization (Kintzios 2000). Recent commercialization of the 'natural products' concept (medicines, food additives and essential oils) has further increased the rate of sage exploitation. As already detailed in **Section 2.7**, a complete inventory of chemical compounds occurring in a plant is referred to as the plant's metabolome (Sumner et al. 2003). This is the basis of metabolomics. Metabolomics is a set of techniques used for global profiling and sometimes identification of chemical compounds in an organism. In phytochemistry, metabolomics breaks down crude extracts into constituent parts by using various separation techniques illustrated in **Section 2.7** (Weckwerth and Morgenthal 2005) with the ultimate goal of isolating biologically active compounds.

Although traditional medicine uses unprocessed plant material and crude herbal extracts, modern medicine and pharmaceutical standards demands knowledge of all compounds in the metabolome. Since metabolomics is based on separating the metabolome into constituent parts, it is a crucial link between modern western medicine and traditional medicine. It simultaneously elucidates the efficacy of traditional plant preparations and gives a scientific understanding of botanical remedies.

Comprehensive insight into herbal preparation is critical because quality and quantity of chemicals vary with genetics, environment and their relationship (Francisco et al. 2011). Changes in ambient conditions, herbivory and pathogen activity trigger production of secondary metabolites, whilst the genetic makeup of the plant determines the appropriate response to these environmental cues. Also, variation in the genomes within and between plant species and the level of plant-environment interaction

[5] Chapters 5 and 6 are going to be published as paper with the following title "Effects of over-expressing the AgGPPS2 gene in *Salvia stenophylla* on terpenoid biosynthesis" in the journal called Metabolic Engineering. The manuscript is currently in preparation.

determines the level of response and thus amount of secondary compounds synthesized. The resultant phytochemical polymorphism makes it difficult to standardize processing and quality of botanicals.

In this study, the geranyl diphosphate synthase gene from *Abies grandis* (*AgGPPS2*) was introduced in *S. stenophylla* and molecular analysis confirmed integration of the gene (see **Section 5.3.3**) thus verifying creation of a new *S. stenophylla* line possibly with a different metabolite profile. Effects of heterologous gene expression was summarized earlier in **Table 2.2**. Introduction of a single gene can affect biosynthetic activities of more than one pathway, warranting a thorough investigation of secondary compound profiles. Most importantly, an all-inclusive analysis is paramount as it determines the market value of herbal preparations. Detailed chemical analyses are also vital because they form the basis for further biotechnological manipulations of metabolic pathways.

While it is difficult to give a complete detailed profile of a plant's metabolome, this chapter attempts to make use of these separation methods (LC- and GC-MS) to give a comprehensive description of the volatile and polar secondary compounds found in *S. stenophylla*. It compares metabolite profiles of transgenics bearing the *AgGPPS2* gene with wild type plants whilst trying to document the chemical variation induced by different growth conditions.

6.2 Materials and methods

6.2.1 Plant material

Plantlets were maintained *in-vitro* as described in **Chapter 3** and transgenic plants were generated following the procedure reported in **Section 5.2.4**. Transgenic and wild type plants were harvested at six weeks for *in-vitro* cultured plants whilst the greenhouse acclimated plants were harvested ten weeks after acclimatization and at 12 weeks when they were flowering. All greenhouse plants were acclimated as detailed in **Section 3.2.2** and their age was determined starting from the day of transfer from tissue culture and this was taken to be day 0.

6.2.2 Essential oil extraction

The essential oil was produced by hydro-distillation using a Clevenger type apparatus (Clevenger 1928). Briefly, a 1 kg of fresh aerial plant material was heated in 250 mL of distilled water at 40 ℃ for 8 h. Harvested oil was collected in glass vials and was kept in the dark at -20 ℃ until use.

6.2.3 Extraction of polar compounds

For this analysis, *in-vitro* plant material was divided into aerial parts (leaves and stems combined) and roots. Aerial parts of 8 and 10 week-old (flowering) acclimated plants were also used for the extraction. At least 100 mg of plant material was ground in liquid nitrogen using a mortar and pestle. Metabolites were extracted with 5 mL chloroform: methanol (1:1; v/v) solvent. The mixture was sonicated for 30 min with intermittent shaking and was filtered twice through Whatman No. 1 filter paper. The filtrate was then dried in the Genevac® EZ2 Personal Evaporator (Genevac LTD, Ipswich, England) and the extract was resuspended in methanol to a concentration of 5 mg mL^{-1} which was used for LC-MS analysis.

6.2.4 GC-MS

Two different methods were used for the relative quantification of volatile compounds using GC-MS:

1) ***HS-SPME-GC-MS*** was carried out in a similar manner as was reported in **Chapter 3**. Briefly, directly after harvesting, 0.4 g of leaf material was placed inside 20 ml headspace glass vials. Each container was sealed with an aluminium-coated silicone rubber septum. The extraction of the volatiles was facilitated by heating samples for 15

min at 80 ℃. Released volatiles were adsorbed by the SPME fiber [DVB/ Carboxen/PDMS, StableFlex (Supelco)]. A 1:20 (v/v) split injection ratio was used to introduce compounds into the Waters GCT Premier instrument fitted with a HP5 column (30 m, 0.25 mm i.d., 0.25 lm film thickness).

2) ***Liquid injection:*** Essential oils were diluted with GC-MS grade dichloromethane (DCM) at a ratio of 1: 5 (v/v). One millilitre of the diluted sample was injected for a splitless run with an initial temperature of 40 ℃ and a maximum oven temperature of 250 ℃. In both cases samples were run together with the known chemical standards and the following compounds were used; (-)-α-bisabolol, camphene, (1R)-(+)-camphor, (+)-3-carene, β-caryophyllene, (1R)-(+)-α-pinene, R-(+)-limonene and were confirmed using the NIST05 library.

6.2.5 LC-MS

Non-volatile compounds were analysed using LC-MS and LC-UV based on the time of flight principle. Metabolites were separated on the Waters ACQUITY UPLC™ system was used for using a binary phase with solvent A (0.1 % [v/v] formic acid) and B (acetonitrile) according to the gradient shown in **Table 6.1** and each run lasted 20 min. The system was also equipped with an ACQUITY UPLC™ AutoSampler and PDA Detector LC-UV analysis.

Table 6.1 A gradient chart for a 20 min LC run with 0.1 % (v/v) formic acid (A) and acetonitrile (B) made up the mobile phase with a constant flow rate of 400 µL min^{-1}

Time (min)	% A	% B
0.00	90.0	10.0
0.30	90.0	10.0
15.00	0.0	100.0
17.00	0.0	100.0
17.10	10.0	90.0
20.00	10.0	90.0

6.2.6 Data analysis

For the GC-MS analysis, peaks were identified and quantified using the MassLynx 4.0 software linked to the NIST05 library and chromatograms were drawn using the mass to charge ratio (*m/z*). Mean relative abundance data of different treatments were compared using one-way analysis of variance (ANOVA). The MassLynx 4.1 software was used for analysis of the LC-MS data and differences in metabolite profiles between transgenic and wild plants were assessed using the principal component analysis (PCA). Both negative and positive electron spray ionization (ESI) were used for mass separation of analytes utilizing the Waters SYNAPT™ G2 MS (Manchester, England). Mass data were collected between 93 and 1500 m/z.

6.3 Results and discussion

6.3.1 GC-MS

The presence and abundance of volatile compounds was probed by using HS-SPME-GC-MS. About 88.89 % of the peaks observed were positively identified using

commercial standards and the NIST05 library. These had a similarity index of greater than 85 % when compared with compounds in the NIST05 database. The remaining 11.11 % were not conclusively identified (similarity index was less than 85 %). Relative abundance was variable with a few compounds not being affected by changes in the genotype or environment (**Table 6.2**). The secondary compound profile seemed similar when comparing tissue cultured non-transgenic plants and *in-vitro* transgenic plants (**Figure 6.1**). Heterologous gene expression had an impact on metabolite quantity, especially on the levels (-)-α-bisabolol, τ-terpinene, α-pinene and (+)-4-carene (**Table 6.3**). Upon introduction of *AgGPPS2* gene, accumulation increased from 19.0 % to 31.1 % in *S. stenophylla* leaves. (+)-4-Carene and α-pinene were significantly reduced in plants with an extra copy of the heterologous *AgGPPS2*. The sesquiterpene (-)-α-bisabolol was improved considerably (**Table 6.3**). Economically, this is a beneficial response as this sesquiterpene is essential to the pharmaceutical and cosmetic industries (Kamatou and Viljoen 2010). Volatility of monoterpenes may also explain the low levels of D-limonene, camphene and β-pinene. The analysis could be further improved by using real-time chromatography tools to monitor volatile metabolites as they are being emitted (Steeghs et al. 2004, Zaikin 2011).

Changes in metabolite composition and accumulation were also noted when the propagation environment was changed. Greenhouse acclimatization had caused no change in the relative abundance of (-)-α-bisabolol and α-terpineol, but (+)-4-carene, δ-3-carene, β-pinene, camphor and α-phellandrene were increased significantly (**Figure 6.2**). On the other hand, heavier molecules such as cis-lanceol, caryophyllene, and manool became less abundant *ex-vitro*. Monoterpenes (β-pinene, δ-3-carene, (+)-4-carene and α-phellandrene) form the crucial plant-environment interface presented as a sweet and pungent odour, responsible for deterring and attracting predators and pollinators respectively (Kessler and Baldwin 2004, Hare and Sun 2011).

Table 6.2 An ANOVA comparing mean relative abundances of major volatile compounds from *S. stenophylla.* Means for *in-vitro* transgenic and wild type plantlets, and greenhouse acclimated wild type plants at 10 weeks and at 12 weeks (flowering) were compared

Compound	DF	Sum of Squares	Mean Squares	F-ratio	*p*-value
(-)-α-Bisabolol*	3	231.33	77.11	17.70	0.001
(+)-4-Carene*	3	14.46	4.82	17.70	0.00
B-Bisabolene*	3	15.08	5.03	5.34	0.03
Borneol*	3	0.94	0.31	0.37	0.78
Camphene	3	0.72	0.24	2.84	0.12
Camphor*	3	2.87	0.96	5.89	0.03
Caryophellene*	3	12.30	4.10	6.61	0.02
cis-Lanceol	3	9.37	3.12	4.24	0.05
D-Limonene	3	9.67	3.22	2.69	0.13
Manool	3	17.36	5.79	2.05	0.20
Ocimene*	3	0.46	0.15	8.88	0.01
Thujene	3	0.01	0.00	4.53	0.05
α-Farnesene	3	0.43	0.14	0.81	0.53
α-Phellandrene*	3	8.25	2.75	8.21	0.01
α-Terpineol	3	0.35	0.12	3.14	0.10
β-Farnesene*	3	2.20	0.73	14.35	0.00
β-Pinene*	3	15.90	5.30	35.36	0.00
σ-3-Carene*	3	43.82	14.61	8.11	0.01
τ-Terpenene*	3	2.07	0.69	12.35	0.00

Asterisk (*) denotes compounds that showed significant variation in metabolite accumulation between treatments

