

**Influence of strigolactones and auxin on
Sutherlandia (Lessertia) frutescens in vitro plant
tissue cultures**

by

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Science in the Faculty of Science at Stellenbosch University



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Declaration by student

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Declaration by supervisors

We hereby declare that we acted as Supervisors for this MSc and regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the Faculty of Science for examination by the University-appointed examiners.

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Abstract

Sutherlandia frutescens (L.) R. Br., also known as *Lessertia frutescens*, is a leguminous shrub indigenous to southern Africa. Traditionally this plant has been used for the treatment of various ailments; current interest in this plant has escalated after it was announced that extracts could aid in the relief and treatment of HIV/AIDS. These extracts contain an array of metabolites, including sutherlandins, sutherlandiosides L-arginine, L-canavanine, asparagine, gamma-aminobutyric acid (GABA), and various other amino acids, which have been linked to medicinal uses. This study focused on the use of hormones to promote the growth and metabolite production of *S. frutescens in vitro* cultures. The growth promoting substances used in this study were synthetic analogues of strigolactones, GR24 and Nijmegen-1, and auxins, indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA).

The first part of this study focused on the effects strigolactones and auxins, alone and combined, had on the growth of *S. frutescens in vitro* nodal explants. The *S. frutescens* nodal explants had the most significant improvement in growth with treatments that contained 1 mg/L NAA. These treatments increased growth *via* fresh and dry mass and plant length. The metabolite content of these nodal explant cultures was evaluated using liquid chromatography/mass spectrometry (LC/MS) metabolite analysis. The treatments that contained 1 mg/L NAA differed in metabolite composition and showed an increase in metabolite quantity. The SU1 content of the treated plants was also quantified using LC/MS techniques and a combination of 1 mg/L NAA and Nijmegen-1 doubled the amount of SU1.

The effect of strigolactones was also studied using hairy root cultures of *S. frutescens*. Strigolactones alone slightly inhibited the formation of lateral transgenic roots, but when these chemicals were used in combination with auxins, significant reduction in dry mass and lateral

root outgrowth resulted. Of the treatments tested in this study, 0.1 mg/L IBA caused noticeable alterations to the metabolite pool, with amino acids such as GABA and arginine accumulating at higher levels than the control explants.

The exploitation of hormones to up-regulate the growth and metabolism of the medicinally important plant, *Sutherlandia frutescens*, proved successful in this study. The use of *in vitro* nodal explants along with hairy root cultures has assisted in the establishment of a stable system for the up-regulation of metabolites.

Opsomming

Sutherlandia frutescens (L.) R. Br., ook bekend as *Lessertia frutescens*, is 'n peulagtige struik inheems tot suider Afrika. Tradisioneel is die plant vir 'n groot verskeidenheid van kwale gebruik; huidige belangstelling in die plant het toegeneem nadat dit bekend gemaak was dat ekstraksies vanaf hierdie plant verligting kan bied vir MIV/VIGS. Hierdie ekstrakte bevat 'n verskeidenheid van metaboliete, insluitend sutherlandins, sutherlandiosiede L-arginien, L-kanavanien, asparagien, gamma-aminobottersuur (GABS), asook verskeie ander aminosure wat medisinale gebruike het. Die studie het gefokus op die gebruik van hormone om die groei en metaboliete van *S. frutescens in vitro* kulture te vermeerder. Die groei reguleerders wat in hierdie studie gebruik was, was die sintetiese analoë van strigolaktoon, GR24 en Nijmegen-1, asook die ouksiene, indool-3-bottersuur (IBS) en naftaleen asynsuur (NAS).

Die eerste deel van die studie het gefokus op die effek van strigolaktoon en ouksien, alleen en in kombinasie, op die groei van *S. frutescens in vitro* nodale mikrostringels. Die *S. frutescens* nodale mikrostringels wat behandel was met 1 mg/L NAS het die aansienlikste toename in groei getoon. Hierdie behandeling het groei bevorder deur middel van vars en droë massa en plant lengte. Die metaboliet inhoud van die behandelde mikrostringels was met behulp van vloeistofchromatografie/massa spektrometrie (VC/MS) ondersoek. Al die behandelinge wat 1 mg/L NAS bevat het, het in metaboliet samestelling verskil en het ook 'n toename in metaboliet hoeveelheid getoon. Die SU1 inhoud van die behandelde plante was ook met behulp van VC/MS tegnieke gekwantifiseer en dit was gevind dat 'n kombinasie van 1 mg/L NAS en Nijmegen-1 die hoeveelheid SU1 verdubbel het.

Die effek van strigolaktoon op harige wortel kulture van *S. frutescens* was ook ondersoek. Strigolaktoon alleen het die formasie van laterale transgeniese wortels effens inhibeer, maar

wanneer hierdie chemikalieë saam met ouksiene gebruik was, was die aansienlike afname van die massa en inhibisie van die laterale wortel uitgroeisels meer prominent. Van al die behandelinge wat in hierdie studie getoets is, het 0.1 mg/L IBS die mees merkbare veranderinge in metaboliete meegebring en aminosure soos GABS en arginien het teen hoër vlakke versamel.

Die uitbuiting van hormone om groei en metaboliet produksie te bevorder in die belangrike medisinale plant, *Sutherlandia frutescens*, was suksesvol in hierdie studie. Die gebruik van nodale mikrostringels asook harige wortel kulture het bygedra om 'n stabiele sisteem te vestig vir die vermeerdering van metaboliete.

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Publications

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Abbreviations

ABA	-	Abscisic acid
ABP1	-	Auxin binding protein 1
AIDS	-	Acquired immunodeficiency syndrome
BCE	-	Before Common Era
C	-	Carbon
CCD	-	Carotenoid cleavage dioxygenase
cm	-	Centimeter
DNA	-	Deoxyribonucleic acid
ELSD	-	Evaporative light scattering detector
FT-IR	-	Fourier transform infrared
g	-	Gram
GABA	-	γ -aminobutyric acid/ gamma-aminobutyric acid
GC	-	Gas chromatography
HIV	-	Human immunodeficiency virus
HIV-RT	-	Human immunodeficiency virus-reverse transcriptase
HPLC	-	High performance liquid chromatography

HPTLC	-	High performance thin layer chromatography
IAA	-	Indole-3-acetic acid
IAN	-	Indole-3-acetonitrile
IBA	-	Indole-3-butyric acid
IGP	-	Indole-3-glycerol phosphate
IPA	-	Indole-3-pyruvic acid
kV	-	Kilovolts
L	-	Liter
LC	-	Liquid chromatography
LC-MS	-	Liquid chromatography-mass spectrometry
LC-UV	-	Liquid chromatography–ultra violet
LSD	-	Least significant difference
M	-	Molar
mg	-	Milligram
min	-	Minute
mL	-	Milliliter
mm	-	Millimeter
m/v	-	Mass per volume

μm	-	Micrometer
μL	-	Microliter
μg	-	Microgram
MS	-	Murashige and Skoog
<i>m/z</i>	-	Mass-to-charge ratio
N	-	Nitrogen
n	-	Sample size
NAA	-	Naphthalene acetic acid
NIRS	-	Near infrared spectroscopy
NM-1	-	Nijmegen-1
NMR	-	Nuclear magnetic resonance
NO	-	Nitric oxide
NVP	-	Nevirapine
PCA	-	Principal component analysis
PCT	-	Picrotoxin
PDA	-	Photo diode array
PGPS	-	Plant growth promoting substances
pH	-	Hydrogen ion concentration

PTZ	-	Pentylentetrazole
Ri-plasmid	-	Root-inducing plasmid
RNA	-	Ribonucleic acid
rpm	-	Revolutions per minute
SADC	-	South African Development Community
SANBI	-	South African National Biodiversity Institute
SE	-	Standard error
SU	-	Sutherlandioside
SU1	-	Sutherlandioside B
STZ	-	Streptozotocin
TAM	-	Tryptamine
T-DNA	-	Transfer DNA
TL	-	T-DNA left region
TLC	-	Thin layer chromatography
TR	-	T-DNA right region
UNAIDS	-	Joint United Nations Program on HIV/AIDS
UNICEF	-	United Nations International Children's Fund
UPLC	-	Ultrapformance liquid chromatography

V	-	Volts
v/v	-	Volume per volume
WHO	-	World Health Organisation

CHAPTER 1:

General Introduction

1.1 MEDICINAL PLANTS AND BIOTECHNOLOGY

Throughout the ages mankind has used plants for shelter, food, clothing, fragrances and medicine. Medicinal plants have formed the basis of traditional medicines and this knowledge has been passed on from generation to generation (Gurib-Fakim, 2005). The earliest known writings of medicinal plants were found six thousand years ago on Sumerian clay tablets, listing roughly 300 medicinal plants (Sumner, 2000). The Egyptians listed more than 850 medicinal plants on a papyrus scroll that dates back to 1500 BCE. About 3000 years ago, the Chinese emperor Shen Nung wrote the first herbal book, Pen Tsao, which illustrated the gathering, preparation and use of 365 medicinal plants (Sumner, 2000).

Today the use of medicinal plants still exists. The uses of traditional medicine in developing countries is widespread, while the use of complementary and alternative medicine in developed countries is increasing (WHO, 2005). Natural products based on medicinal plants, as well as derivatives thereof, represent more than 50% of the total clinically-used drugs in the world (Gurib-Fakim, 2005). This suggests that natural products are an important source for drug development. According to the World Health Organisation (WHO, 2005), up to 80% of people in Africa and 40% of individuals in China use traditional medicine to meet their health care needs. With this growing need for medicinal plants and plant natural products, new techniques should be applied and developed to ensure an adequate supply of plants and their products to the consumer. With the assistance of biotechnology, these needs can, in part, be fulfilled.

Biotechnological approaches can be applied to help sustain and improve medicinal plant growth and natural product production. The micropropagation and cultivation of plants can be used for sustainability in order to discontinue wild harvesting and destruction (Ramawat et al., 2004). Natural products produced by plants can be chemically synthesised and used in modern drug development. The biosynthetic pathways of plants can either be genetically modified or altered through exogenous application of growth promoting factors to promote the production of specific natural products (Ramawat et al., 2004; Julsing et al., 2007). With these techniques, medicinal plants can be used efficiently for the treatment of various diseases worldwide without the possibility of endangering indigenous plant species.

1.2 MEDICINAL PLANTS OF SOUTHERN AFRICA

South Africa has more than 24 000 indigenous plant species, of which 771 species are used as medicines (Mander et al., 2007). The trade of medicinal plants in South Africa is estimated to be worth about R2.9 billion per annum, with about 70% of South Africans consulting traditional health practitioners for their health care needs (Department of Health, 2008). With this large number of consumers, it is estimated that approximately 20 000 tonnes of plant material are used per year as medicines. Only about 50 tonnes of this harvested material is from cultivated plants, with the rest of it being harvested from the wild. This indicates a need to establish easy cultivation systems to ensure that wild plant populations will not be decimated. Devastatingly, 86% of the plant parts harvested will cause the death of the entire plant (Mander et al., 2007). This destruction caused the near extinction¹ of *Siphonochilus aethiopicus*, commonly known as African Wild Ginger (Mander et al., 2007), and almost 20% of medicinal plant species have now been added to South African National Biodiversity Institute's (SANBI) Red List (<http://redlist.sanbi.org>, accessed 2013-10-29).

¹ Regarded as extinct in the wild in KwaZulu-Natal, South Africa

Only about 38 medicinal plant species in southern Africa have been commercialised (Van Wyk, 2008). Some of the commercially important medicinal plants in southern Africa include: *Agathosma betulina* (round leaf buchu) which is enjoyed as a general health tonic; *Aloe ferox* (bitter aloe) is used as a laxative medicine; *Aspalathus linearis* (rooibos tea) is utilized as a health drink and also as an ingredient in cosmetics; *Hoodia gordonii* (hoodia) is consumed to suppress hunger and thirst; *Harpagophytum procumbens* (devil's claw) is used to counter arthritis, painful joints and loss of appetite; *Pelargonium sidoides* (rabas) is used to treat bronchitis and upper respiratory tract infections; and *Sutherlandia frutescens* (cancer bush) is used to treat an extensive range of illnesses including cancer, fever, poor appetite, diabetes, kidney and liver conditions, heart failure, wasting diseases, stress and anxiety, to name but a few (Van Wyk, 2008).