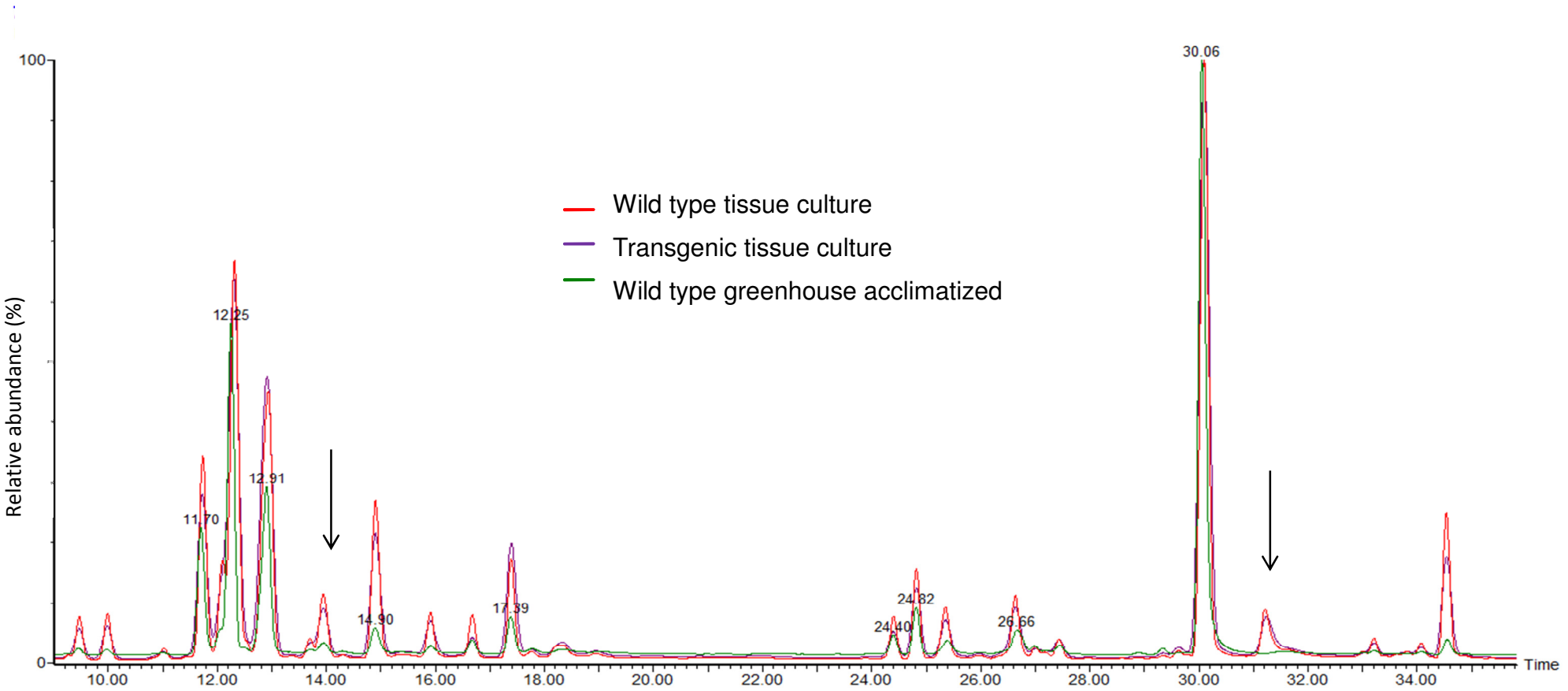

Figure 6.1 Metabolite profiles for tissue cultured transgenic and wild type and *in-vitro* derived greenhouse acclimated wild type *S. stenophylla.* Profiles did not differ significantly except for peaks shown with arrows which only appeared in tissue cultured plants (both transgenic and wild type). However, there was notable difference in relative accumulation of specific compound between transgenic and non-transgenic tissue cultured plants

Table 6.3 Mean relative abundance of the major volatile compounds found in transformed and non-transformed *S. stenophylla* leaves. Non-transformed plants were grown in tissue culture or in the greenhouse. Greenhouse plants were harvested at flowering and prior to flowering

Compound	Transgenic (tissue culture)	Wild type (tissue culture)	Acclimated (10 weeks)	Acclimated flowering (12 weeks)
(-)-α-Bisabolol*	**31.1 ± 0.17 b**	**19.0 ± 0.93 a**	**19.1 ± 1.31 a**	**19.80 ± 1.58 a**
(+)-4-Carene*	**1.27 ± 0.31 a**	**2.27 ± 0.22 b**	**4.13 ± 0.21 c**	**4.02 ± 0.44 c**
Borneol*	**2.50 ± 0.17 b**	**2.22 ± 0.46 b**	**1.87 ± 0.51 a**	**2.62 ± 0.71 a**
Camphene	0.50 ± 0.01 a	1.09 ± 0.21 a	1.12 ± 0.23 a	1.23 ± 0.04 a
Camphor*	**0.20 ± 0.04 a**	**0.74 ± 0.29 b**	**1.66 ± 0.18 c**	**1.18 ± 0.27 c**
Caryophellene*	**1.25 ± 0.02 a**	**3.52 ± 0.81 b**	**1.09 ± 0.14 a**	**1.13 ± 0.24 a**
cis-Lanceol	3.39 ± 0.57 a	4.13 ± 0.80 a	1.98 ± 0.16 a	2.11 ± 0.30 a
Cis-Ocimene*	**0.20 ± 0.05 a**	**0.41 ± 0.04 b**	**0.73 ± 0.08 c**	**0.69 ± 0.10 c**
D-Limonene	5.86 ± 1.14 a	5.22 ± 0.36 a	6.76 ± 0.27 a	7.62 ± 0.87 a
Manool	5.86 ± 2.94 a	5.77 ± 0.34 a	3.28 ± 0.47 a	3.28 ± 0.29 a
ß-Bisabolene*	**2.56 ± 0.27 b**	**4.35 ± 1.00 c**	**1.69 ± 0.23 a**	**1.53 ± 0.15 a**
ß-Farnesene*	**1.76 ± 0.16 b**	**2.16 ± 0.07a**	**1.25 ± 0.17 a**	**1.05 ± 0.13 a**
ß-Pinene	4.04 ± 0.43 a	4.36 ± 0.22 a	6.36 ± 0.24 a	6.86 ± 0.07 a
Thujene	0.07 ± 0.01 a	0.14 ± 0.02 a	0.15 ± 0.02 a	0.15 ± 0.00 a
α-Farnesene	2.61 ± 0.57 a	2.94 ± 0.22 a	2.83 ± 0.22 a	2.45 ± 0.02 a
α-Phellandrene*	**1.35 ± 0.34 a**	**1.98 ± 0.27 a**	**3.43 ± 0.33 c**	**3.40 ± 0.41 c**
α-Terpineol	0.06 ± 0.01 a	0.31 ± 0.12 a	0.59 ± 0.16 a	0.35 ± 0.07 a
σ-3-Carene*	**11.90 ± 1.61 a**	**10.40 ± 0.90 a**	**14.50 ± 0.48 b**	**15.20 ± 0.46 b**
τ-Terpenene*	**0.37 ± 0.08 a**	**0.86 ± 0.09 b**	**1.46 ± 0.12 c**	**1.48 ± 0.12 c**

Asterisk (*) denotes compounds that showed significant variation in metabolite accumulation between treatments. Different letters in the same row denote significant statistical differences in the mean relative abundance.

The glasshouse atmosphere, unlike the *in-vitro* environment, is a less controlled setup and had more real life, physical disturbances (air draughts and watering) and thus there were more challenging interactions for the *in-vitro* derived plantlets. These might have generated the increase in demand for monoterpenes and the subsequent accumulation. The constant air circulation results in movement of volatiles away from the plant, creating a need for additional biosynthesis and the consequent increase in accumulation of these terpenes. The reactive nature of monoterpenes means they readily react with O_3 and OH molecules to form biogenic secondary organic aerosols (SOAs), a worrying point in climate change research as SOAs tend to absorb and scatter thermal radiation and thereby influence the global energy balance (Gao et al. 2010). This characteristic of monoterpenes is nonetheless beneficial as it allows emitting plants to cope better with heat stress by having some level of control of their immediate environment. It follows that when exposed to high greenhouse temperatures in comparison to the controlled *in-vitro* growth conditions, *S. stenophylla* produced more monoterpenes to cope with the highly fluctuating temperatures. Production of secondary compounds as protectants against heat stress has also been documented by Copolovici et al. (2005). Invasive species in Hawaii enhance their survival by emitting more monoterpenes when compared to the native plants, affording themselves protection from multiple stresses (Llusià et al. 2010).

Secondary compounds are generally affected by stage of growth (Ben Taarit et al. 2009, Lange et al. 2000). At the time of analysis, most glass house plants were approaching the flowering stage in which they produce brightly coloured mauve seemingly insect pollinated flowers and the olfactory stimulants are produced in to attract pollinators. The metabolite accumulation before and during flowering was not significantly affected by flowering (**Table 6.3**). Naturally sesquiterpernes should be in higher concentrations during flowering because they are normally synthesised by floral tissue, dominating the floral scent to attract pollinators (Nieuwenhuizen et al. 2010), but with *S. stenophylla* the flowers are self-pollinating and do not produce any scent. For commercial production of sesquiternes, it is thus not necessary to wait for the plant to flower since the highest levels are attained from week 10.

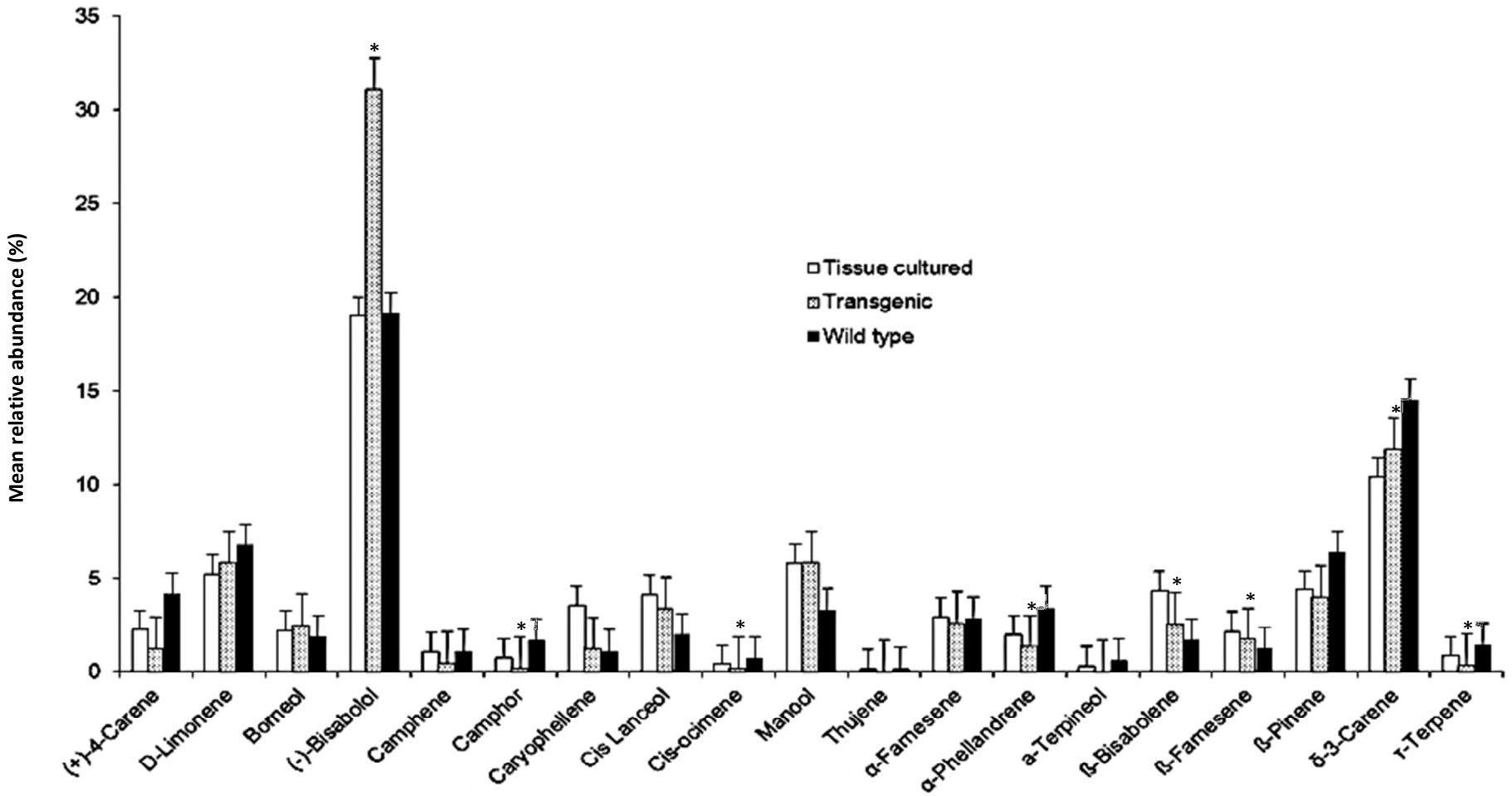

Figure 6.2 Mean relative abundance (%) of major secondary compound synthesised by *S. stenophylla*. Plants were cultivated in-vitro (tissue cultured), or in the garden (wild type) and some were micropropagated but carried the gene *AgGPPS2* (transgenic).Mean abundances for transgenic and tissue cultured plants were compared with the wild type plants using an ANOVA at 95% confidence level. Asterix (*) denote compounds that showed significant differences between genotypes.

In essential oil production application of these findings provides a platform on which farmers can decide a system and time of production based on the target compound since each chemical may tend to vary with time of stage of growth, genotype and growth conditions. One of the challenges met by farmers intending to venture into medicinal plant cultivation, especially when considering local *Salvia* species as potential crops, is the absence of information on agronomic principles, growth patterns, and their impact on essential oil accumulation. This study, together with the **Chapters 4** and **5** provide information that can make such ventures more feasible as the best growth conditions form essential oil production are outlined. This is the first time that this data is presented in primary literature, illuminating the need for further studies of this nature.

6.3.2 LC-MS

Solvent extracts analysed via LC-MS were subjected to both positive and negative electron spray ionization (ESI) mass spectrometry to deduce the mass to charge ratios. The results showed little variation regarding the metabolite profiles. There was notable variation in the number of compounds detected using different ionization modes, between different treatments (**Table 6.4**). Wild type and transgenic extracts had almost similar chemical profiles when analysed using both negative and positive mode suggesting a balance between acidic and basic compounds in the solvent extract.