The expanding practice of using medicinal plants is not only prevalent in developing countries; reliance on plants for health purposes is also observed in developed countries as well. This clearly shows that there is a dire need for research into the safety of use and the cultivation of medicinal plant species. So much is still not known about medicinal plants and their products that the cure to cancer, tuberculosis and HIV/AIDS may lie in these plants.

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CHAPTER 2:

Literature Review

2.1 *SUTHERLANDIA (LESSERTIA) FRUTESCENS*

Sutherlandia frutescens (also known as *Lessertia frutescens*) is an upright, southern African, leguminous shrub, more commonly known as the cancer bush (or kankerbossie in Afrikaans), that forms part of the *Fabaceae* family. This perennial, but short-lived shrub, reaches up to 2.5 meter in height with bright red flowers that appear from spring to mid-summer (Van Wyk and Albrecht, 2008). This plant is restricted to drier areas of southern Africa (Figure 2.1) where it grows in the savannah and hillsides near streams in drier areas. This plant may also be found on rocky sandy soils along coastal areas (South African National Biodiversity Institute, 2012).

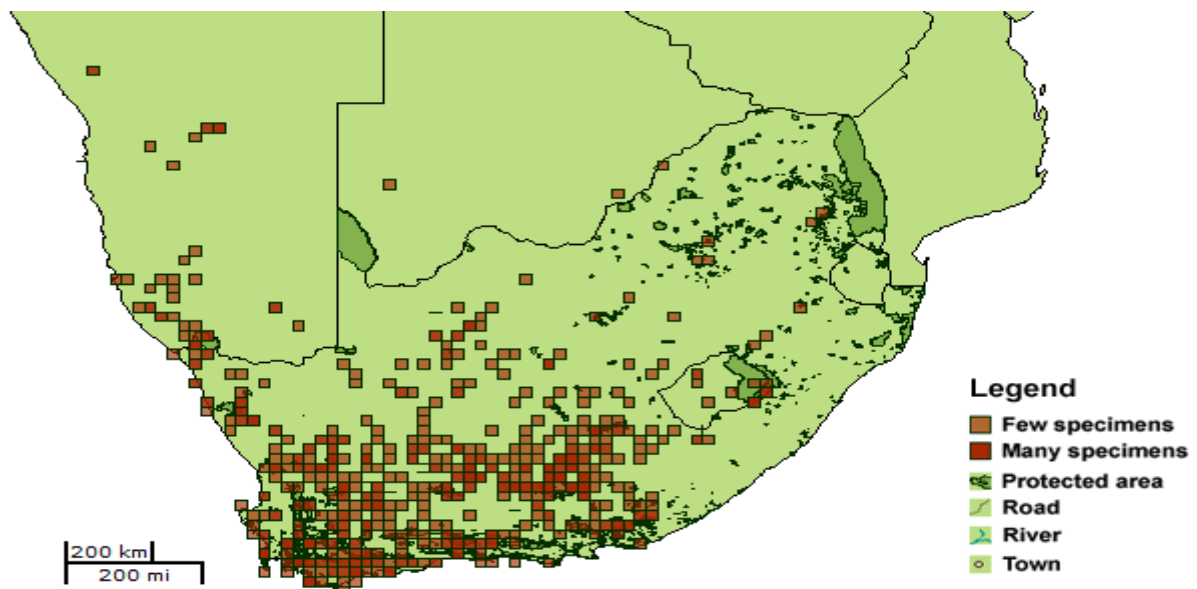


Figure 2.1: Distribution map of *Sutherlandia frutescens* (South African National Biodiversity Institute, 2012-06-06)

2.1.1 Medicinal applications

Sutherlandia frutescens has been widely used by local traditional cultures for the treatment or relief of diseases such as cancers, HIV/AIDS, diabetes, stress and anxiety, inflammation, pain, and wounds (Van Wyk and Albrecht, 2008; Harnett et al., 2005). These applications make *S. frutescens* a perfect plant to study in order to reveal if and how the extracts are responsible for the treatment or relief of these diseases. The mechanism(s) of action is still not known.

2.1.1.1 Cancer

Worldwide, about 12.7 million people were diagnosed with cancer and almost 7.6 million deaths resulted from this disease in 2008 (Ferlay et al., 2010). With this alarmingly high fatality rate, it is necessary to develop new drugs to cure or inhibit this deadly disease. *Sutherlandia frutescens* has been used historically to treat certain cancers. *Sutherlandia* extracts have shown antiproliferative² effects on human breast and leukaemia tumor cell lines *in vitro* (Tai et al., 2004). Although the precise compounds responsible for the antiproliferative effects are not clear, it was thought for a time that canavanine (Section 2.1.2.1) or GABA (Section 2.1.2.4) might be responsible. Recent studies show that sutherlandins and sutherlandiosides (Section 2.1.2.6) that are solely produced by *Sutherlandia* are actually responsible for the antiproliferative effects (Van Wyk and Albrecht, 2008). The plant extracts induced apoptosis³ of Chinese Hamster Ovary (CHO) cells and neoplastic cells (cervical carcinoma) (Chinkwo, 2005), as well as an oesophageal cancer cell line (Skerman et al., 2011). Tumorigenic breast adenocarcinoma cells died as a result of apoptosis and autophagy⁴ after being exposed to *S. frutescens* water extracts (Stander et

²Used or tending to inhibit cell growth

³A genetically determined process of cell self-destruction that is activated either by the presence of a stimulus or by the removal of a stimulus or suppressing agent

⁴A catabolic process involving the degradation of the cell's own components through the lysosomal machinery

al., 2009). These studies validate the potential of *Sutherlandia* to be used as a therapeutic agent for the cure of certain cancers.

2.1.1.2 HIV/AIDS

The Human Immunodeficiency Virus (HIV) gradually destroys the immune system, making it harder for the body to fight off infections. Acquired Immunodeficiency Syndrome (AIDS) is the final stage of HIV infection which causes severe damage to the immune system. It was estimated that 34 million people worldwide lived with HIV/AIDS in 2010 and approximately 1.8 million deaths in the same year resulted from the syndrome (WHO, UNAIDS and UNICEF, 2011). Extracts made from *Sutherlandia* leaves and flowers inhibit HIV-RT and glycohydrolase enzyme activity which reduces the infectivity of the HIV virion (Harnett et al., 2005). A possible compound responsible for this effect is L-canavanine. According to a patent, this amino acid destroyed 95% of HIV-infected lymphocytes *in vitro* (Green, 1988). Currently, a double blind, placebo-controlled study of the safety and efficacy of *Sutherlandia* capsules on HIV-infected patients is being investigated (Clinical Trials website, accessed 2012-06-13). *Sutherlandia* capsules were also tested on healthy adults and confirmed to be safe to use. The medication also improved appetite of patients, which could relieve the loss of appetite experienced by HIV/AIDS patients (Johnson et al., 2007). Recently, a drug-herb interaction study on rats warned that administration of *S. frutescens* and nevirapine (NVP), a prescribed anti-retroviral drug, could lead to therapeutic failure by enhancing the intrinsic hepatic clearance⁵ of NVP (Minocha et al., 2011).

⁵ Quantifies the loss of drug during its passage through the liver

2.1.1.3 Diabetes

Diabetes is a chronic disease occurring when the pancreas cannot produce enough insulin, the hormone which regulates blood sugar (type I diabetes), or when the body cannot effectively use the insulin being produced (type II diabetes). When diabetes is not controlled it can result in raised blood sugar, which may cause severe damage to the nerves and blood vessels (World Health Organisation website, accessed 2012-06-13). Over 346 million people have diabetes worldwide, of which 90% is type II diabetes, according to the WHO.

The anti-diabetic properties of *S. frutescens* extracts were tested on streptozotocin (STZ)-induced diabetes mellitus rats with chlorpropamide⁶ (250mg/kg) being used as a reference drug (Ojewole, 2004). The *Sutherlandia* aqueous shoot extracts (50 – 800 mg/kg) caused a significant improvement in hypoglycaemia and also maintained a low blood glucose concentration for longer (Ojewole, 2004). A similar study was conducted on Wistar rats fed a diabetogenic diet. The rats that received the plant extracts had normalised insulin levels and enhanced glucose uptake into muscle and adipose tissue, with a lower intestinal glucose uptake (Chadwick et al., 2007). These studies show that *S. frutescens* extracts have the potential to be used for the treatment of type II diabetes, but clinical trials will need to be conducted for a definite conclusion.

2.1.1.4 Other ailments

Inflammation can be treated by consumption of *Sutherlandia* water extracts, due to the antioxidant proficiency of the extracts. A study by Fernandes et al. (2004) demonstrated that the antioxidants have superoxide and hydrogen peroxide scavenging abilities, which may contribute to the anti-inflammatory effect of hot water extracts of *S. frutescens* subsp. *microphylla* powdered plant material.

⁶ A drug used for the treatment of type II diabetes

The use of *Sutherlandia* crude extracts lessened the onset of seizures induced by pentylenetetrazole (PTZ) and picrotoxin (PCT) in mice (Ojewole, 2008). Thus the use of *Sutherlandia* crude extracts in the management and potential treatment of epilepsy and convulsion is plausible.

2.1.2 Important compounds produced by *Sutherlandia*

Important metabolites which accumulate in *S. frutescens* include both primary metabolites such as arginine, asparagine, proline, canavanine and γ -aminobutyric acid (GABA), and secondary metabolites such as flavonol glycosides and triterpene glycosides (Van Wyk and Albrecht, 2008). These metabolites are now thought to be responsible for the relief or treatment of the above-mentioned illnesses.

2.1.2.1 L-Canavanine

L-Canavanine is a non-protein amino acid derived from glutamate via canaline (Figure 2.2) and used by the plant as a nitrogen source, but also used as an allelochemical⁷ to defend against insects and other herbivores (Rosenthal, 1990). This non-protein amino acid is found in the leaves and seeds of most leguminous plants, including *Sutherlandia* (Moshe, 1998). Canavanine is also a potent arginine (protein amino acid) antagonist, since it shares the same biosynthetic precursor and is a guanidinoxy structural analogue (Figure 2.2; Rosenthal, 1977).

When this amino acid is consumed in high amounts by herbivores, canavanine, instead of arginine, can be mistakenly incorporated into proteins. Incorporation results in structurally

⁷A chemical produced by a living organism that exerts a detrimental physiological effect on individuals of another species when released into the environment

abnormal proteins, since canavanine is unable to form crucial ionic interactions with acidic residues. These abnormal proteins do not function properly and as a result, cell growth is inhibited (Bence and Crooks, 2003). Through this mechanism, plants that produce high amounts of canavanine can protect themselves from insects and other pests. This mode of action makes this amino acid a promising anti-cancer agent in humans, particularly for the treatment of pancreatic cancer (Crooks and Rosenthal, 1994). The use of canavanine as an anti-retroviral has been patented (Green, 1988) and this also makes canavanine a possible constituent for the treatment of HIV.

2.1.2.2 L-Arginine

In plants, the essential amino acid, L-arginine, is synthesised from glutamate via ornithine (Figure 2.2). This amino acid is used in protein synthesis and additionally is a precursor to polyamines and alkaloids (Slocum, 2005). Arginine is also a direct precursor to nitric oxide (NO), which is an important molecule to protect the plant against oxidative stress and is a key signalling molecule (Ferreira and Cataneo, 2010). Nitric oxide is not only a valuable molecule in plants but also in humans and has been suggested to have functions in pathophysiology, trauma and wound repair (Vissers et al., 2004).

In mammals, arginine plays a key role in several metabolic pathways. It is the precursor to polyamines such as putrescine, spermine and spermidine (Section 2.1.2.3) which are involved in cell growth and differentiation (Cynober, 1994). Arginine is an indirect precursor, via arginase, for collagen formation which, with NO, is involved in the production of extracellular matrix molecules important in wound healing (Schäffer et al., 1997; 1999). Supplement studies have also shown that arginine stimulates excretion of growth hormones as well as insulin (Cynober, 1994). The regulation of T-cell function has also been linked to the availability of arginine. The lack of L-arginine blocks T-cell proliferation and also leads to

a reduced production of cytokines (Rodríguez et al., 2007), which can ultimately lead to cancer.

2.1.2.3 Polyamines

The main polyamines found in plants include putrescine, spermidine and spermine (Martin-Tanguy, 2011). These molecules are important in plant growth and development (Hunter and Burritt, 2012) and also in stress tolerance (Edreva, 1996; Kuznetsov and Shevyakova, 2007). Polyamines are poly-cations and are thus able to bind negatively-charged molecules such as DNA (Basu et al., 1990), proteins and membrane phospholipids (Martin-Tanguy, 2011). Polyamine biosynthesis in plants occurs via two pathways; the first is the decarboxylation of ornithine and the second pathway involves the decarboxylation of arginine (Figure 2.2; Martin-Tanguy, 2001). The degradation of polyamines leads to the formation of pyrroline which can be further catabolised into GABA (Section 2.1.2.4) (Martin-Tanguy, 2011).

In human health, polyamines are involved in cell growth, proliferation and even cell death (Cynober, 1994; Thomas and Thomas, 2001). Interestingly the synthesis of polyamine diminishes with age (Larqué, 2007). Thus, intake of polyamines should be increased with age to minimise some of the features associated with ageing.

2.1.2.4 γ -Aminobutyric acid (GABA)

Gamma-aminobutyric acid (GABA) is a four-carbon, non-protein amino acid known for its involvement in biotic and abiotic stress in plants (Mayer et al., 1990; Bolarín et al., 1995; Ramputh and Brown 1996; Kinnersley and Turano, 2000). In plants, GABA is synthesised

via the GABA shunt, which refers to the pathway that converts glutamate to succinate (Shelp et al., 1999; Figure 2.2). Gamma-aminobutyric acid is also a breakdown product of polyamines (Martin-Tanguy, 2001; Figure 2.2).

In mammals, GABA is mainly known for its involvement in neurotransmission (Maitre, 1997). Due to its involvement in the central nervous system, it has been suggested that GABA, also found in *Sutherlandia* extracts, may play a role in the improvement of the mood and well-being (Abdou et al., 2006), which can indirectly reduce wasting in HIV/AIDS and cancer patients (Tai et al., 2004). Gamma-aminobutyric acid can also be beneficial for lowering high blood pressure (Abe et al., 1995). An additional role for GABA in mammals is that it inhibits the migration of tumour cells (Ortega, 2003).

2.1.2.5 Asparagine

Asparagine was the first amino acid to be isolated from plants more than 200 years ago (Vauquelin and Robiquet, 1806). This amino acid amide has a N:C ratio of 2:4, making it an effective molecule for the storage and transportation of nitrogen, especially in legumes (Lea et al., 2006). Asparagine also accumulates under various stress conditions, either directly or indirectly via restriction of protein synthesis (Stewart and Larher, 1980; Lea et al., 2006). Asparagine in plants is synthesised in a two-step reaction. The amino group from glutamate is transferred to oxaloacetate with the aid of aspartate aminotransferase to form aspartate and 2-oxoglutarate. The second step results in the synthesis of asparagine when the amino group from glutamine is transferred to aspartate by asparagine synthetase to form asparagine and glutamate (Taiz and Zeiger, 2006; Figure 2.2). Asparagine functions in mammals include protein synthesis (Li et al., 2007) and a role in the metabolism of ammonia (Owen and Robinson, 1962).

2.1.2.6 Sutherlandins and Sutherlandiosides

In an attempt to find chemical constituents in *Sutherlandia*, it was discovered that this plant contains cycloartane glycosides known as sutherlandiosides (SU) (Fu et al., 2008) which are thought to inhibit cancer cell growth, given that they have a similar structure to other cycloartanes that exhibit this mode of action (Van Wyk and Albrecht, 2008). Flavonoid glycosides known as sutherlandins were classified by Fu and colleagues (2010). In an attempt to find chemical markers for *S. frutescens*, a Liquid Chromatography-Ultra Violet coupled to a Evaporative Light Scattering Detector (LC-UV/ELSD) analytical method was developed to detect both sutherlandins and sutherlandiosides from aerial parts of *S. frutescens* (Avula et al., 2010). With the use of these chemical markers, *S. frutescens* growing at different localities in South Africa was shown to have different chromatographic patterns according to the levels of these markers. Thus plants that differ due to geographic positioning can be identified easily based on the chemical profiles (Albrecht et al., 2012), as populations growing in the Karoo region contain higher levels of SU compounds whereas those found in the Gansbaai area lack these chemicals.

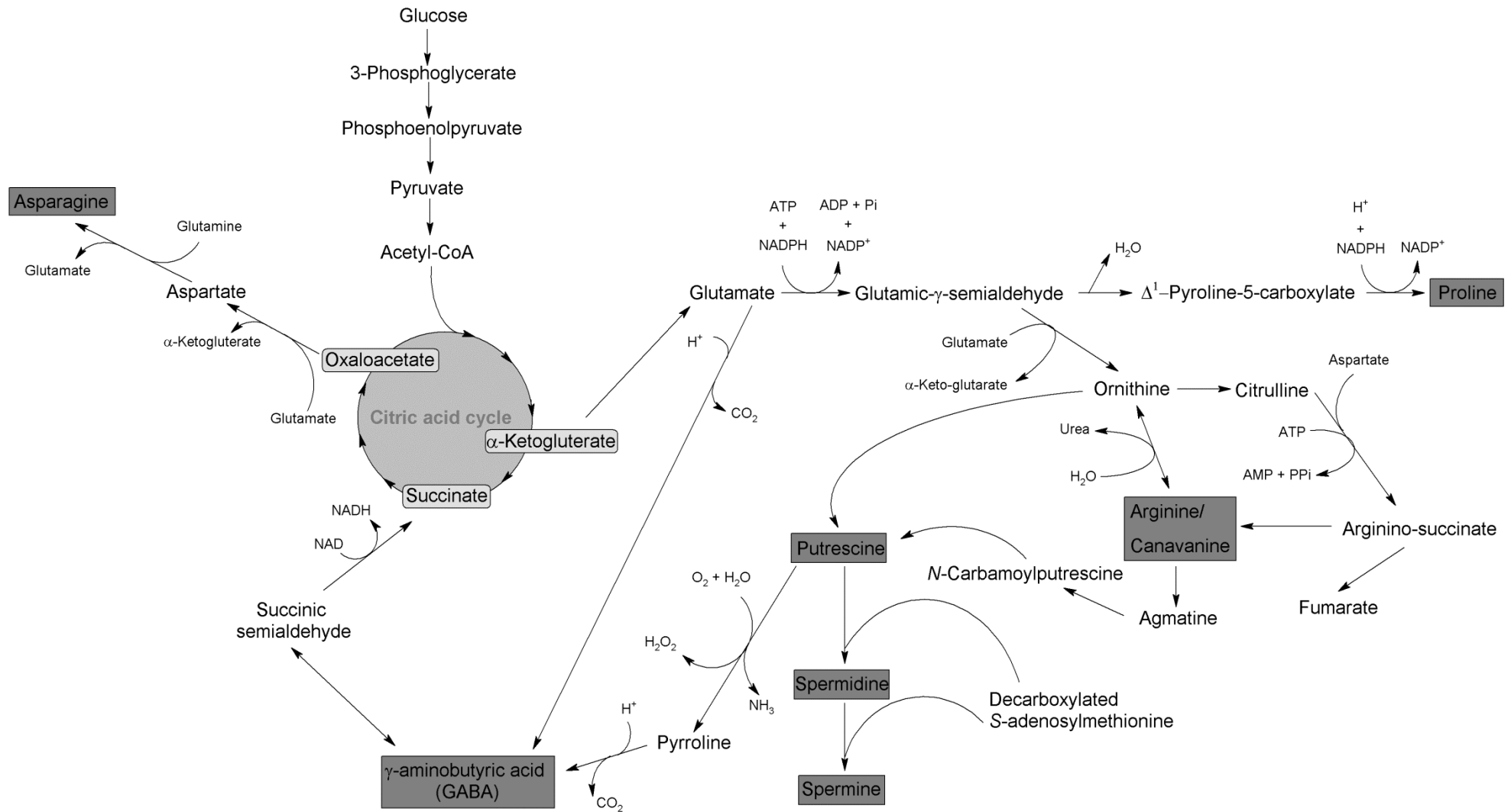


Figure 2.2: Biosynthesis of asparagine, proline, arginine, canavanine, putrescine, spermidine, spermine and gamma-amino-butyric acid from the citric acid cycle. Compiled from Sieciehowics et al. (1988), Rosenthal (1990), Brown and Shelp (1997), Shelp et al. (1999), Martin-Tanguy (2001), Taiz and Zeiger (2006).

2.2 GROWTH PROMOTING SUBSTANCES

Plant growth promoting substances (PGPS) are signal molecules that interact with specific protein receptors and are used to signal growth and alter plant metabolism to best adapt to their direct environment (Taiz and Zeiger, 2006). These growth promoting substances play key roles in shoot elongation, plant architecture, seed and fruit development and root elongation, to name a few (Ross and Reid, 2010). Through exogenous application of PGPS to plants, changes in growth and metabolism can be studied extensively. In this study, the effect of exogenously-applied synthetic strigolactones (Section 2.2.1), GR24 and Nijmegen-1, and auxins (Section 2.2.2), indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA), on *Sutherlandia frutescens* were investigated to monitor possible changes in plant growth and metabolism.

2.2.1 Strigolactones

Strigolactones are a group of terpenoid lactones, known to be derived from carotenoids (Matusova et al., 2005; Alder et al., 2012), which are involved in both above and below ground plant architecture (Yamaguchi and Kyozuka, 2010). Above ground, strigolactones are involved in shoot apical dominance via the inhibition of shoot branching, as has been demonstrated in a variety of plant species (Klee, 2008). Below ground, strigolactones stimulate the germination of parasitic plants' seeds (Matusova et al., 2005) and also stimulate interactions with symbiotic arbuscular mycorrhizal fungi (Besserer et al., 2006; Gomez-Roldan et al., 2008). Under optimal conditions, root architecture is also affected by strigolactones which reduce lateral root formation and improve the length of root hairs (Kapulnik et al., 2011a; 2011b). Strigolactone levels rise under suboptimal conditions, such as low phosphate levels, to help adapt the plant to its current conditions (Kohlen et al., 2011). The adaptation to low phosphate levels is normally achieved by adapting root

architecture to enhance phosphate uptake. For this reason, the formation of lateral roots (Ruyter-Spira et al., 2011) and root hairs (Mayzlish-Gati et al., 2012) are enhanced under phosphate limiting conditions which are linked to the formation of strigolactones. Under phosphate stress, strigolactones also promoted the growth of crown roots in rice (Arite et al., 2012).

Strigolactone biosynthesis (Figure 2.3) starts with the isomerisation of an all-trans- β -carotene by D27, an iron-binding polypeptide with catalytic properties. This isomerisation yields the formation of 9-cis- β -carotene which is cleaved by carotenoid cleavage dioxygenase 7 (CCD7). The resultant 9-cis- β -apo-10'-carotenal is converted by another carotenoid cleavage dioxygenase, CCD8 into carlactone (Alder et al., 2012). Carlactone is converted into a mobile product or might act as the mobile product itself and is transported out of the plastid. Outside the plastid the mobile product is finally converted into a strigolactone by a cytochrome P450 monooxygenase (Booker et al., 2005; Matusova et al., 2005; Umehara et al., 2008; Alder et al., 2012).