Table 6.4 Number of peaks detected using LC-MS of solvent extracts of transgenic and non-transgenic *S. stenophylla* extracted from different tissue and at different growth periods. WT and TG represent wild type and transgenic *in-vitro* plants respectively, WTR and TGR denotes wild type and transgenic root extracts respectively and WTO and TGO are 10 week old greenhouse acclimatized plants

ESI Mode	Genotype					
	Wild Type			Transgenic		
	WT	WTR	WTO	TG	TGR	TGO
+ve	86	84	87	89	96	88
-ve	86	79	70	83	85	86

Acidic compounds are better analysed using the negative ESI setup whilst basic metabolites are more accurately analysed using positive ESI. Compounds A – M [**Figure 6.3 (1)** and A – P **Figure 6.3 (2)**] were common in both clones.

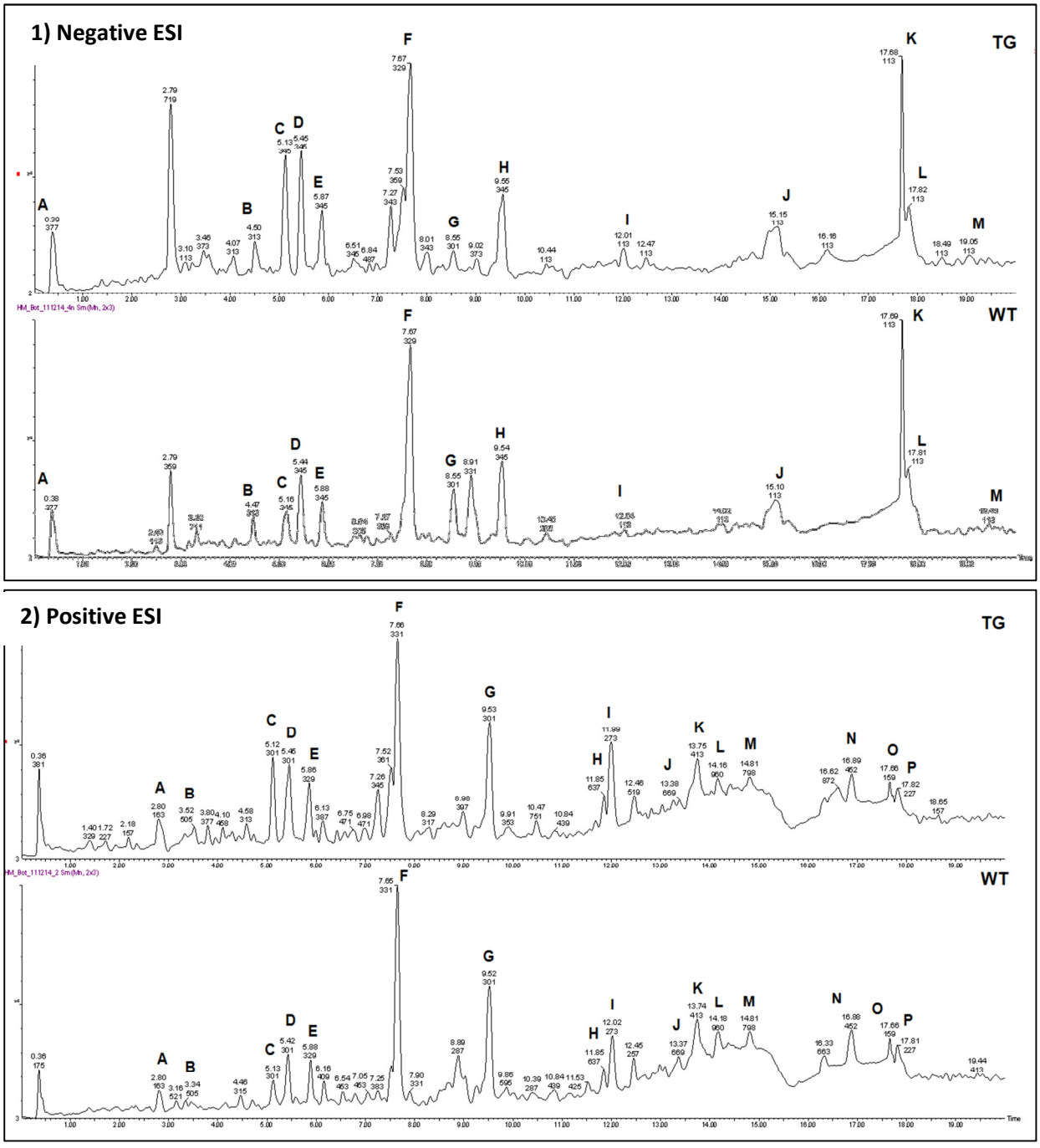

Figure 6.3 LC-MS ESI Total ion chromatograms showing metabolite profiles for transgenic (TG) and non-transgenic (WT) plants cultivated *in-vitro* for a period of 10 weeks analysed using both negative (**1**) and positive (**2**) ESI. Peaks with similar letters in each ionization mode denote compounds of similar structure and ion fragmentation pattern**.**

Fewer peaks were detected from the root extract and only six compounds were common in the root, stem and leaf extracts, possibly explaining why traditional healers and herbalists use aerial parts for disinfecting rituals and healing purposes. The stage of growth also proved to affect non-polar compound accumulation. Both transgenic and non-transgenic eight week old greenhouse plants showed different profiles when compared with their respective *in-vitro* counterparts. Upon carrying out a PCA, 8-week old plant clustered together whilst the *in-vitro* derived analytes grouped to the tissue type (**Figure 6.4**), confirming that some chemicals are under strict developmental and organ specific control.

Rosmarinic acid was detected in the positive mode (ESI+) and is probably one of the reasons why the profiles for root extracts were different from the above ground plant parts as shown in both transgenic and wild type plants it was below detectable limits. It was more abundant in transgenic (5.75 %) than in wild type (2.83 %). Rosmarinic is a plant defence compound derived from phenylpropanoids (Petersen et al. 2009, Sánchez-Campillo et al. 2009), a class of plant chemicals known to be produced in response to microbial attack (Dixon et al. 2002). It is a strong antioxidant often associated with the Lamiaceae family.

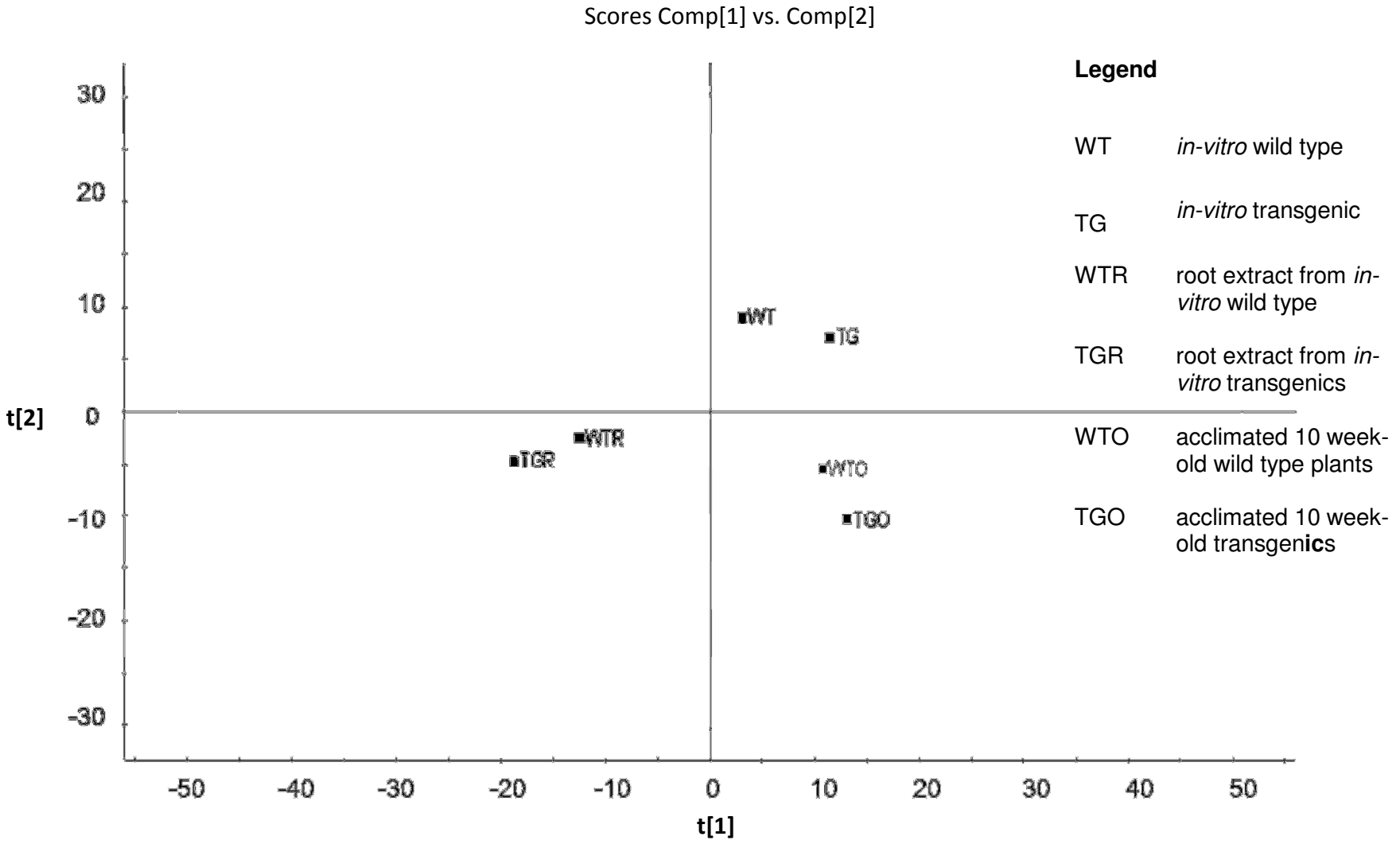

Figure 6.4 A PCA plot of LC-MS (ESI-) profiles showing variations in metabolite accumulation between *in-vitro* wild type and transgenic, young and old, and root and aerial part plant polar extracts.

6.4 Conclusion

This is the first attempt to modify terpene biosynthesis with the aid of *Agrobacterium* in *S. stenophylla.* The results showed that the metabolome can be altered through genetic engineering. Presence of an additional copy of *AgGPPS2* caused changes in the accumulation of individual metabolites but maintained the phytochemical integrity of the plant. Compound composition was altered in both the essential oil fraction and in the non-polar component and the environment proved to be a significant force in determining metabolome quality and accumulation. It was also interesting to note that there was a marked diversity in root and aerial parts derived chemicals. Full elucidation of phytochemicals found in all clones is required to better understand the global effect of transgenesis. Further fractionation of extracts is essential to fully understand the nature of the difference in metabolite quality between genotypes, and especially, between root and leaf extracts. This will elucidate the compounds making the difference between aerial part and root extracts. Consequently, it is also necessary to investigate the phytochemistry of the progeny of the first filial generation of the transgenic line to ascertain stability and inheritance through Mendelian genetics which may affect phytochemical accumulation.

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Chapter Seven

General conclusion and recommendations

7.1 Conclusion

The overall aim of this research was to determine the effect of heterologous *AgGPPS2* gene expression on *in-vitro* secondary compound biosynthesis in *Salvia. stenophylla* with special interest on terpenes. To achieve this, a micropropagation system for *S. stenophylla* was established and optimised prior to determining the global effect of transgene expression. The transgene was mobilized into *S. stenophylla* with the aid of *Agrobacterium tumefaciens* (EHA105) carrying a pCAMBIA-*AgGPPS2* construct.

Medicinal flora of South Africa can be conserved using biotechnology and production of essential products can be improved by applying the same principles. The first part of this study showed the usefulness of smoke and the removal of seed coat in the *in-vitro* germination process of *S. stenophylla.* Such information is important when considering field cultivation of this species using seeds as starting material. The microplants generated from tissue culture, both *in-vitro* and when acclimatized in the greenhouse, had similar metabolite profiles when compared to naturally growing wild plants, thus providing an alternative source for pharmaceutical bioactives. This is of benefit to both the natural products and environmental conservation sectors as it allows sustainable use of botanical resources and *in-situ* conservation, as the increasing demand for plant material might threaten wild population with over-harvesting. The germ-free plantlets from tissue culture are also crucial in genetic manipulation experiment as this technology requires sterile plant material. Ease of greenhouse acclimatization of tissue culture derived plant means that this technique can be used for production of planting material in commercial farming systems producing essential oil and other compounds from *S. stenophylla.* Superior lines can be replicated using this method.

One of the major outputs from this study was the determination of *in-vitro* optimal levels of potassium and nitrogen and the effect of changing them on metabolite accumulation. This work concluded that the manipulation of potassium and nitrogen levels in growth media sometimes influences the secondary compound profile. Most fertilizer application regimes are based on nitrogen and potassium supplementation. Plant nutrition is an important agronomic consideration and information of the correct nutrient requirements is essential when promoting farming of local sages. This study provides the platform on which further research on other mineral requirements and their subsequent effect on the production of essential oils and other phytochemicals from *Salvia stenophylla* can be launched.

Heterologous gene expression proved to be a potent technique in altering the secondary compound production. It produced viable plants with similar morphological features as non-transgenic plant and most importantly the transgenic plants exhibited superior (-)-α-bisabolol accumulation when compared to wild type plants. The latter is an indication of how metabolite engineering can be used to improve essential oil composition of local *Salvias.* Subsequent metabolite analysis corroborated that polar compounds were also affected by heterologous expression of *AgGPPS2* with rosmarinic being detected at more than twice the concentration in non-transgenic plants.

In summary, results from this project not only further emphasise how third generation biotechnology tools can be used to improve production and provide alternative molecular systems for farming of the medicinal *S. stenophylla*, but also open new avenues for research into the secondary pathways that yield useful phytochemicals in plants. This is the first original research on heterologous gene expression in South African sages. This industry is still in its infancy in South Africa and the continent as a whole. In conclusion, this application of science generated in this study forms a base for secondary chemical processing industry development in South African and the regional pharmaceutical sector.

7.2 Recommendation

The justification for this study was founded on the need to alter terpenoid metabolite yield and quality in plants of pharmaceutical importance and at the same time not exert pressure on natural ecosystems with harvesting of floral material for processing from the veld. This study was able to improve and provide viable alternatives to wild harvesting sage for industrial processing, but there are still other methods that can be explored to ensure maximum benefits from exploitation of biotechnology tools. From the tissue culture plants, initiation of cell suspension cultures for production of non-volatile compound could be assessed. Phenolic compounds, for instance, may accumulate during callus formation and influencing the callus to produce a particular phenolic of interest is a worthwhile study. In instances where metabolites are synthesized in specialised organs, whole plant systems will be most convenient. These cultures are easier to manage and metabolite harvesting should is less expensive as some of the metabolites will be in the liquid media.

Since industrial micropropagation requires intensive capital investment, the essential oil industry can profit in the short term if there is also work done on field cultivation of *S. stenophylla* and indeed other South African sages. Field cultivation is much easier to establish and can easily be adopted by resource poor farmers living in some eco-regions where *Salvia* is endemic, and thus it provides prospects for better livelihoods for these communities. In such cases, research centres and the corporate sector can come in with micropropagation to generate and dispatch plantlets for commercial field farming, and in the process create jobs and promote the biopharmaceutical sector based on plant natural products.

This study was centred on increasing yield and finding alternative forms of production of *S. stenophylla* phytochemicals. This resulted in changes in accumulation and in some instances metabolite profiles were altered. It would be valuable to establish the effects of these changes on pharmacological activity of *S. stenophylla* volatiles and solvent extracts. It is possible that the changes might bring about beneficial synergestic interactions and further studies on the phytochemistry and pharmacology

will elucidate existence of such relationships. Equally important was also to determine the subsequent change in enzyme activity in response to *AgGPPS2* over-expression, but the cost was prohibitive. It follows that research still needs to be done to develop cheaper routine enzyme assay methods for isoprenoid synthases.

Lastly, it is paramount that more genes in the terpene biosynthesis pathway be over-expressed in *S. stenophylla.* The *AgGPPS2* gene over-expressed catalyses the reaction producing diphosphate, the backbone for the whole monoterpenes family. This can also be extended by condensation of another isoprene molecule to form the precursor molecule for sesquiternes. However, specific terpenes are produced when these precursors undergo a cyclization step, with the aid of cyclases and synthases, resulting in different end products with different bio-activity depending on the position and nature of cyclization. Therefore, a more specific approach to gene modification can potentially yield more of specific targeted isoprenoids. Enzymes like limonene synthase, (-)-bisabolol synthase, and (+)-3-σ-carene synthase are worthy of further investigations to elucidate their activity when over-expressed simultaneously with *AgGPPS2* and farnesyl diphosphate synthase genes. Nonetheless, conclusions from this study present novel findings which encourage application of high throughput biotechnology for sustainable exploitation of medicinal flora.

Addendum I

Abstracts of articles published from this thesis

A. *Salvia stenophylla* micropropagation

Plant Growth Regul (2010) 61:287–295
DOI 10.1007/s10725-010-9476-7

ORIGINAL RESEARCH

In vitro seed germination and cultivation of the aromatic medicinal *Salvia stenophylla* (Burch. ex Benth.) provides an alternative source of α-bisabolol

Hannibal T. Musarurwa · Johannes van Staden · Nokwanda P. Makunga

Received: 29 October 2009 / Accepted: 13 April 2010 / Published online: 30 April 2010

Abstract The aromatic medicinal plant *Salvia stenophylla* contains α-bisabolol, making this plant an important contributor to the aromatherapy and cosmetic industries in South Africa. Due to its commercial importance, the cultivation of this plant using an in vitro system was considered. Firstly, seedlings were raised in vitro after breaking dormancy with light, smoke-water or chemical scarification treatments. Germination improved when seeds were smoke-treated or soaked in 70% (v/v) H_2SO_4. Vigorous plantlet regeneration was achieved when seedling explants were cultured on Murashige and Skoog (1962) medium with 5.7 µM IAA and 8.9 µM BA. The potential regeneration capacity for this protocol was estimated and over 1,000 plantlets can be produced from a single shoot (6.67 cm with 4–6 nodes) over a period of 3 months. Plants rooted easily regardless of their growth medium. This was followed by their successful rapid establishment and normal growth out of culture (75%). Finally, the volatile compounds in in vitro plants were compared to ex vitro plants via headspace solid phase microextraction linked to gas chromatography–mass spectrometry. The chemical complexity of microplants was similar to wild plants with in vitro plants continuing to produce α-bisabolol (21%) at high levels.

Keywords α-Bisabolol · Essential oil · Lamiaceae · Plant tissue culture · Seed dormancy · Smoke-induced germination

Abbreviations

2,4-D	2,4 Dichlorophenoxyacetic acid
ANOVA	Analysis of variance
BA	N_6-benzylaminopurine
HS–SPME–GC–MS	Headspace solid phase microextraction–gas chromatography–mass spectrometry
IAA	Indole-3-acetic acid
LSD	Least significant difference
MS	Murashige and Skoog (1962) medium
NAA	α-Naphthaleneacetic acid
PAR	Photosynthetically active radiation
PGR	Plant growth regulator

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Introduction

Salvia stenophylla (Burch. ex Benth.) (family Lamiaceae) is a South African sage found in almost all biomes in the country (Germishuizen et al. 2006; Jequier et al. 1980) showing that it is tolerant to a number of different environments. *S. stenophylla*, commonly known as the blue mountain sage is an upright perennial growing up to 40 cm with narrow linear oblong to oblong lanceolate aromatic leaves (Viljoen et al. 2006). Its square stem is covered with sparse hairs and brightly coloured pinkish-blue axial flowers. Traditionally, *S. stenophylla* is used as a

B. Optimization of potassium and nitrogen requirements for *in-vitro* cultivation of *Salvia stenophylla*

J Plant Growth Regul (2012) 31:207–220
DOI 10.1007/s00344-011-9232-x

Chemical Variation in Essential Oil Profiles Detected Using Headspace Solid-Phase Microextraction Gas Chromatography Spectrometry in Response to Potassium, Nitrogen, and Water Available to Micropropagated Plants of *Salvia stenophylla* (Burch. *ex* Benth.)

Hannibal T. Musarurwa · Lerí Koegelenberg · Nokwanda P. Makunga

Received: 4 February 2011 / Accepted: 1 July 2011 / Published online: 24 September 2011

Abstract The effects of nitrogen, potassium, water stress, and phytohormones were studied using a *Salvia stenophylla* (Burch. *ex* Benth.) microplant system. In vitro regeneration was monitored and then followed with volatile secondary metabolite profiling through headspace solid-phase microextraction gas chromatography. Plantlet growth was most prolific on half-strength Murashige and Skoog medium without phytohormones. An increase in macronutrients supplied to the microplants enhanced accumulation of the commercially important (−)-α-bisabolol, while no significant changes to the relative abundance of β-bisabolene, α-muurolene, α-patchoulene, and D-limonene (among others) became apparent. Water-stressed plants, treated with sorbitol and polyethylene glycol, had a lowered rooting capacity in vitro. Overall, as a plant production system, micropropagation did not have deleterious effects on the biochemistry of *S. stenophylla* as no significant differences in metabolic profiles existed between conventional garden plants and in vitro propagules, regardless of phytohormone treatment. We also show that nutrient manipulation can be used efficiently as a strategy for positively altering secondary metabolism. This will ultimately benefit the domestication of this commercially important medicinal herb.

Electronic supplementary material The online version of this article (doi:10.1007/s00344-011-9232-x) contains supplementary material, which is available to authorized users.

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Keywords (−)-α-Bisabolol · Essential oil · Metabolite profiling · Secondary metabolites · Polyethylene glycol · Sorbitol

Introduction

Plant secondary metabolism is biochemically and genetically complex. It is a function of spatial, temporal, and phenological factors, with seasonal changes and species growth patterns impacting both metabolite synthesis and subsequent accumulation. Therefore, metabolite heterogeneity within species is often reliant on biotic and abiotic factors. *Salvia stenophylla* Burch. *ex* Benth. (Lamiaceae) (Fig. 1a), or blue mountain sage, is one of 26 indigenous South African species (Kamatou and others 2005; Germishuizen 2006). This small, bushy perennial herb (average height = 40 cm), with flowers varying from blue to purple and which bloom from August to February (Jequier and others 1980), has a wide geographical distribution in South Africa (Germishuizen 2006).

Similar to other sages, *S. stenophylla* produces a diverse range of volatile compounds of commercial significance (Kamatou and others 2008) consisting primarily of monoterpenes and sesquiterpenes along with their oxygenated derivatives, which could be alcohols, aldehydes, esters, ethers, ketones, phenols, or oxides (Longaray Delamare and others 2007), rendering the oil aromatic. Apart from (−)-α-bisabolol, an important component of dermaceutical and cosmeceutical products due to its anti-inflammatory, anti-irritant, antimicrobial, and cicatrizant activity (Kamatou and others 2010), major compounds of industrial importance include manool, limonene, δ-3-carene, α- and β-pinene, camphor, and camphene, among many others. These biochemicals play a major role in the quality of

C.

Figure A1.1 Flowering transgenic *Salvia stenophylla* plant acclimatized in the green house featured on the cover page of the June issue of the Journal of Plant Growth Regulation (31:2).

Addendum II

Abstracts from conference contributions from this thesis

A. International Organization of Chemical Sciences in Development (IOCD) conference 2011, Cape Town, South Africa

Musarurwa HT, Koegelenberg L, Makunga NP (2011) *In-vitro* increase of potassium and nitrogen availability improves (-)-α-bisabolol accumulation in *Salvia stenophylla* (Burch. ex Benth; Family Lamiaceae) microplants

Plant Secondary compounds are produced to facilitate plant-environment interactions. Commercially, these are highly desired for cosmeceutical and pharmaceutical industries but are synthesized in minute quantities to meet current demands, posing an environmental stress on wild populations as cultivation is negligible. Micropropagation was established for *Salvia stenophylla* to aid conservation and domestication, providing an easy-to-manipulate biotechnological tool to study effects of nitrogen, potassium, osmotica and phytohormonal supplementation on plantlet productivity, and volatile secondary metabolism. Headspace-solid phase microextraction gas chromatography mass spectrometry was used for metabolomic assessment and an optimal split injection ratio of 1:20 with heating at 15 min at 80 ºC for releasing volatiles. Wild plants, *in-vitro* plants and greenhouse acclimated plants had similar metabolomes, validating use of this alternative production system (Musarurwa et al. 2010). *In-vitro* organogenesis and growth was more prolific when plantlets where cultured on medium, free of phytohormone supplementation, with half the complement of Murashige and Skoog (1962) salts. A superior rooting capacity was the most dramatic developmental change noted *in-vitro*. A three-fold increase in potassium and nitrogen supply elevated (-)-α-bisabolol accumulation from 51.87% and 48.08%, to 61.01% and 61.14%, respectively. Otherwise, only insignificant changes to β-bisabolene, α-muurolene, α-patchoulene and D-limonene concentrations were apparent. α-Guaiene, δ-guaiene, guaiol, α-copaene, humulene, muurolene were detected only in sorbitol-treated microplants, possibly indicating significance of water deprivation and added carbon availability to their biosynthesis. Apart from showing that tissue culture has insignificant deleterious impacts for *S. stenophylla*, we also show for the first time that nutrient manipulation can successfully be used as a strategy for improving secondary metabolism in this herbal species.