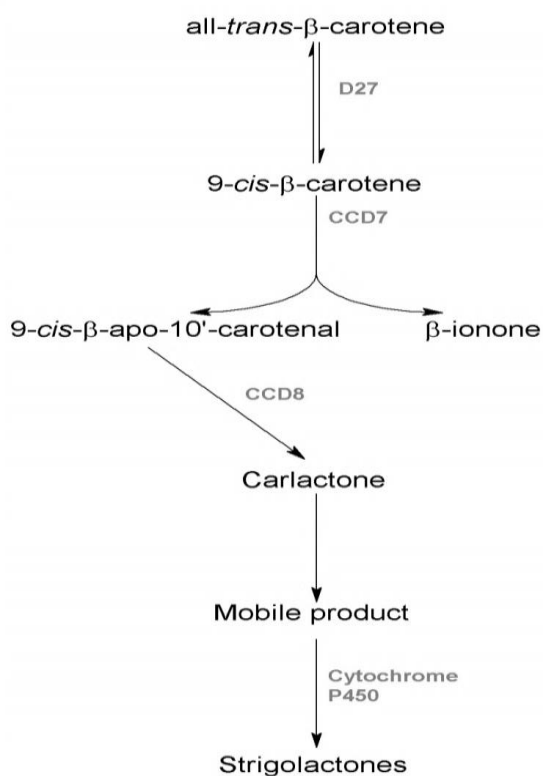


Figure 2.3: Biosynthesis pathway of strigolactones. Enzymes involved in the synthesis are indicated in grey. The synthesis starts with the isomerisation of C9-C10 double bond in all-trans- β -carotene by D27 to form 9-cis- β -carotene. This is then cleaved by CCD7 into 9-cis- β -apo-10'-carotenal and β -ionone. The 9-cis- β -apo-10'-carotenal is then converted to carlactone by CCD8. A mobile product is finally converted by a cytochrome P450 into strigolactones. Adapted from Alder et al., 2012.

The naturally occurring strigolactones identified thus far all have a similar four-ring backbone and differ from one another only in terms of the saturation of the rings (Gomez-Roldan et al., 2008; Umehara et al., 2008). These terpenoid lactones consist of four rings; the tricyclic lactone (A, B and C rings) connected to an α,β -unsaturated furanone moiety (D-ring) via an enol-ether bridge (Humphrey et al., 2006). Zwanenburg et al. (2009) reported that the enol-ether bridge and the furanone ring (D-ring) are vital for the activity of strigolactones, with the mode of action thought to be as follows: The furanone ring stimulates a nucleophilic attack by an electron-rich species, resulting in the D-ring being eliminated with the ABC part binding covalently to the receptor (Zwanenburg et al., 2009). The synthetic strigolactone, GR24, displays the same structure as naturally-occurring strigolactones, in that it also contains a tricyclic lactone connected to a furanone moiety (Figure 2.4). Another synthetic strigolactone, Nijmegen-1 (Figure 2.4), in contrast only has three rings (open C-ring), but the same α,β -unsaturated furanone ring (D-ring) and the enol-ether bridge. Thus the furanone ring is able to stimulate the nucleophilic attack and this strigolactone is still active.

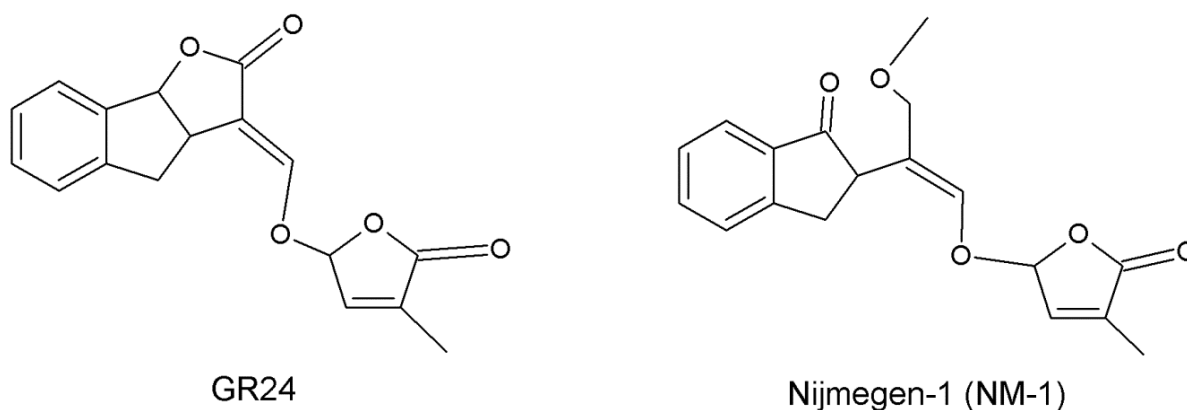


Figure 2.4: Chemical structure of the synthetic strigolactones, GR24 and Nijmegen-1. Redrawn from Zwanenburg et al., 2009.

2.2.2 Auxin

Auxins were the first group of hormones that were studied in plants and are mainly known for their ability to induce stem elongation and root formation (Taiz and Zeiger, 2006). Various auxins have been found in plants, but the most abundant natural auxin is indole-3-acetic acid (IAA). Auxins are relatively simple molecules; they contain an aromatic ring and a carboxyl group (Taiz and Zeiger, 2006). The structural similarity between auxins and the amino acid tryptophan suggests that auxins are synthesised from this amino acid. Various biosynthesis pathways have been proposed in plants using tryptophan as the precursor for auxin biosynthesis. These pathways include the indole-3-pyruvic acid (IPA) pathway, the tryptamine (TAM) pathway and the indole-3-acetonitrile (IAN) pathway (Normanly et al., 1995; Figure 2.5). The IPA pathway is the more common tryptophan-dependent pathway in plants; however, plants that do not utilise this pathway make use of the TAM pathway, with the exception of tomato which utilises both. The IAN pathway is found in only a few plant families, for the reason that this pathway uses a specific enzyme, nitrilase, which converts IAN to IAA (Taiz and Zeiger, 2006). Auxins may also be synthesised via a tryptophan-independent pathway (Wright et al., 1991; Normanly et al., 1993). In this pathway, it is hypothesised that IAA is synthesised either from indole-3-glycerol phosphate (IGP) or indole (Zhao et al., 2002).

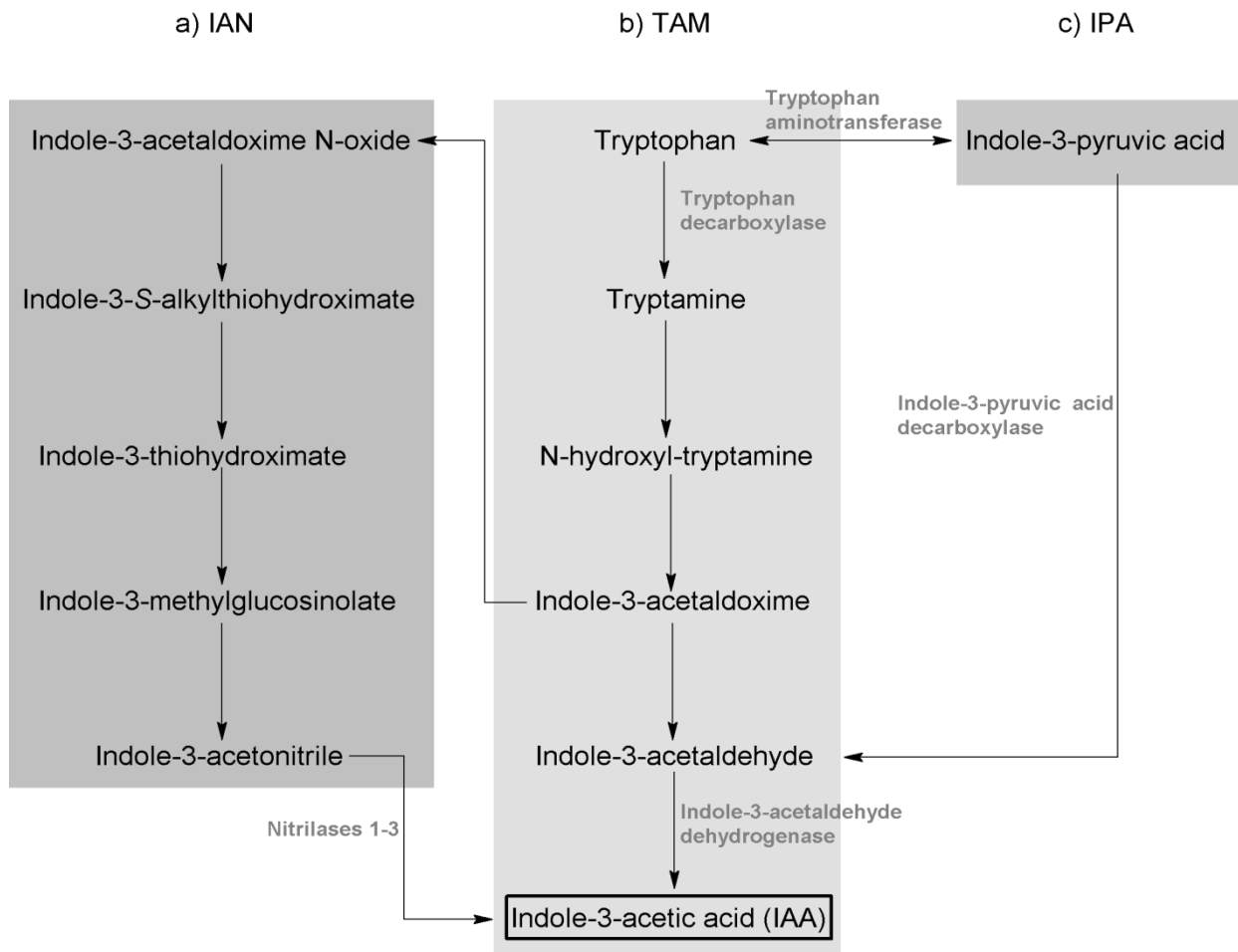
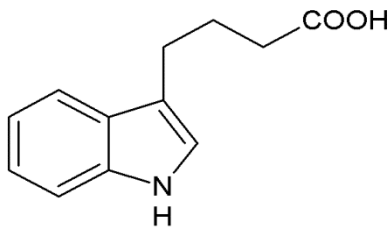
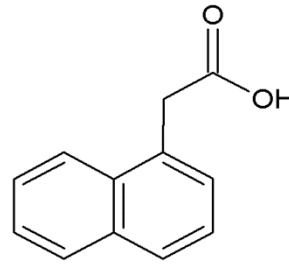


Figure 2.5: Biosynthesis pathways of Indole-3-acetic acid (IAA). Three pathways for IAA exist in plants namely a) the indole-3-pyruvic acid (IPA) pathway, b) the tryptamine (TAM) pathway and c) the indole-3-acetonitrile (IAN) pathway. The known enzymes involved in the synthesis of IAA are indicated in grey. Adapted from Taiz and Zeiger, 2006.

In this study, the natural auxin, indole-3-butyric acid (IBA; Figure 2.6), was utilised for the reason that IBA, in most cases, is more effective in stimulating root formation than IAA (Ludwig-Müller, 2000). Indole-3-butyric acid is also much more stable than IAA in MS medium, thus it is better to use in *in vitro* studies. Another synthetic auxin, naphthalene acetic acid (NAA; Figure 2.6), was also used to establish whether different auxins have different effects on growth and metabolism of *S. frutescens*.



Indole-3-butyric acid (IBA)



Naphthalene acetic acid (NAA)

Figure 2.6: Structure of the synthetic auxins, indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA).

The observed elongation effect following auxin treatment might be explained through auxin action which promotes stem elongation through the elongation of cells. This is also known as the acid growth theory (Rayle and Cleland, 1992). Two alternative theories exist; the activation theory and the synthesis theory. The activation theory proposes that auxin binds to an auxin binding protein (ABP1) which is a luminal endomembrane protein (Sauer and Kleine-Vehn, 2011). This ABP1-IAA complex then interacts with the plasma membrane H^+ -ATPase to stimulate proton pumping. The synthesis theory suggests that IAA stimulates secondary messengers to activate the expression of the genes that encodes the plasma membrane H^+ -ATPase. This multiplies the amount of plasma membrane H^+ -ATPase in the cell. With both theories, proton extrusion is ultimately amplified. The high concentration of protons in the cell wall stimulates acid-induced cell wall loosening, which is thought to be mediated by expansins (Figure 3.2; Taiz and Zeiger, 2006; Sauer and Kleine-Vehn, 2011). The synthetic auxin, NAA, can move through the cell wall *via* diffusion more easily than IAA (IBA must be converted by plants to IAA to function) and it seems that IAA uses different influx and efflux carriers than NAA (Normanly et al., 1995; Rashotte et al., 2003; Campanoni and Nick, 2005).