References

Murashige T. and Skoog F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15:473–497

Musarurwa, H.T., van Staden, J. and Makunga, N.P. 2010. *In-vitro* seed germination and cultivation of the aromatic medicinal *Salvia stenophylla* (Burch. ex Benth.) provides an alternative source of α-bisabolol. Plant growth regulation 21: 287-295

Figure 1 Relative accumulation of *Salvia stenophylla* metabolites in response to different nutrient concentration *in-vitro* after a six week growth period on semi-solid MS medium. Stacked columns represent metabolites identified for each treatment and the maximum value shows the total number of compounds identified.

B. South African Association of Botanists Conference 2011, Grahamstown, South Africa

Makunga NP, Musarurwa HT, Koegelenberg L, Ndimande GS (2011) Altered volatile compounds of *in-vitro* microplants *Salvia stenophylla* (Burch. *ex* Benth)

Aromatic medicinal plants often display essential oil polymorphism and this is a function of the genotype and environmental factors. Through using an *in-vitro* system, we were able to determine the effects of nitrogen, potassium, water stress and phytohormones on the essential oil chemicals of *Salvia stenophylla* (Burch. ex Benth) microplants. Leaf volatiles were monitored using headspace solid phase microextraction gas chromatography spectrometry (HS–SPME–GC-MS) and significant changes to the accumulation of the commercially important (–)-α-bisabolol were detected due to changing *in-vitro* conditions whilst minor changes to the relative abundance of β-bisabolene, α-muurolene, α-patchoulene and D-limonene (amongst others) were apparent. Concomitantly, transgenic clones of S. stenophylla, generated through *Agrobacterium*-transformation, over-expressing a heterologous geranyl diphosphate synthase gene (GPS) exhibited as higher levels of (–)-α-bisabolol. Pinene and δ-3-carene were negatively affected by the introduction of the transgene. Transformation, on the other hand, proved a valuable system for improving metabolite quality affecting terpenoid biosynthesis. (–)-α-Bisabolol accumulation was directly correlated to potassium and nitrogen availability. However, little to no impact was detectable on other secondary compounds. Plants generated in this study may serve as an alternative commercial source of secondary metabolites produced *by S. stenophylla.*

C. ANALITIKA 2010, Stellenbosch, South Africa

Musarurwa HT, Makunga NP (2010) Effect of tissue culture, genetic and nutrient manipulation of secondary compound profile of *Salvia stenophylla*

Volatile compounds of aromatic medicinal plants often determine their efficacy and quality. Their relative abundance is a function of growth conditions and the genotype of individuals resulting in essential oil polymorphism. Through altering the genome of *Salvia stenophylla* using Agrobacterium-transformation, we aimed at over-expressing a heterologous geranyl diphosphate synthase (GPS) gene. Non-transgenic plants were subjected to varying *in-vitro* growth conditions so as to improve metabolite yield and quality through manipulation of nitrogen and potassium concentrations prior to gas chromatography mass spectrometry (GC-MS). The complexity of essential oil compounds was determined after solid phase micro-extraction (SPME) or hydro-distillation of foliage followed by direct liquid injection. Metabolite profiles of transgenic and non-transgenic microplants showed significant variation based on nutrient levels in growth medium. Caryophellene was detected in microplants but not in wild type. Bisabolol (an anti-allergenic skin care additive) showed a 6% increase whilst pinene and δ-3-carene were negatively affected by transgenesis. Overall, fewer metabolites were evident with use of liquid injection with monoterpenes (84.21 %) and sesquiterpenes (15.79 %) being the major compunds. Through SPME-GC-MS indicated higher diversity of secondary volatiles where monoterpenes (54.55 %), sesquiterpenes (36.36 %) and diterpenes (9.09 %) composed the largest fraction of metabolites.Tricvclene and O-cymene were only detectable through use of liquid injection of the hydrodistillate. Bisabolol accumulation was correlated to potassium and nitrogen availability but mineral nutrition had little to no effect on other secondary compounds. Transformation, on the other hand, proved a valuable system for improving metabolite quality affecting terpenoid biosynthesis.

D. Student Conference on Conservation Science, 2010, New York City, USA

Musarurwa HT, Ndimande SG, Makunga NP (2010) Conservation of a South African Sage using Biotechnology

Salvia stenophylla secondary compounds are used as additives in cosmeceuticals and medicines in southern Africa and their demand is on the increase compared to sensitive synthetic compounds. However, like most secondary metabolites they are synthesized in low quantities and there is little cultivation of these species leading to most of the plant material being wild-harvested. We aimed at increasing the terpene production in *S. stenophylla by* over-expressing the heterologous geranyl diphosphate synthase gene (*AgGPPS2*) from *Abies grandis*, using *Agrobacterium tumefaciens* (EHA105). This led to the development of a new micropropagation protocol for *S. stenophylla*. The plants proliferated best on medium supplemented with 5.7 µM indole acetic acid (IAA) and 8.9 µM N-6-benzyladenine (BA). Metabolite profiling through solid phase microextraction-gas chromatograpy mass showed that transformed *S. stenophylla* plants had 6% increase in (-)-α-bisabolol accumulation whilst 3-δ-carene, pinene and camphor were lowered compared to non-transgenics. Tissue cultured plants synthesized 'new' compounds that were not detected in the wild type during analysis. This suggested that changing the growth conditions can be a potential tool for improving metabolite composition which could be modified for industrial application. Over-expression of the *AgGPPS2* gene positively influences the essential oil production of *S. stenophylla* which are of pharmaceutical importance.

E. South African Association of Botanists conference 2009, Stellenbosch, South Africa

H.T. Musarurwa, N.P. Makunga (2009) Establishing a tissue culture system to increase secondary metabolite production: Over-expression of geranyl diphosphate synthase to up-regulate production of terpenes in *Salvia stenophylla*

Salvia stenophylla is known to have medicinal properties that are important in the cure of a number of skin diseases. This is largely due to the presence of essential oils, which are derived from terpenoid synthesis regulated by the enzyme geranyl diphosphate synthase. The study is aimed at improving the levels of secondary compounds of the essential oil produced by this plant by over-expressing the gene coding for geranyl diphosphate synthase using *Agrobacterium* transformation. Such improvements have conservation and economic benefits as they will not only reduce wild harvesting of the plant, but also provide a basis for *in-vitro* production of metabolites of interest. Germination in *S. stenophylla* seeds was investigated and seeds were subjected to four different treatments (chemical scarification, smoke extract treatment, smoke extract and scarification). The control was left untreated. Smoke-treated and scarified seeds exhibited a high germination under both dark and light conditions and had similar effect germination ($P>0.05$). This suggests that *S. stenophylla* seed germination is depended on smoke as a germination cue and the removal of the seed coat. However, a combination of both treatments had no effect on germination ($P<0.05$). Some of the germinating seeds were used to establish a tissue culture system. Upon establishment of *in-vitro* seedlings, *Agrobacterium*-mediated transformation with the *AgGPPS2* synthase gene (Burke and Croteau, 2002) was examined using hypocotyl and cotyledon explants. The *AgGPPS2* gene was sub-cloned into a pCAMBIA vector and *Agrobacterium tumefaciens* EHA105 transformation was conducted using standard methods. Selection of putative transformants was done using kanamycin-supplemented medium and transformation was tested using PCR analysis.

Addendum III

Media for *in-vitro* plant cultivation and bacterial growth

Table A3.1 Murashige and Skoog medium (1962)

Components	**Amounts (mg L^{-1})**
$MgSO_4 7H_2O$	370
KH_2PO_4	170
KNO_3	1900
NH_4NO_3	1650
$CaCl_2$	440
Micronutrients	
H_3BO_3	6.2
$MnSO_4 4H_2O$	22.3
$ZnSO_4 7H_2O$	8.6
$Na_2MoO_4 2H_2O$	0.25
$CoCl_2 6H_2O$	0.025
KI	0.83
$FeSO_4 7H_2O$	27.8
$NaEDTA_2H_2O$	37.3
Organic supplements	
myo-inositol	100
glucose	30 000

Table A3.2 Bacterial media

Component	Amount (g L^{-1})
LB medium	
Yeast extract	5
Tryptone	10
Sodium chloride	10
Agar	15
YMB medium	
yeast extract	5
KH2PO4	0.1
Mannitol	10
Magnesium sulphate	0.1
Agar	15

Addendum IV

Transformation vector construct and sequence of inserted *AgGPPS2*

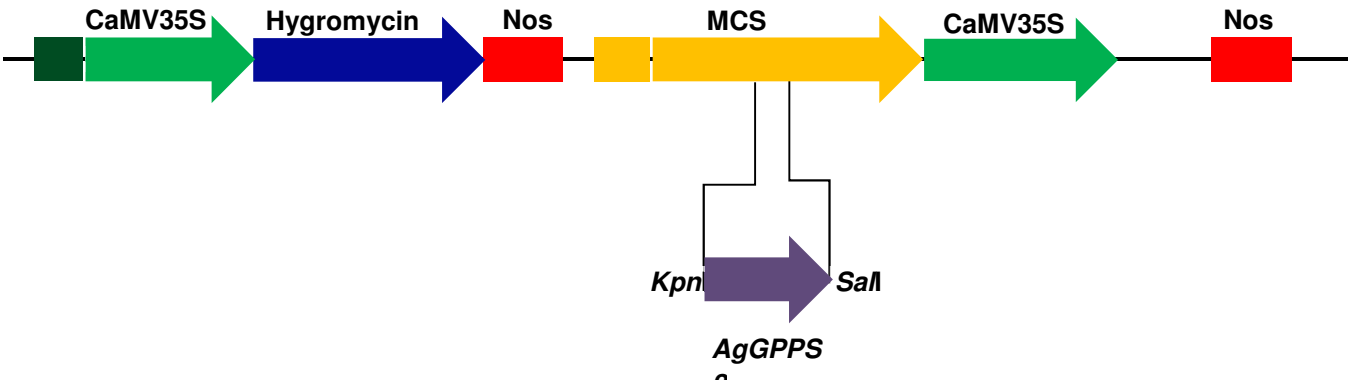

Figure A4.1 A diagrammatic representation of pCAMBIA1301 plant transformation vector used for transforming *Salvia stenophylla*. The vector is harboring the CaMV35S promoter, AgGPPS2 gene and had the GUS gene knocked out.

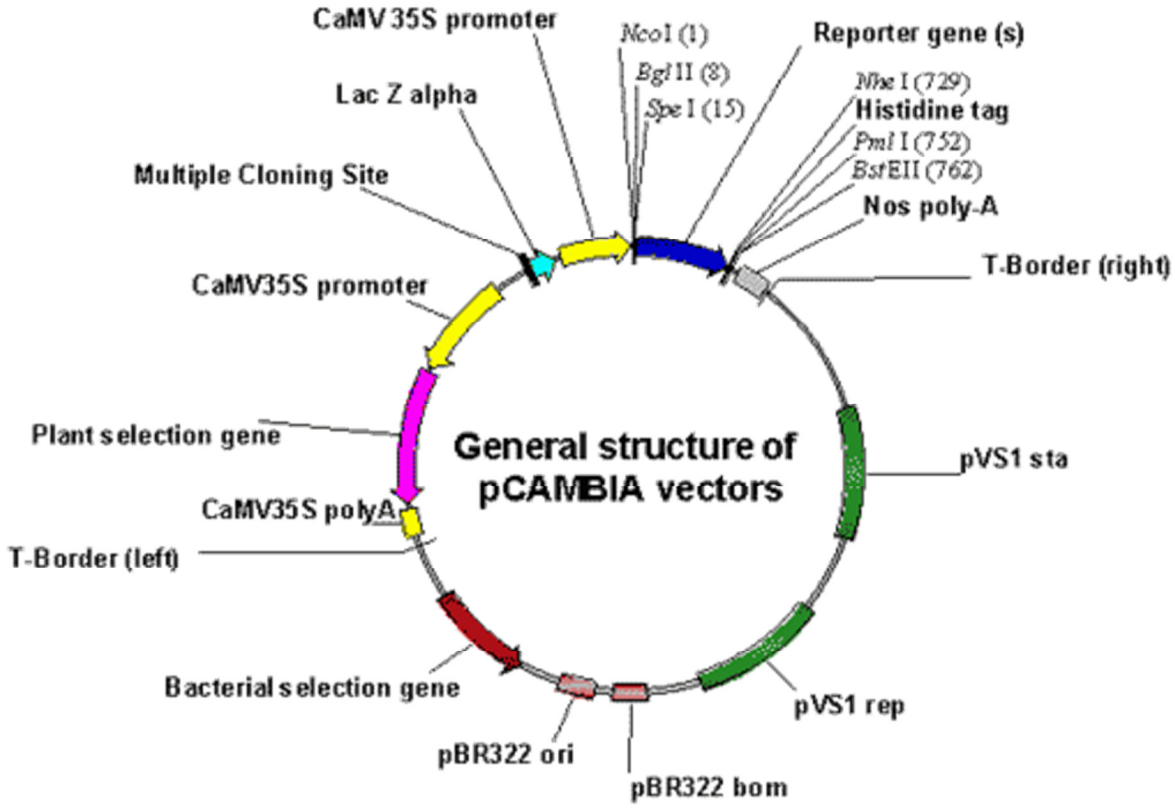

Figure A4.2 The pCAMBIA transformation prior to modification to insertion of *AgGPPS2* (http://www.cambia.org/daisy/cambia/2046/version/1/part/4/data/pCAMBIA1301.pdf?branch=main&language=default)