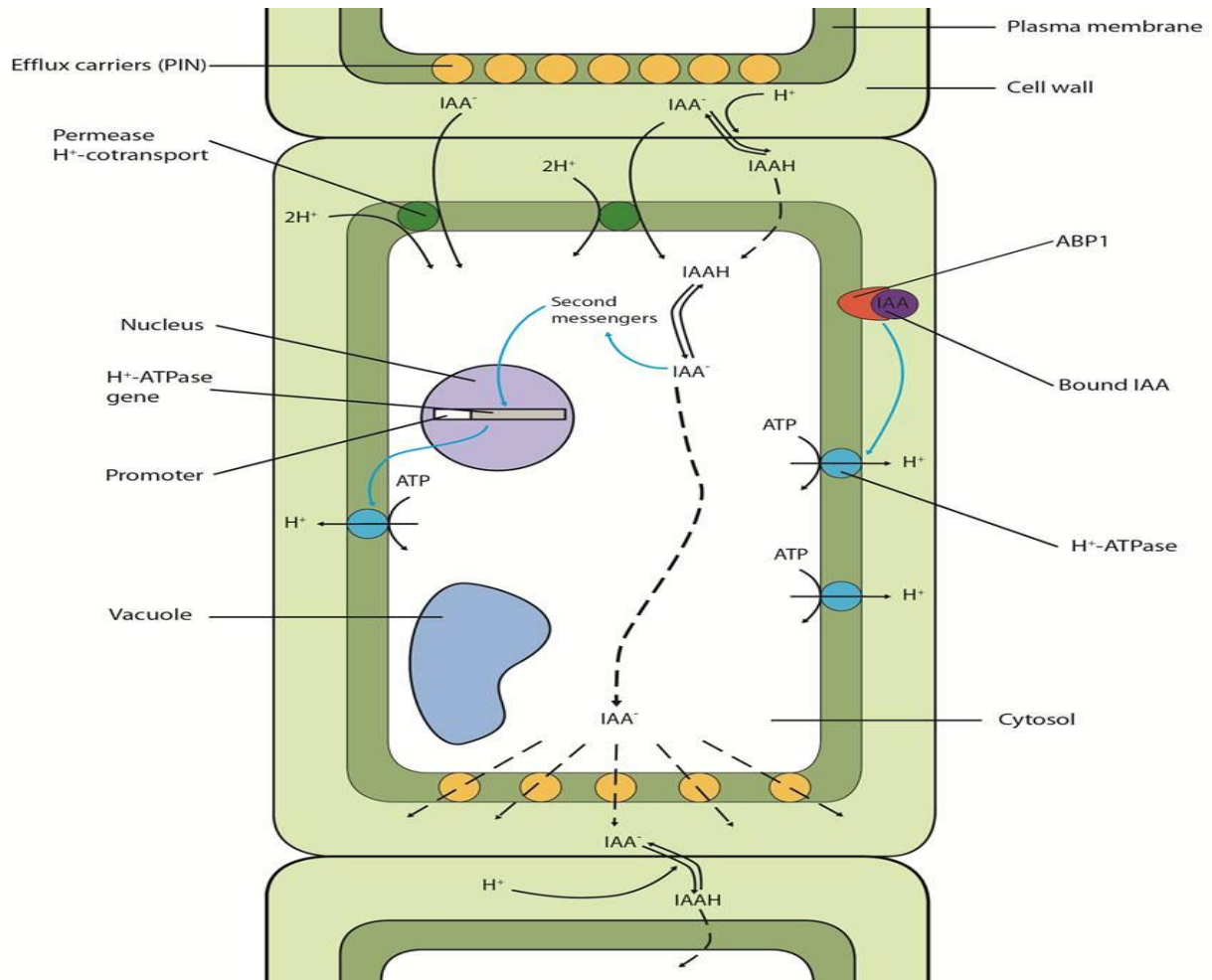


Figure 2.7: Illustration of polar auxin transport (indicated by black arrows) and auxin-mediated cell elongation (indicated by blue arrows). Auxin primarily moves into the cells through influx carriers (permease H⁺-cotransport). Auxin can also passively enter the cells in an undissociated form (IAAH). Once in the cytosol auxin reverts to the anionic form and can then be transported out of the cell via anion efflux carriers (PIN) which are concentrated at the basal ends of each cell. Auxin-mediated cell elongation occurs by one of two mechanisms. The first mechanism is that auxin binds to an auxin binding protein (ABP1) which is situated in the plasma membrane. This IAA-ABP1 complex stimulates the H⁺-ATPase pump to pump H⁺ out of the cytosol and into the cell wall. The second mechanism is that auxin in the cytosol activates second messengers that stimulate the synthesis of H⁺-ATPase pumps. The multiplication of H⁺-ATPase pumps will enhance the amount of proton pumping. With both mechanisms the pH is lowered in the cell wall. This lower pH stimulates expansins to loosen the cell wall which results in cell elongation. Adapted from Taiz and Zeiger (2006) and drawn with Adobe Illustrator CS5 and Photoshop CS5 (2010).

2.3 HAIRY ROOTS

Plants produce a vast number of primary and secondary metabolites. Primary metabolites are essential for the survival of the plant, whereas secondary metabolites are not essential for primary growth and development processes, but are produced for their antibiotic, anti-fungal, anti-viral, UV protection and numerous other properties (Bourgau et al., 2001). Secondary metabolites are of considerable interest because they serve as an important source of pharmaceuticals.

Hairy root disease is a result of *Agrobacterium rhizogenes* infection. The *Agrobacterium* transfers a specific segment of DNA (called T-DNA) from its root-inducing (Ri) plasmid into the host-cell genome (Gelvin, 1998). The DNA segment is flanked by 25 base pair DNA sequences, in the same orientation, called the left (TL) or right (TR) T-DNA borders or regions (Gelvin, 1998; Tzfira and Citovsky, 2006). The Ri-plasmid also contains most of the virulence (*vir*) genes used to deliver its single-stranded T-DNA into the plant cell (Tinland et al., 1994; Tzfira and Citovsky, 2006). Genetic transformation methods have made it possible to use *A. rhizogenes* to establish genetically-modified hairy root cultures. This is done by disabling the native T-DNA in the Ri-plasmid of the *A. rhizogenes*. A small vector containing the desired and modified T-DNA can then be mobilised into *Agrobacterium*, typically via tri-parental mating using *Escherichia coli* (Karimi et al., 1999). The modified *Agrobacterium* can now be co-cultured with the plant material to initiate infection. The desired T-DNA region is then inserted into the plant's genome (refer to Tzfira and Citovsky [2006] for a detailed review of the infection process). The plant will start to produce what are now known as hairy roots.

Hairy roots exhibit a few typical phenotypes in that they lack geotropism and have a high occurrence of lateral branching (Shen et al., 1988). Hairy roots contain the *rolA*, *rolB*, *rolC* and *rolD* genes as transgenes, which are generally located in the TR region of the *Agrobacterium* vector prior to transformation. These genes are responsible for the induction

of lateral roots (Nilsson and Olson, 1997). The *rol* genes have been found to be activators of secondary metabolism in certain plant species (Guillon et al., 2006b; Bulgakov, 2008). The *rolA* gene has a stimulatory effect on nicotine production and the *rolA* protein forms part of the DNA-binding proteins (Bulgakov, 2008). The *rolB* gene is thought to be the most powerful inducer of secondary metabolism but also suppresses cell growth, whereas the *rolC* gene promotes growth. The *rolC* gene stimulates the production of alkaloids in transformed plants (Bulgakov, 2008). The *aux* gene in the TL region is responsible for the hairy root phenotype of the transformed roots (Nilsson and Olson, 1997).

Hairy roots of *Lotus corniculatus* were more sensitive to exogenously-applied auxin than normal untransformed roots (Shen et al., 1988). The lateral root number of hairy root cultures of *Pueraria lobata* was slightly stimulated by exogenously applied IBA, but IBA repressed or had no effect on primary root and lateral root length (Liu et al., 2002).

The establishment of hairy root cultures serves as a mechanism to produce secondary metabolites in abundance. Secondary metabolites contain the active compounds found in medicinal plants and thus, through the establishment of hairy root culture systems of medicinal plants, secondary metabolites can be obtained to a greater extent than from normal wild-type medicinal plant roots (Guillon et al., 2006a).

2.4 PROBLEM STATEMENT

The current interest in the pharmacological activity of *S. frutescens* extracts and the statements by the WHO (2005) and SADC (2003) on the use of medicinal plants for the treatment of HIV/AIDS (amongst others) have put *Sutherlandia* in the spotlight. Thus the demand for *Sutherlandia* and *Sutherlandia*-based products is higher than before. In an attempt to fulfil this demand, methods for faster plant growth, plant biomass improvement

and/or amplification of certain metabolites will have to be developed. In this thesis, attempts to enhance plant biomass and metabolites are described.

2.5 AIMS AND OBJECTIVES

The aims and objectives of this study were to increase the biomass and metabolites of *S. frutescens* plants and hairy root cultures, via the application of phytohormones (strigolactones and auxins). The levels of growth and metabolites were assessed to establish which treatment(s) would have potential commercial application.

Auxin and strigolactones were tested in conjunction and alone, because it is believed that strigolactones act as a secondary messenger to auxin to inhibit bud outgrowth of plants (Brewer et al., 2009). The combined outcome of these hormones on hairy roots and their combined effect on primary and secondary metabolism of plants remains largely unknown. The effect that strigolactones might have on the secondary metabolism of hairy roots was also tested alone and in conjunction with auxin. This was done because strigolactones have been shown to be involved in the plant stress response. Secondary metabolism is generally up-regulated in response to stress. Hairy roots are used to evaluate the effect these treatments will have on secondary metabolites since hairy root cultures produce large amounts of secondary metabolites.

Strigolactone application reduces the number of lateral roots and also enhances root hair elongation (Kapulnik et al., 2011b), but under stress conditions, a proliferation in root growth can be expected (Arite et al., 2012). With the use of hairy roots the effect of strigolactones on lateral root growth can be studied more deeply. It was postulated that the strategy applied here would also aid in the understanding of strigolactones' mode of action on root formation.

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CHAPTER 3:

Sutherlandia frutescens in vitro propagation

3.1 INTRODUCTION

3.1.1 Commercial cultivation of medicinal plants and medicinal products

Plants are used as sources for a vast number of natural drugs and several of these medicinal species such as *Taxus* species are illegally harvested from the wild for use in the medicinal plant trade (Roberson, 2008). Consequently, plant species are being depleted at an alarming rate and this may result in a loss of genetic diversity, loss of natural habitat and local extinctions (Canter et al., 2005). Furthermore, with a growing world population, methods for the cultivation of medicinal plants are necessary. A need exists to look into reproductive biology, seed germination and methods of conservation and multiplication (Ramawat, 2004) to protect and improve the domestication of medicinal plants. A biotechnology approach can be taken in order to conserve medicinal plant species.

3.1.2 Biotechnology approach to commercialisation

Different techniques have been developed in which plants or the products they produce can be manipulated to give a higher yield. The greater yield will in turn result in fewer plants being used for medicinal purposes. This also reduces the area needed to commercially grow the improved medicinal plants. The use of biotechnology can assist with increasing plant yield. A few approaches can be taken to obtain higher amounts of a certain active compound

or its desired derivative from medicinal plants, which include: growth optimisation, selection of desired cultivars or cells, cell mutations, biotransformations⁸ through manipulation of biosynthetic pathways and genetic engineering (Ramawat, 2004). The addition of plant hormones and nutrients might enhance growth when compared with wild-grown plants and thus finding the correct concentration of nutrients and hormones would greatly benefit the commercial cultivation of the medicinal plants (Gana, 2010). The yield of plants can also be increased through a simple breeding selection technique in which the plants that grow faster or bigger or have the most metabolites will be chosen to breed with to establish an improved cultivar (Tracy, 2003). A more molecular approach can be taken to improve the yield of plants by mutating plant cells and observing which of these mutated cell lines or regenerated plants from the mutated cells result in higher growth, metabolite levels, grow on limited nutrients or are resistant to insects (Koyama et al., 2000). The mutation of cells can be achieved *via* techniques such as chemical mutations, silencing or inserting specific genes, or exposing seeds of plants to ultra-violet light (Woo et al., 1971). The biotransformation of plant cells and organ cultures can be obtained through the manipulation of the biosynthetic pathways *via* mutation of cells or the genetic transformation of cells (Giri et al., 2001).