Basic local nucleotide alignment using BLASTN for heterologous AgGPPS2 inserted in *S. stenophylla*

A. Reverse sequence

Score = 1291 bits (699), Expect = 0.0

Identities = 746/766 (97%), Gaps = 18/766 (2%)

Strand=Plus/Minus

```
Query  394   TGAGTATTGCTCGCCTTGAATCACGGCCAGTCGGCCTGTARACGGGCCATCGACTGAATA  453
              |||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  1524  TGAGTATTGCTCGCCTTGAATCACGGCCAGTCGGCCTGTAGACGGGCCATCGACTGAATA  1465

Query  454   TCCGGTTTGAGTTTGCAGTTGGACTAGATTTCACAATTAAAATGCTGTGCTCAAGTTTTC  513
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1464  TCCGGTTTGAGTTTGCAGTTGGACTAGATTTCACAATTAAAATGCTGTGCTCAAGTTTTC  1405

Query  514   ACAATACAAGAAGTTGCAGTGCTACATCATAGATAGTATACGGAGGGCAGGGAACGCAGG  573
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1404  ACAATACAAGAAGTTGCAGTGCTACATCATAGATAGTATACGGAGGGCAGGGAACGCAGG  1345

Query  574   TCTAACTCTGTTACTTTAAAAAACTATTAAACCAAGATTATCCACTCTAACAAACAAGAA  633
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1344  TCTAACTCTGTTACTTTAAAAAACTATTAAACCAAGATTATCCACTCTAACAAACAAGAA  1285

Query  634   CGCTTTGAGGAGGCTCTTCAATTTTGTCTGAATGCCACGTAATCTGCAAGACCCAACAGA  693
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1284  CGCTTTGAGGAGGCTCTTCAATTTTGTCTGAATGCCACGTAATCTGCAAGACCCAACAGA  1225

Query  694   GGTGCTGCCTTCACTGGATCGAAGCAAGATAACTCTCCCTTAGCTCTGTTCAACAATTCA  753
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1224  GGTGCTGCCTTCACTGGATCGAAGCAAGATAACTCTCCCTTAGCTCTGTTCAACAATTCA  1165

Query  754   TCAGAAAACTCCTTTGCTTTCTCCAAACCCATGAGCTTTGGATAAGTTGCCTTATCACTA  813
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1164  TCAGAAAACTCCTTTGCTTTCTCCAAACCCATGAGCTTTGGATAAGTTGCCTTATCACTA  1105

Query  814   ATCAAATCCTTTCCTGCAGTCTTGCCCAGTTCGTCTGATGATTTCGTGACATCGAGTATG  873
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1104  ATCAAATCCTTTCCTGCAGTCTTGCCCAGTTCGTCTGATGATTTCGTGACATCGAGTATG  1045

Query  874   TCATCCACAACCTGAAAAAGAAGCCCCACGCAACGGGCATACCTTCGAGCTCTCTCGATC  933
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1044  TCATCCACAACCTGAAAAAGAAGCCCCACGCAACGGGCATACCTTCGAGCTCTCTCGATC  985

Query  934   ACAATCTCCGAAGCACCACCGATGATCGCCCCACACACAACCGAGCACTCCAAGAGCATT  993
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  984   ACAATCTCCGAAGCACCACCGATGATCGCCCCACACACAACCGAGCACTCCAAGAGCATT  925

Query  994   GCAGTCTTGTGA-TATGA-TCCATTCCAGAGTCTGA-GGTCAATAGA-GGATCCC-TTCG  1048
              |||||||||||| ||||| |||||||||||||||| |||||||||| ||||||| ||||
Sbjct  924   GCAGTCTTGTGAATATGAATCCATTCCAGAGTCTGAAGGTCAATAGAAGGATCCCCTTCG  865


Query  1049  CTGGCAATATCGAC-ATCTGGCCACCCATA-CCCCT-CAGAGC-TGT-GCTCTACCCAGT  1103
              ||||||||||||| ||||||||||||||| ||||| |||||| ||| ||||||||||||
Sbjct  864   CTGGCAATATCGACCATCTGGCCACCCATAACCCCTTCAGAGCCTGTTGCTCTACCCAGT  805

Query  1104  -CAGATAC-ATC-TCAA--TC-TATCAGCCCCM-CAGTTT-GCTTG  1141
               ||||||| ||| ||||  || ||||||||||  |||||| |||||
Sbjct  804   TCAGATACCATCCTCAAAATCCTATCAGCCCCCACAGTTTTGCTTG  759
```

B. Forward sequence

Score = 1949 bits (1055), Expect = 0.0

Identities = 1091/1108 (98 %), Gaps = 10/1108 (1 %)