Another biotechnology approach to amplify the growth or metabolites of a certain medicinal plant species is by creating new varieties of the plant. This can be accomplished either through genetic manipulation or breeding to acquire a desired trait. These plants are unique when compared to the wild-type and can be propagated continuously without losing the chosen trait (Hartmann et al., 2011). The new variety of the medicinal plant can then be successfully used for commercial growth. Phyto Nova developed such a plant variety of *Sutherlandia frutescens* known as Phyto Nova Sutherlandia SU1TM (Phyto Nova website, accessed 12-12-12). This plant variety yields higher levels of the SU1 metabolite than the normal wild-type *S. frutescens* found in the same area.

⁸ Chemical reactions catalyzed by cells, organs or enzymes

A final step to increase the yield of the optimised, transformed, mutant or new variety of plants is through the use of micropropagation. This is the method used to multiply the number of plantlets and to obtain clones from the same plant within a controlled and aseptic environment (Hartmann et al., 2011). The favoured plant, be it with improved growth or an enhanced production of metabolites, is cultivated using tissue culture techniques in order to acquire a substantial number of clones from the same preferred plant (Debergh and Read, 1991). These can then be used as is, or be transferred to a greenhouse to grow until they reach a useable size. The use of tissue culture techniques ensures the production of exact clones and also helps control the growth conditions and nutrients of the plants. This method has the added advantage of reducing the area used to cultivate these plants, since plants can be stacked on racks in a room and still have sufficient light for growth. Consideration for the potential of somaclonal variation should be taken when growing plants in tissue culture. If somaclonal variation occurs it can lead to negative effects such as poor growth, discolouration or even plant death (Bairu et al., 2011).

This study will focus on the use of hormones to up-regulate growth and metabolites of *S. frutescens* via the use of tissue culture techniques. Plant hormones rarely act alone to induce or inhibit growth and development of plants. Thus this system is quite complex and through the application of hormones in a controlled environment, these complex interactions can be studied to some extent (Gaspar et al., 1996). The study on how the hormones affect growth of the plants, at what concentrations and in which combinations will help to establish which treatments can be used to optimise the growth of *in vitro* explant cultures.

3.1.3 Aim of chapter

In this chapter, the optimisation of the growth of *S. frutescens* explants through the addition of different growth promoting substances using micropropagation methods is described. Different combinations and concentrations of strigolactones and auxins were used to establish the best treatment for growth promotion. These explants were measured for various growth parameters including biomass accumulation, bud/shoot production and stem elongation.

3.2 MATERIALS AND METHODS

3.2.1 *In vitro* culture conditions

Nodal explants with an axillary bud from a continuous *in vitro* *S. frutescens* plantlet culture (Colling et al., 2010) were placed in full strength Murashige and Skoog (1962) medium solidified with 0.8% (m/v) agar (pH 5.8) containing 3% (m/v) sucrose and 0.1 g/L myo-inositol. Conditions were kept similar to those described by Colling et al. (2010) for the continuous culture system.

Ten nodal explants of approximately 2 cm in length and containing an axillary bud were placed in glass vessels (90 x 50 mm) containing MS medium with the hormone treatments described in Table 3.1. All explants were kept at 18/6h light-dark conditions (25±3°C at a light intensity of 50 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (400 – 700 nm wavelength) using two Osram L58W/640 Energy saver lamps). The explants were left to grow for 28 days. After 28 days, plants were removed from the treatments and the stem elongation, bud outgrowth, and fresh and dry mass were measured (Figure 3.1 and 3.2a). The samples were frozen in liquid nitrogen (-196°C) and kept at -80°C until further analysis. This experiment was repeated three times.

Table 3.1: Growth promoting substances used for the treatment of *S. frutescens* in vitro nodal explant cultures growing on Murashige and Skoog (1962) medium. The untreated medium contains no added plant growth promoting substances.

Treatment	Untreated	0.1 mg/L IBA (4.92×10^{-7} M)	1 mg/L IBA (4.92×10^{-6} M)	0.1 mg/L NAA (5.37×10^{-7} M)	1 mg/L NAA (5.37×10^{-6} M)
Untreated	X	X	X	X	X
1×10^{-7} M GR-24	X	X	X	X	X
1×10^{-7} M Nijmegen-1	X	X	X	X	X

3.2.2 Data and statistical analysis

Quantitative analysis is represented as mean values and the reproducibility of the results is conveyed as standard error. The normality of the data was determined using a Shapiro Wilk's *W* test. Depending on the normal distribution of the data, either a *Post Hoc* Fisher least significant difference (LSD) test was performed for normally distributed data or a Kruskal-Wallis test was performed for abnormally distributed data. These tests were used to established significant differences between treatments. The differences between means reaching a minimal confidence level of 95% were considered as being statistically significant. All data were analysed with the use of the Statistica Release 8 program (Statsoft Inc., 2007). Graphs were plotted using Microsoft Excel 2010 (Microsoft Corporation, 2010).

3.3 RESULTS

3.3.1 Effect of plant growth promoting substances on growth of *Sutherlandia in vitro* plant cultures

3.3.1.1 Stem length

To test whether strigolactones affect stem length elongation of *S. frutescens*, explants were treated with strigolactones for four weeks. It is known that the synthetic strigolactone, GR24, inhibits hypocotyl elongation of *Arabidopsis thaliana* in the presence of light (Nelson et al., 2010). Thus it is possible that GR24 can have an effect on the stem length of *S. frutescens* explants. Strigolactones were also used with the addition of auxin to establish if an elongation effect is observed in the presence of auxin and if strigolactone also inhibits auxins' action of stem elongation. The stem length of the explants was measured after four weeks of treatment.

The explants responded positively to the addition of 1 mg/L IBA, 0.1 mg/L NAA and 1 mg/L NAA. These treatments showed a significant increase ($p=0.004739$, $p=0.045025$, $p=0.002234$ respectively) with regards to stem elongation when compared with the untreated control (Figure 3.1a). Strigolactones alone had no effect on explant elongation (Figure 3.1a, Figure 3.2b). The combination of strigolactones and auxin generally decreased the length of the explants (Figure 3.1a). When GR24 and IBA were combined, the decline in growth was significant when compared with the use of only 0.1 mg/L IBA ($p=0.00718$) or 1mg/L IBA ($p=0.000051$) respectively (Figure 3.1a). This decrease in growth is also prominent visually (Figure 3.2b treatments 2, 3, 7, 8, 12, 13). The reduction in growth was only observed when the strigolactone-auxin-treatment was compared to the auxin-treatment and not to strigolactone-treatment or untreated explants. Combinations of 1 mg/L NAA and either strigolactone improved the stem length when compared to the untreated or strigolactone-

treated explants (Figure 3.1a, Figure 3.2b). The treatments that contained GR24 in conjunction with auxin (Figure 3.2b treatments 6 – 10) were a greater inhibitor of stem elongation than the treatments that contained a mixture of Nijmegen-1 and auxin (Figure 3.2b treatments 11 – 15).

3.3.1.2 Bud outgrowth

Strigolactones were first categorised as plant hormones when it was found that these molecules inhibit shoot branching (Gomez-Roldan et al., 2008). Since then, numerous studies on this hormone have been carried out. To test whether strigolactones inhibited bud outgrowth in *S. frutescens* explants, the total number of bud outgrowths was counted after four weeks of treatment with strigolactones and auxin. Auxin was used in conjunction with strigolactones because it was previously thought that auxin inhibits bud outgrowth through a secondary messenger (Dun et al., 2009). This secondary messenger is now known to be strigolactones (Brewer et al., 2009). Thus the aim was to establish whether a combination of strigolactones and auxin have an additive effect.

The outgrowth of buds was significantly lower with added GR24 ($p=0.015618$) when compared with the untreated control. The GR24 in combination with auxin also inhibited the bud outgrowth significantly when compared to their respective auxin controls (Figure 3.1b). This inhibition was most noted when GR24 and NAA were used in conjunction, resulting in a significant reduction of bud outgrowth when 0.1 mg/L ($p=0.022735$) or 1 mg/L ($p=0.008882$) NAA was used (Figure 3.1b). Nijmegen-1 did not significantly affect bud outgrowth, either singly or in combination with the auxins (Figure 3.1b). In this experiment, treatment with auxin did not show a reduction in bud outgrowth as was expected from auxin application.

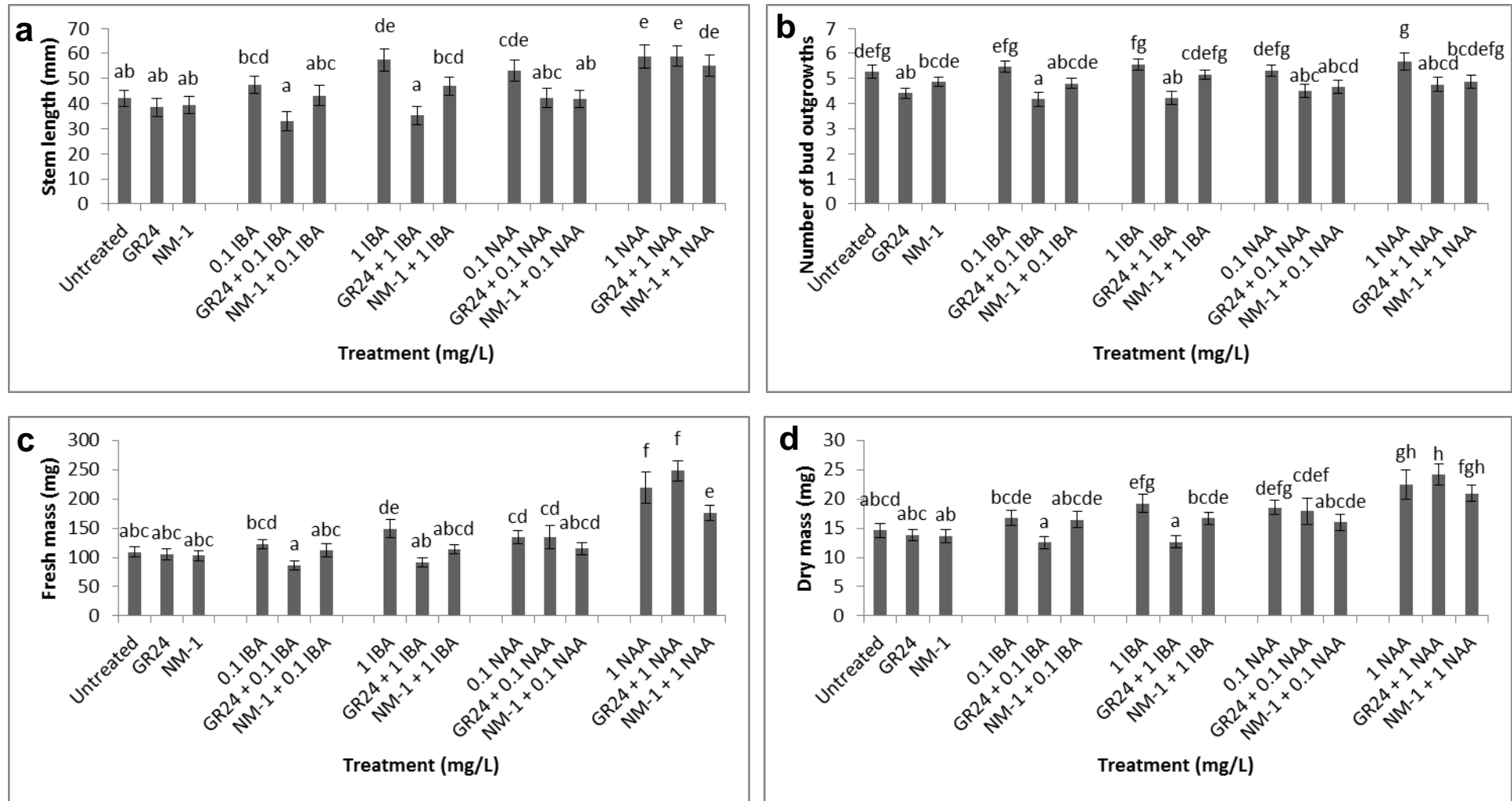


Figure 3.1: *Sutherlandia frutescens* in vitro growth after four weeks on treatments containing strigolactones and/or auxin or no hormones. The bars represent the means \pm standard error ($n=30$) and different letters indicate a statistical significant difference at the 95% confidence level. a) Stem length measured in millimetres (mm). b) Bud outgrowth. c) Fresh mass measured in milligrams (mg). d) Dry mass measured in milligrams (mg). NM-1: Nijmegen-1; IBA: Indole-3-butyric acid; NAA: Naphthalene acetic acid.

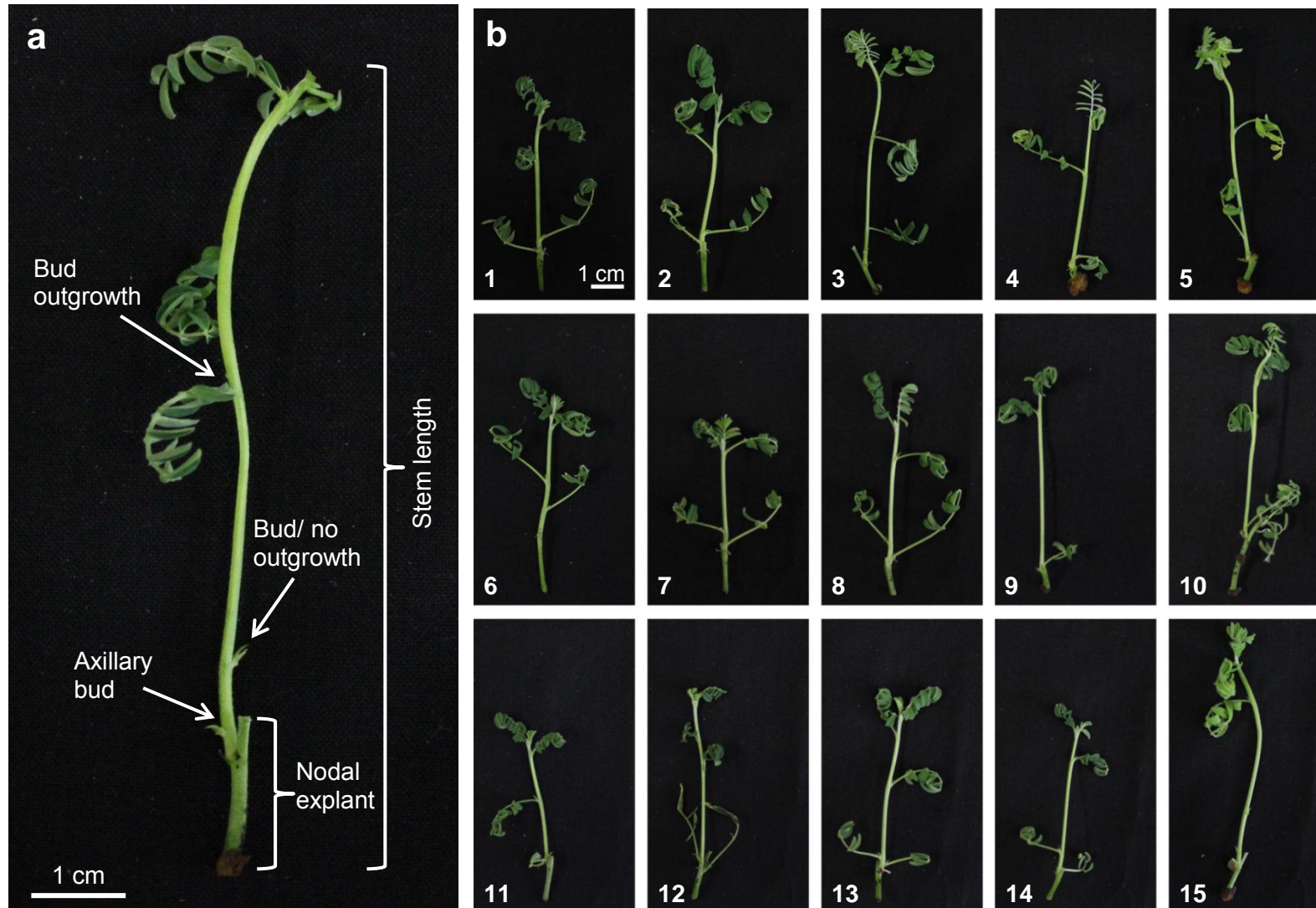


Figure 3.2: Four week old tissue cultured *S. frutescens* nodal explant with and without growth substances. a) Visualisation of growth measurements. Stem length indicates the stem that was measured for stem elongation. Bud outgrowth and no bud outgrowth is indicated. Nodal explant and axillary bud refers to the starting material. b) Growth of explants after treatment with 1) untreated 2) 0.1 mg/L IBA 3) 1 mg/L IBA 4) 0.1 mg/L NAA 5) 1 mg/L NAA 6) GR24 7) GR24 + 0.1 mg/L IBA 8) GR24 + 1 mg/L IBA 9) GR24 + 0.1 mg/L NAA 10) GR24 + 1 mg/L NAA 11) NM-1 12) NM-1 + 0.1 mg/L IBA 13) NM-1 + 1 mg/L IBA 14) NM-1 + 0.1 mg/L NAA 15) NM-1 + 1 mg/L NAA. NM-1: Nijmegen-1; IBA: Indole-3-butyric acid; NAA: Naphthalene acetic acid.

3.3.1.3 Mass accumulation

Mass of the treated plants was measured to determine if strigolactones and auxin had an effect on mass accumulation and if this can be correlated to the bud outgrowth (Figure 3.1b) and stem elongation (Figure 3.1a). The comparison of fresh mass to dry mass should show the amount of water in the cells. The treatment containing 1 mg/L IBA significantly promoted the fresh mass ($p=0.027864$; Figure 3.1c) as well as the dry mass ($p=0.024548$; Figure 3.1d) of the plants when compared with the untreated control. Treatment with the addition of GR24 to IBA lowered the fresh mass significantly when compared with treatment with 0.1 mg/L IBA ($p=0.046894$) and 1 mg/L IBA ($p=0.001765$; Figure 3.1c) alone. This significant reduction in mass was also apparent with the dry mass when compared with 0.1 mg/L ($p=0.044029$) and 1 mg/L IBA ($p=0.001388$) alone (Figure 3.1d). The use of 1 mg/L NAA resulted in a significant increase in both fresh (Figure 3.1c) and dry (Figure 3.1d) mass, whether applied alone or in combination with either GR24 or Nijmegen-1.

3.4 DISCUSSION

Strigolactones have been shown to inhibit hypocotyl elongation of *A. thaliana* when grown in light (Nelson et al., 2010), but in this experiment strigolactones only inhibited shoot lengthening in the presence of IBA. It is well-known that at certain concentrations, auxin promotes the growth of stems (Yang et al., 1993). With exogenously-applied synthetic auxin, shoots would generally be expected to show an elongation in stem length when compared with the untreated control. Achieving stem elongation with auxin application depends on the amount of auxin which is supplied to the plant. Too much or too little will not cause stem elongation and might even inhibit normal stem elongation. In pea seedlings, exogenously applied indole-3-acetic acid (IAA) stimulated stem length elongation at concentrations ranging from 10^{-6} M to 10^{-3} M (Yang et al., 1993). Dose-response curves of auxin have been developed and differ slightly between plant species (Yang et al., 1993; Taiz and Zeiger,

2006). Thus two different concentrations of two different synthetic auxins were used in this experiment, since certain auxins can have a more prominent effect in a species than others (Khunachak et al., 1987; Kollárová et al., 2005). In *S. frutescens*, treatment with NAA at a concentration of 1 mg/L resulted in the largest increase (59 mm) in stem elongation, with 1 mg/L IBA-treated explants being slightly shorter (57 mm) (Figure 3.1a). It is interesting to note that even though a higher growth elongation was observed with the NAA treatments, the strigolactones did not show the elongation inhibition effect noted with IBA (Figure 3.1a). The addition of strigolactones to IBA inhibited the elongation effect of IBA, making these plants similar to the untreated and strigolactone-treated controls. This might indicate that different auxins have different effects on strigolactone's mode of action. When referring to the acid growth theory (Section 2.2.2) it seems plausible that strigolactones (with GR24 being more effective) can inhibit the action of ABP1 or the influx carriers associated with IAA, and thus the inhibition of stem elongation is more noticeable with IBA than with NAA since the NAA can still be taken up in the cell. Thus the auxin does not need to bind to ABP1 to stimulate the H⁺-ATPase pumping, but can activate second messengers that initiate the synthesis of plasma membrane H⁺-ATPase.

Along with auxin and cytokinin, strigolactones are involved in regulating shoot branching (Gomez-Roldan et al., 2008, Umehara et al., 2008, Ferguson and Beveridge, 2009). It is thought that strigolactones inhibit shoot branching (Gomez-Roldan et al., 2008) by acting as a long-distance inhibitory signal which is regulated by auxin (Brewer et al., 2009). Thus the addition of auxin to strigolactone treatment is expected to show a decline in bud outgrowth, as was observed when a combination of GR24 and IBA was used for treatment (Figure 3.1b).

The mode of action on how strigolactones possibly inhibits the outgrowth of buds is thought to be by inhibiting the mode of action of the PIN proteins (Lazar and Goodman, 2006). Application of GR24 resulted in a decline in auxin levels of *Arabidopsis* leaf material (Ruyter-Spira et al., 2011) and it is thought to be due to presumed GR24-mediated reduction of PIN1

cycling (Prusinkiewics et al., 2009). These PIN proteins are efflux carriers of auxin which transports auxin out of the cells (Petrášek et al., 2006). Thus it is proposed that due to inhibition of the PIN proteins the auxin cannot be transported out of the cells located in the bud, causing auxin build-up. This build-up results in the inhibition of bud outgrowth. It is possible that strigolactones can inhibit bud outgrowth without causing an inhibition in stem elongation with the addition of NAA, because the PIN proteins in the buds are inhibited to a greater degree than in the stems (Lazar and Goodman, 2006). Thus NAA can be normally transported out of the cells in the stems to result in stem elongation, but in the buds it cannot be transported out of the cells which then results in the inhibition of bud outgrowth. The NAA can move more easily out of the cell via diffusion than IBA and thus NAA does not build-up in the buds to the same extent as the IBA. Thus, treatment with IBA should have a greater effect on bud inhibition than NAA.

The inhibitory effects of strigolactones on bud outgrowth were considerably greater for GR24 than for Nijmegen-1. This may be due to GR24 inhibiting the function of PIN proteins more than Nijmegen-1 does (Figure 3.1b) and it may also explain why Nijmegen-1 does not inhibit stem elongation as much (Figure 3.1a). Parasitic weed seed germination was stimulated better by GR24 application than by Nijmegen-1 application. Only at higher concentrations of Nijmegen-1 did it stimulate the germination of parasitic weeds (Wigchert et al., 1999). The open C-ring structure of Nijmegen-1, compared with the closed C-ring structure of GR24 and all known naturally occurring strigolactones, has been suggested to cause Nijmegen-1 to be less active than GR24 (Zwannenburg et al., 2009). Treatment with NAA increased the fresh mass significantly when compared to the untreated, GR24 and Nijmegen-1 treatments. This higher yield might be partially explained by the significant improvement in stem length. A further contributing factor may be that auxin treatment often results in shoot thickening of the plants and recently strigolactones have also been linked to the shoot thickening process (Agusti et al., 2011). This effect is also known as secondary growth and is mediated by the vascular cambium which exhibits a cell-proliferation activity (Snow, 1935). Even though

treatment with Nijmegen-1 and 1mg/L NAA showed an improvement in fresh mass when compared to the Nijmegen-1 control, it showed a decrease when compared to 1mg/L NAA (Figure 3.1c). This is peculiar since the stem length and bud outgrowth did not differ when Nijmegen-1 with 1mg/L NAA was compared to 1mg/L NAA. Thus it seems that Nijmegen-1 might not have the same effect as GR24 on the cambium activity and might actually limit cambium proliferation to some extent. This might explain why treatment with Nijmegen-1 had more or less the same results as treatments that include GR24, even though the other growth factors seemed to show slight differences between these two strigolactones.