Strand=Plus/Plus

```
Query  72    GATTTTCCCTGCTACAAAAATCTATGTATTGTTTCTTCAGTTGCATTCAAAGATCTATAG  131
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     GATTTTCCCTGCTACAAAAATCTATGTATTGTTTCTTCAGTTGCATTCAAAGATCTATAG  60

Query  132   AATTTCGAGCTTTGAATTTGTTTTAAGAGTTTATAGCAGGAGCTAATCATATAAAGAGGA  191
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    AATTTCGAGCTTTGAATTTGTTTTAAGAGTTTATAGCAGGAGCTAATCATATAAAGAGGA  120

Query  192   CATGGCTTACAGTGCTATGGCAACCATGGGTTACAATGGTATGGCAGCTAGCTGCCATAC  251
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   CATGGCTTACAGTGCTATGGCAACCATGGGTTACAATGGTATGGCAGCTAGCTGCCATAC  180

Query  252   CCTGCATCCTACCAGCCCATTAAAACCTTTTCATGGAGCTTCAACCTCACTGGAAGCTTT  311
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   CCTGCATCCTACCAGCCCATTAAAACCTTTTCATGGAGCTTCAACCTCACTGGAAGCTTT  240

Query  312   TAATGGCGAGCATATGGGCCTCCTCCGAGGGTATTCGAAGAGGAAGCTATCTTCATATAA  371
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Sbjct  241   TAATGGCGAGCATATGGGCCTCCTCCGAGGGTATTCGAAGAGGAAGCTATCTTCATATAA  300

Query  372   AAATCCGGCATCTAGATCCTCAAACGCTACAGTTGCCCAGTTGCTTAATCCTCCACAAAA  431
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Query  852   TAGGATTTTGAGGATGGTATCTGAACTGGGTAGAGCAACAGGCTCTGAAGGGGTTATGGG  911
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Sbjct  841   TGGCCAGATGGTCGATATTGCCAGCGAA-GGGGATCCTTCTATTGACCTTCAGACTCTGG  899
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Sbjct  900   AATGGATTCATATTCACAAGACTGCAATGCTCTTGGAGTGCTCGGTTGTGTGTGGGGCGA  959

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Sbjct  960   TCATCGGTGGTGCTTCGGAGATTGTGATCGAGAGAGCTCGAAGGTATGCCCGTTGCGTGG  1019

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Sbjct  1020  GGCTTCTTTTTCAGGTTGTGGATGACATAC-TC-GATGTCACGAAATCATCAGA-CGAAC  1076

Query  1148  TGG-CAAGACTGCAGGAA-GAATTGGAT  1173
             ||| |||||||||||||| | ||| |||
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Addendum V

GC-and LC-MS total ion chromatograms for *Salvia stenophylla*

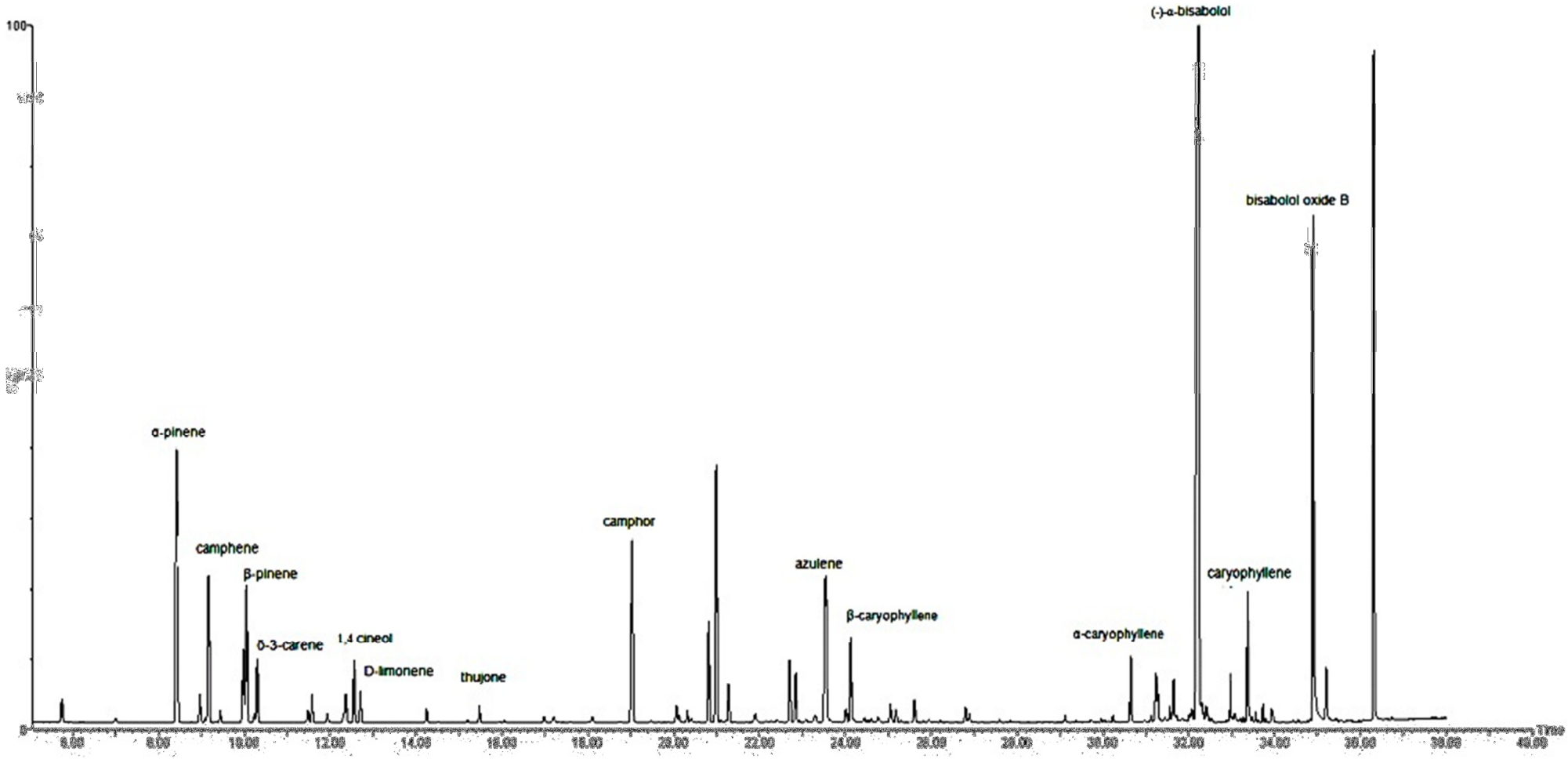

Figure A5.1 GC-MS profile *Salvia stenophylla* essential oil metabolites

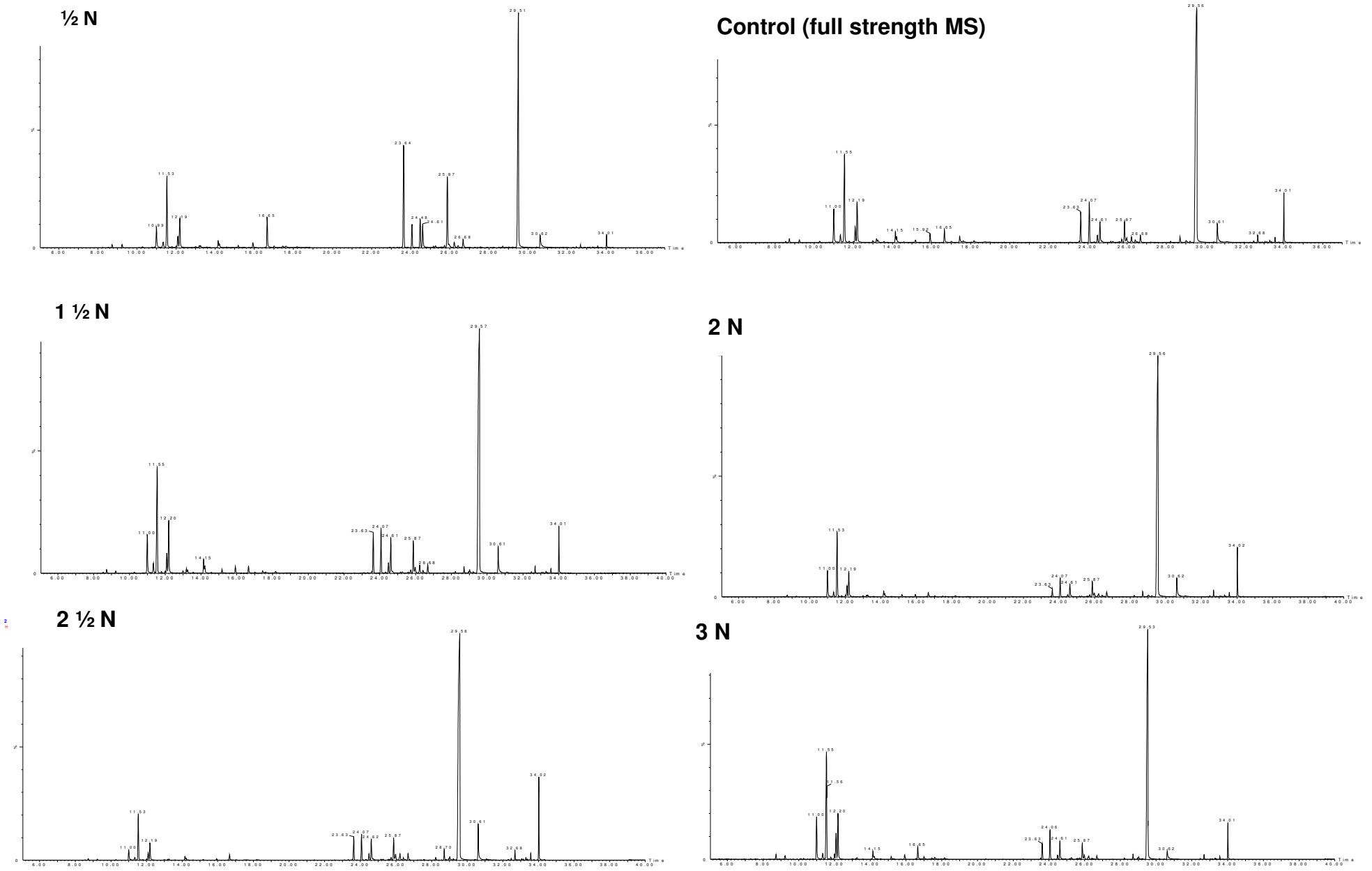

Figure A5.2 Metabolite profile of *S. stenophylla* volatile fraction in response to different nitrogen levels

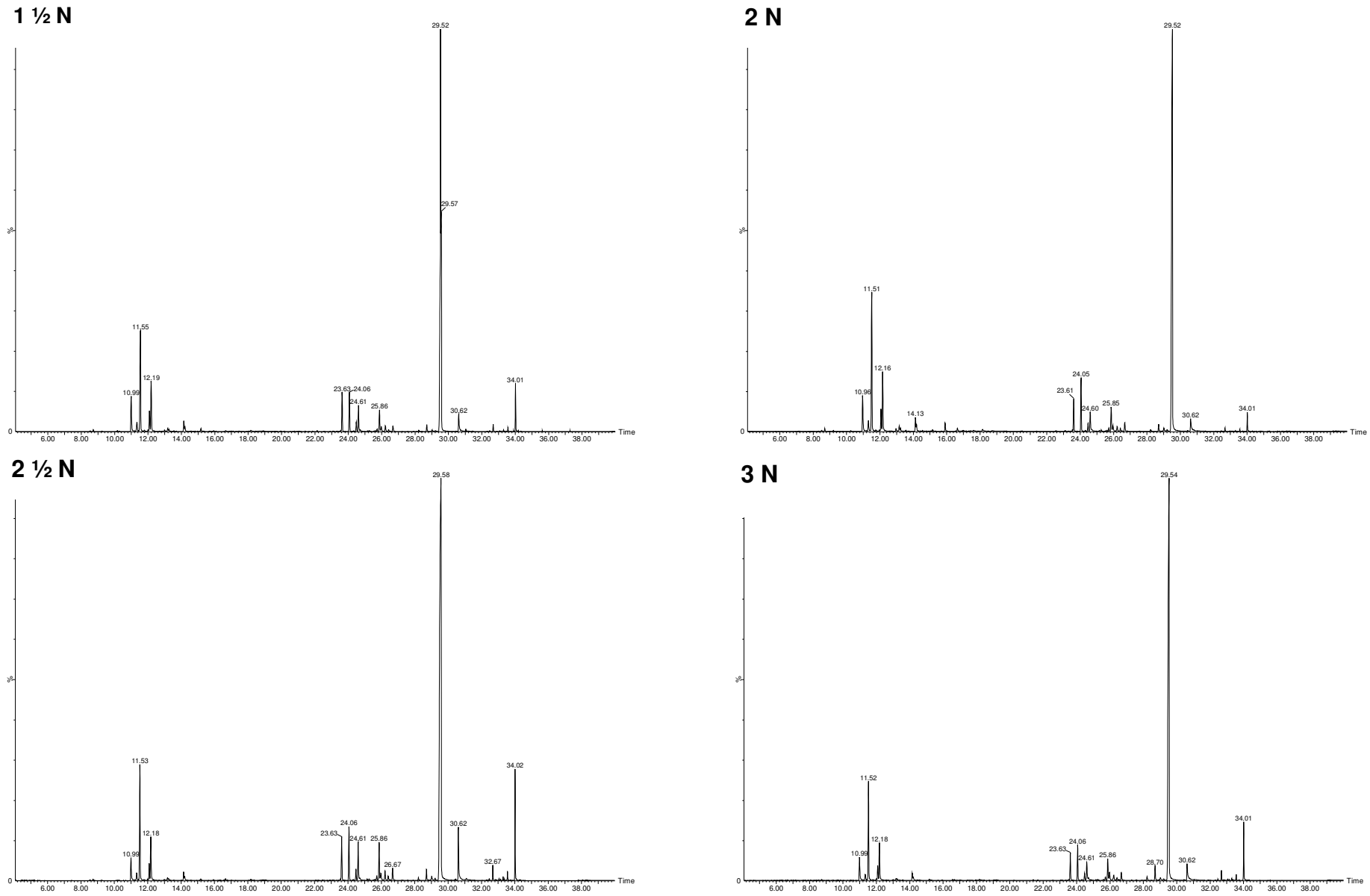

Figure A5.3 Metabolite profile of *S. stenophylla* volatile fraction in response to different potassium levels

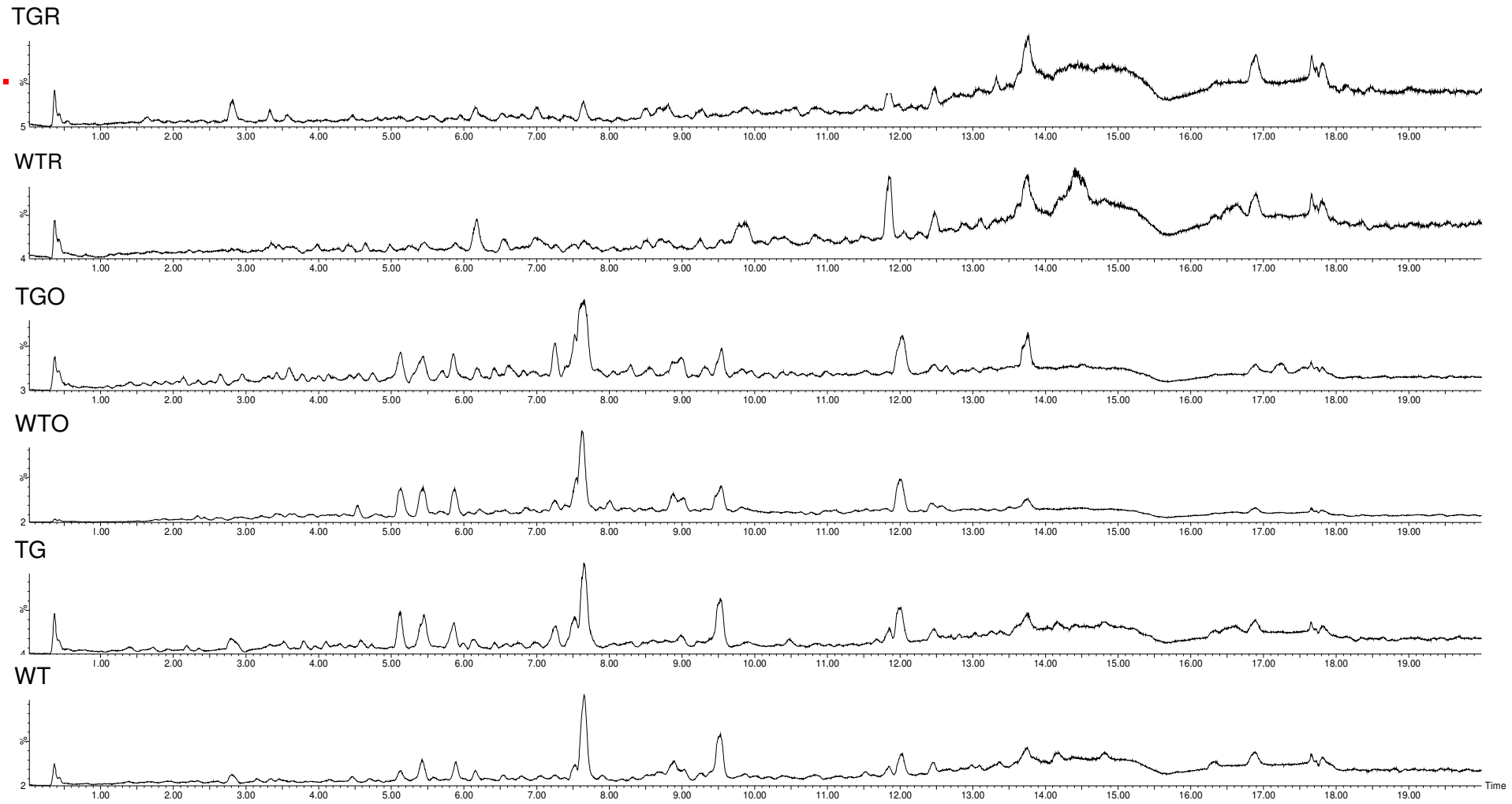

Figure A5.4 LC-MS ESI (+ve) total ion chromatograms showing metabolite profiles for transgenic and non-transgenic plants WT and TG represent wild type and transgenic in-vitro plants respectively, WTR and TGR denotes wild type and transgenic root extracts respectively and WTO and TGO are 10 week old greenhouse acclimatized plants

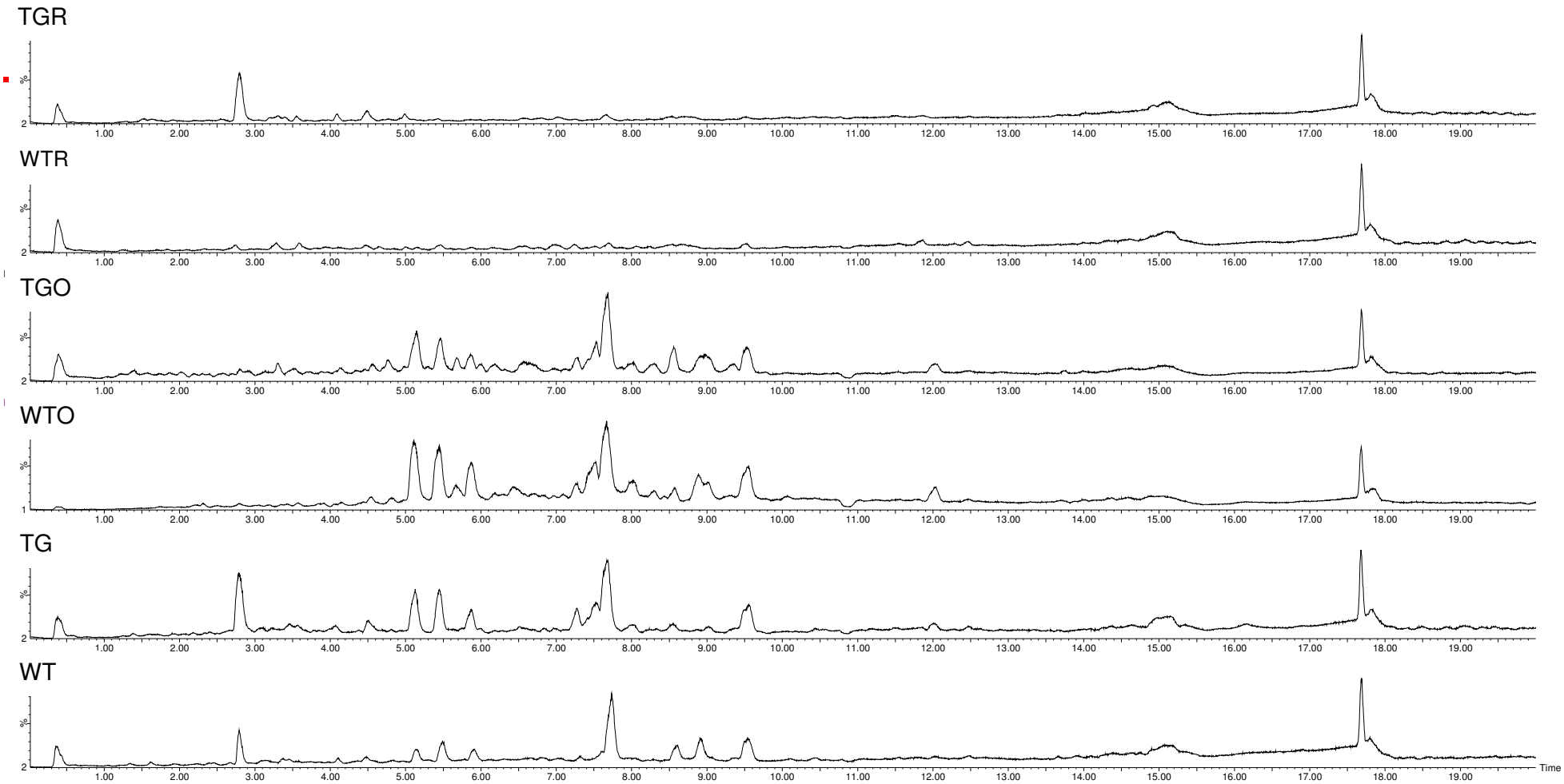

Figure A5.5 LC-MS ESI (-ve) total ion chromatograms showing metabolite profiles for transgenic and non-transgenic plants WT and TG represent wild type and transgenic in-vitro plants respectively, WTR and TGR denotes wild type and transgenic root extracts respectively and WTO and TGO are 10-week old greenhouse acclimatised plants

Addendum VI

Principal component analysis of *Salvia stenophylla* solvent extracts

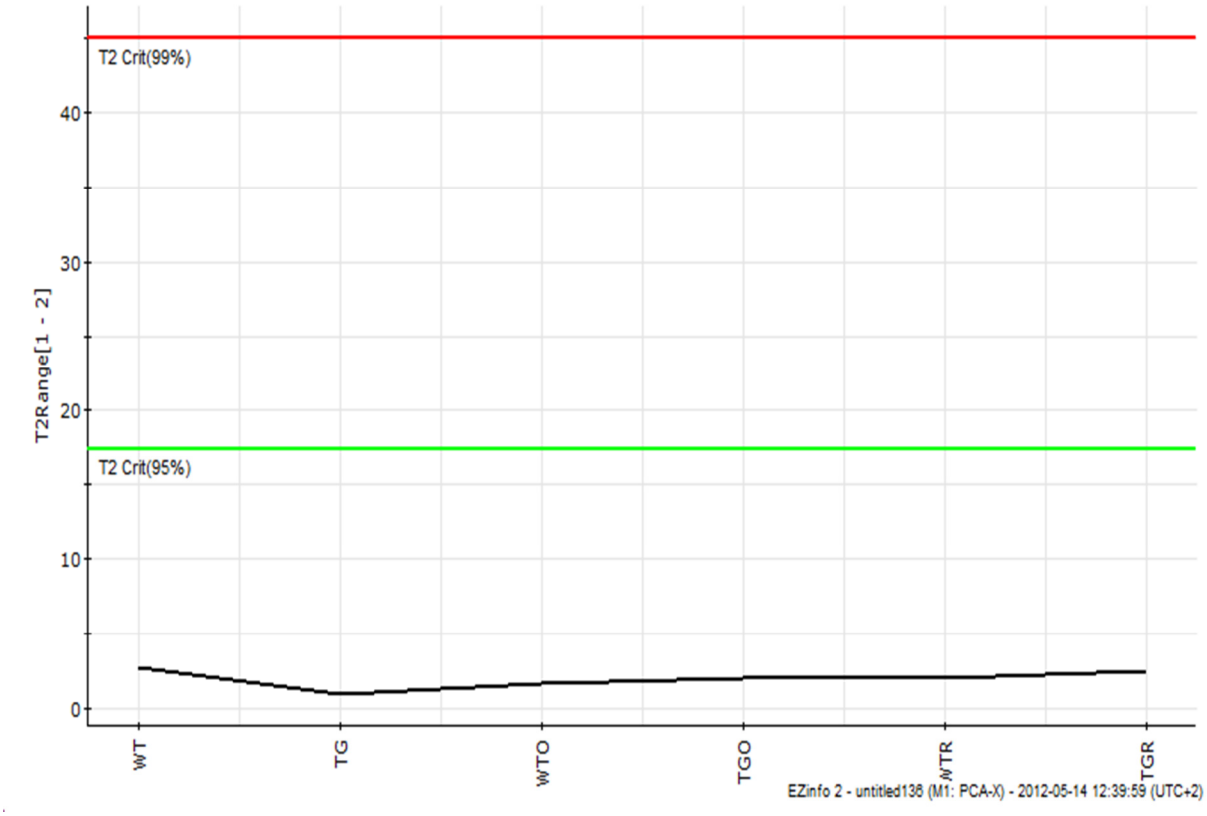

Figure A6.1 Hotelling's T2 range score measuring the distance between factor 1 and factor 2 of the PCA analysis. Scores above the green and red horizontal lines shows the probability that they are similar to each other is less than 5 % and 1 % respectively.

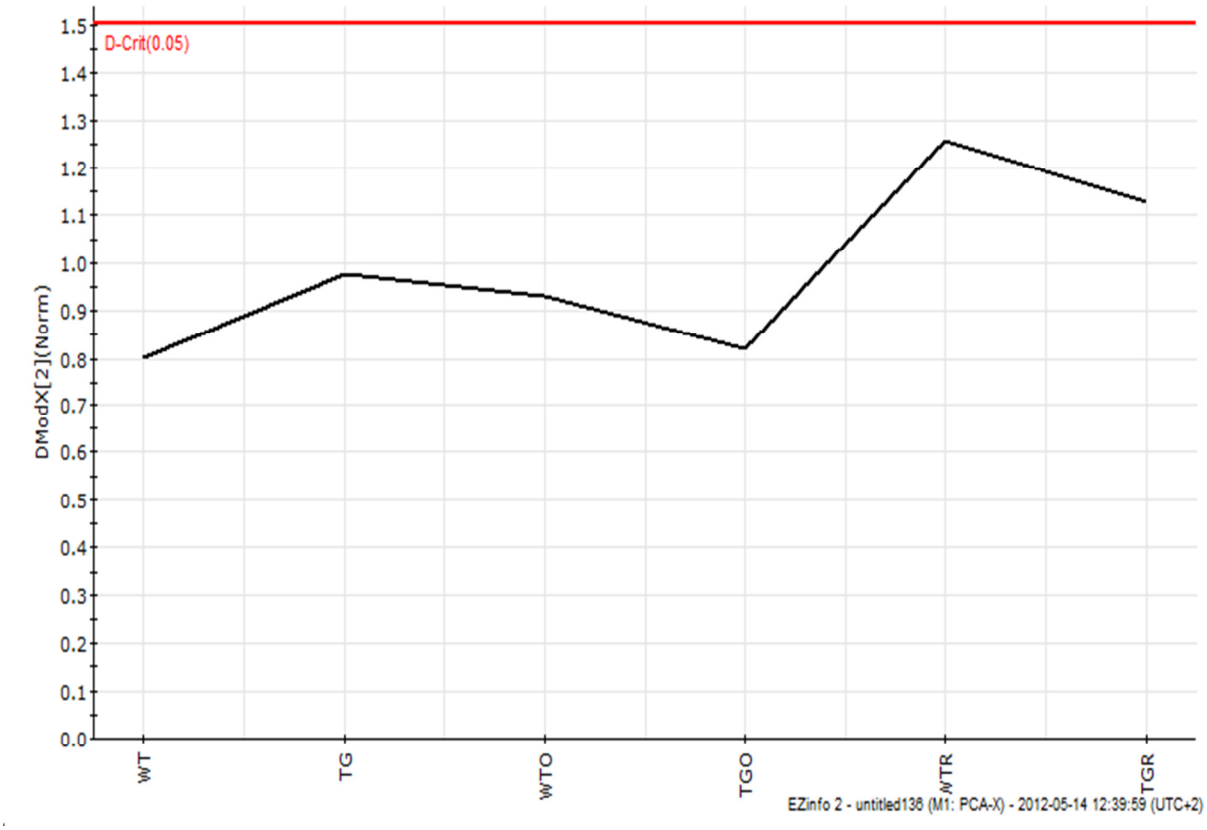

Figure A6.2 Distance model summarizing the noise of observation during the PCA analysis. The larger the noise the less similar is the observation to the others and plots above the red line are significantly dissimilar.

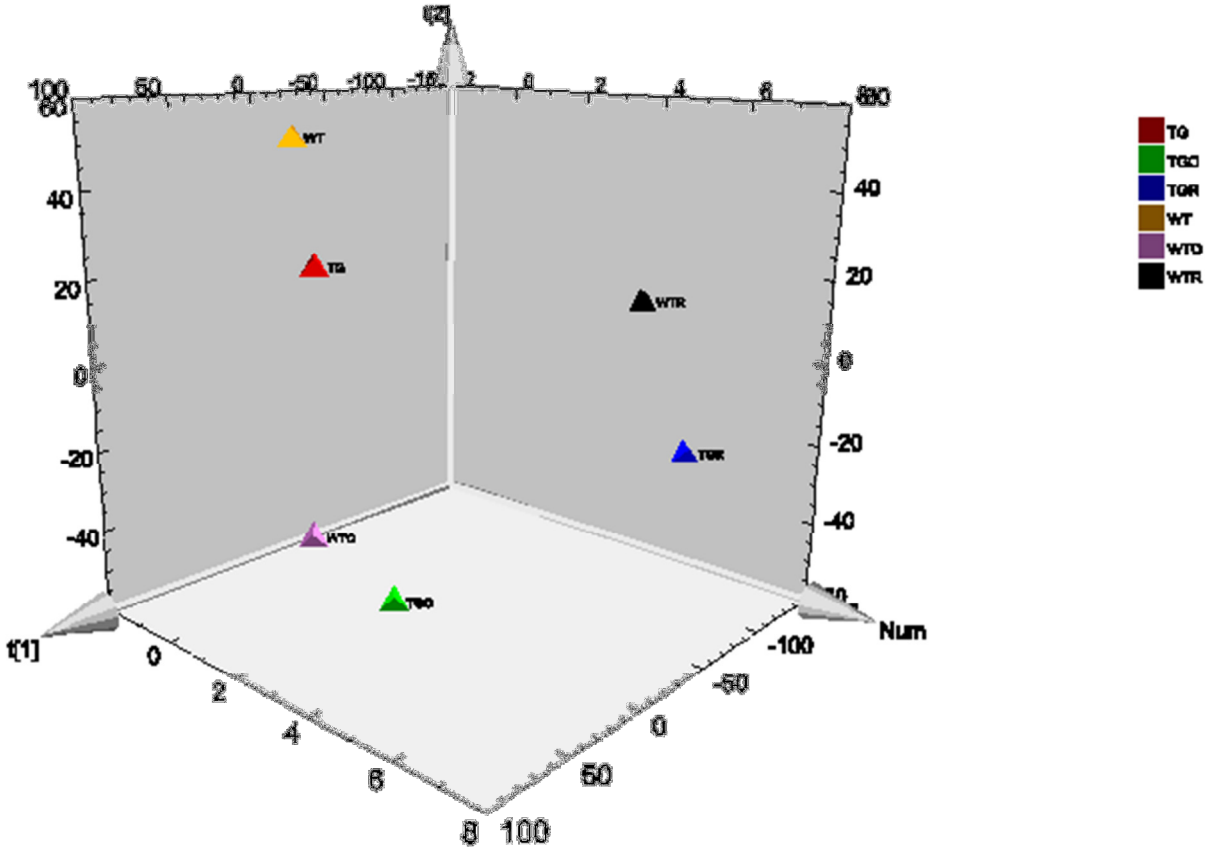

Figure A6.3 A 3D PCA plot (comp [t1] vs. num vs. score [t2]) of LC-MS (ESI-) profiles showing variations in metabolite accumulation between *in-vitro* wild type and transgenic, young and old, and root and aerial part plant polar extracts. The distances of plots from each other depict the difference from one another. The greater the difference the greater the distance between plots