Dry mass is a more accurate measure of plant mass since the fluctuating water content is excluded from the results obtained. Significant promotions in dry masses were observed when 1 mg/L NAA was used in the treatments (Figure 3.1d). The combined treatment of GR24 and 1 mg/L NAA showed the greatest increase in dry mass when compared with the untreated ($p=0.0000$) and GR-24-treated ($p=0.0000$) explants. Thus this treatment resulted in the best improvement in plant mass.

3.5 CONCLUSION

Strigolactones and auxins appear to have a definite effect on each other's mode of action on growth promotion. Of the treatments tested, NAA had the most significant effect on growth. Adding GR24 significantly suppressed bud outgrowth. Addition of strigolactones did not significantly alter the other growth parameters. Future studies on model plant species will assist in examining the interactions of the hormones on a genetic level, since most model species have genetic sequence data available. This will then facilitate in the establishment of yet another role strigolactones play at a molecular level in the control of auxins' mode of action in promoting growth.

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CHAPTER 4:

Influence of strigolactones on metabolites of *in vitro* grown plants

4.1 INTRODUCTION

Metabolomics is a relatively new field of study when compared to its ‘-omics’ equivalents: genomics, transcriptomics and proteomics. Metabolome studies offer us an understanding of the plant’s biochemical status, which can help to monitor and assess gene functions (Fiehn et al., 2000). Not only does a metabolome study facilitate the identification of gene functions, but it also enables the discovery of new metabolites and possibly genes involved in the production of these metabolites.

4.1.1 Techniques to analyse metabolites

Various techniques have been developed to study metabolites, since no one technique alone is sufficient for the visualisation of the entire metabolome. Techniques range from simple thin layer chromatography (TLC) to mass spectrometry (MS), gas chromatography (GC), liquid chromatography (LC or HPLC) and nuclear magnetic resonance (NMR). Mass spectrometry can be combined with either GC or LC to expand the detection limit and also to identify compounds more accurately.

Liquid chromatography coupled to mass spectrometry (LC-MS) is an analytical technique which that has been widely used in differential profiling in order to screen multiple samples to observe phenotypes or discover biomarkers (Katajamaa and Orešič, 2005). This analysis

combines physical separation *via* liquid chromatography (LC) with mass analysis *via* mass spectrometry (MS) to achieve high sensitivity and selectivity. Physical separation is performed by introducing pre-treated extracts of the samples to an LC column. Here the molecules can be separated according to size, affinity to the stationary phase, polarity or hydrophobicity. A retention time is recorded which shows the time between sample injection and the appearance of the compound peak after chromatographic separation. Due to the extracts being a complex mixture of molecules, LC alone is not sufficient enough to distinguish between two compound peaks at the same time point. Thus MS is used with LC to separate the co-elutants according to their mass-to-charge ratio (m/z). These co-elutants are ionized in the LC-MS interface and are introduced to the mass spectrometer where m/z is measured (Katajamaa and Orešič, 2005).

A variety of metabolomic platforms have been used recently to monitor both primary and secondary metabolites of *Sutherlandia* populations which are thought to be important for human health. These includes methods such as HPLC, high performance thin layer chromatography (HPTLC), near infrared spectroscopy (NIRS) and Fourier transform infrared (FT-IR) spectrometry which were utilized to assess the metabolite profiles of wild *Sutherlandia* populations (Mncwangi, 2009). A tremendous amount of variation amongst bioactive compounds is evident within and between populations (Mncwangi and Viljoen, 2012). Therefore, it becomes essential to examine the phytochemical composition, especially for purposes of product development within the phytopharmaceuticals sector. Field-cultivated material may also differ from non-cultivated material, making cultivation practices which aim to increase the levels of pharmacologically active chemicals important.

4.1.2 Key metabolites found in *Sutherlandia frutescens*

Medicinally important metabolites that accumulate in *S. frutescens* include arginine, canavanine, asparagine, γ -aminobutyric acid (GABA), sutherlandiosides and sutherlandins. All of these compounds are thought to contribute to the healing abilities of *S. frutescens* (discussed previously in Section 2.1.2; Mncwangi and Viljoen, 2012). The sutherlandiosides and sutherlandins are the main compounds that are now proposed as the main chemicals that inhibit cancer cell growth (Van Wyk and Albrecht, 2008). These metabolites are also used to assist in quality assurance of *S. frutescens*-based products and they are monitored during the manufacture of *Sutherlandia*-based phytochemicals.

Sutherlandia is regarded as an adaptogen as it has a wide range of health benefitting metabolites including amino acids, vitamins, flavonoids and terpenoids (discussed in Section 2.1.2). The amino acids and non-protein amino acids are key elements that accumulate in the foliage of *Sutherlandia* plants. In plants, the standard amino acids are produced from the precursors (3-phosphoglycerate, phosphoenolpyruvate and pyruvate) as well as from the products of the citric acid cycle (Figure 2.2). The non-protein amino acids are used as intermediates to the standard amino acids. The amino acid ornithine is involved in the production of polyamines (Figure 2.2) which are important in growth and development of plants (Hunter and Burritt, 2012) as well as stress tolerance (Edreva, 1996; Kuznetsov and Shevyakova, 2007).

The accumulation of standard and non-protein amino acids, along with polyamines, can give an indication of the plant's physiological condition. Some amino acids involved in plant stress include proline, arginine, aspartate, alanine, asparagine, aspartate, glutamate and serine (Rai, 2002). The amount of amino acids present in plants can also assist in gaining insight into its physiological state, since many of the amino acids are precursors to other amino acids. Thus an increase in one amino acid can lead to the decrease in another.

Environmental changes contribute to shifts in both the primary and secondary metabolism of plants. Through hormonal application, these changes can be induced and by utilising metabolomics tools, these shifts can be exploited. Such investigations will aid in clarifying how plants function under certain conditions. For example, Planchet et al. (2011) evaluated the nitrogen metabolism of the model legume *Medicago truncatula* after a water deficit treatment or treatment with the hormone abscisic acid (ABA). From this study they found that the plant responds to water scarcity through both ABA-dependent and independent pathways.

4.1.3 Aim of this chapter

Comprehensive investigations at the metabolite level of *Sutherlandia* are rare, and in general, the metabolome of *S. frutescens* has not been well studied. Through the treatment of *S. frutescens* explant cultures with auxins and strigolactones, the metabolome of *S. frutescens* can be altered. Using this approach, an understanding of the influences of these growth promoting agents on metabolite production of this medicinally important plant would be acquired. A recent study indicated that the sutherlandins and sutherlandiosides can be used to identify populations of *S. frutescens* growing in different regions (Albrecht et al., 2012). The localities influenced the levels of the SU compounds and therefore it can be concluded that the production of the SU compounds may be influenced by environmental factors.

4.2 MATERIALS AND METHODS

4.2.1 Plant material

The shoot and leaf plant material that was used for metabolite analysis was obtained from *in vitro* cultured plant stocks cultivated on MS medium (Section 3.2.1). Upon harvesting, the plants were measured for growth (Section 3.2.1). This material was frozen in liquid nitrogen and freeze-dried for a day using the VirTis BenchTop K (model 2KBTES) freeze drier. Material was stored at -80°C until metabolite analysis.

4.2.2 Extraction of metabolites

The extraction of metabolites is an important step in ensuring that the experiment is reproducible and to ensure consistent results. The freeze-dried material was ground to a fine powder with a mortar and pestle and 0.05 gram tissue was used for metabolite extraction.

4.2.2.1 Extraction for LC-MS analysis

Freeze-dried ground plant material was weighed in 2 mL microfuge tubes. Metabolites were extracted with 1 mL acetonitrile:formic acid (50:0.1) (v/v) by sonicating in an ultrasonic bath (Branson B220H) for 60 min. After sonication, the tubes were centrifuged for 10 min at 13 000 x *g* at room temperature. The supernatant was transferred to a clean microfuge tube. These samples were kept at -20°C until analysis.

4.2.2.1.1 Sample preparation for amino acid and polyamine analysis

Samples were derivatised using the Waters AccQ Tag Ultra Derivatization Kit. All samples were analysed twice, firstly undiluted and then 100 times diluted to quantify the amino acids, like arginine, that occur at very high concentrations. The samples were derivatised by adding 10 µL of the prepared undiluted sample (50 µL of internal standard [d_3 -methionine] added to 200 µL of undiluted sample) to the Waters AccQ Tag Kit constituents and placed in a heating block at a temperature of 55°C for ten minutes.

4.2.3 Metabolite analysis

4.2.3.1 LC-MS analysis for general metabolites and sutherlandioside B

Samples were diluted ten times and an injection volume of 3 µL was injected into the LC-MS system. The LC-MS analysis was conducted on a Waters Synapt G2 quadrupole time of flight mass spectrometer (Milford, MA, USA). The instrument was connected to a Waters Acquity ultraperformance liquid chromatograph (UPLC) and Acquity photo diode array (PDA) detector. Ionisation was achieved with an electrospray source, cone voltage of 15V and capillary voltage of 2.5 kV. Nitrogen was used as desolvation gas at 650 L/h and the desolvation temperature was set to 275 °C. A Waters UPLC BEH C18 column (2.1 x 50 mm, 1.7 µm particle size) was utilised. A gradient started with 100%, 0.1% formic acid (solvent A), holding at 100% solvent A for 0.5 min, followed by a linear gradient to 22% acetonitrile (solvent B) over 2.5 min, to 44% solvent B over 4 min and to 100% solvent B over 5 min. The column was kept at 100% solvent B for another 2 min followed by re-equilibration over 1 min to yield a total run time of 15 min. A flow rate of 0.4 mL/min was applied. Sutherlandioside B (SU1) was quantified for each sample using an SU1 standard (kind gift from Professor Albrecht; Cancer Association of South Africa). A reference standard of SU1 was used for quantification of this chemical in *in vitro* leaves. This standard was

isolated by V. Gabrielse (refer also to Van-Wyk and Albrecht, 2008). The SU1 calibration curve had a linear range of 0 to 50 ppm ($R^2 = 0.998$) with a detection limit of 10 ppb.

4.2.3.2 LC-MS analysis for amino acids and polyamines

The LC-MS analysis was performed on a Waters API Quattro Micro (Milford, MA, USA). The instrument was linked to a Waters Acquity ultraperformance liquid chromatograph (UPLC). A negative ionisation was achieved with an electrospray source, cone voltage of 15V and capillary voltage of 3.5 kV. Nitrogen as the desolvation gas (rate of 350 L/h; desolvation temperature of 350 °C) was employed. An injection volume of 1 μ L was introduced into a Waters AccQ Tag C18 column (2.1 x 100 mm, 1.7 μ m particle size). The derivatives were eluted with dilutions of Waters AccQ Tag Ultra Eluent A and Waters AccQ Tag Ultra Eluent B prior to them being quantitated with UV detection.

A gradient was set up and initiated by combining 99.9% eluent A and 0.1% eluent B (holding time of 0.54 min). A linear gradient to 21.2% eluent B over 7.2 min, to 90% eluent B over 0.31 min, to 100% eluent B over 0.45 min then followed. The column was kept at 100% eluent B for another 1 min to yield a total run time of 9.5 min. Throughout the run the flow rate was maintained at 0.7 mL/min.

4.2.4 Data collection and statistical analysis

For the metabolite analysis, five samples were used to measure the Sutherlandioside B (SU1) in the shoots. In order to determine the amount of SU1 in an average four week old *in vitro* grown plant, the amount of SU1 per gram of dry weight was approximated in a minimum of 24 plants. The mean amount of SU1 in an average nodal explant culture of four weeks was calculated. Quantitative analysis is represented as mean values. The normality

of the data was determined using a Shapiro Wilk's *W* test prior to further analysis, depending on the normal distribution of the data, using either a *Post Hoc* Fisher LSD test for normally distributed data or a Kruskal-Wallis test for non-normally distributed data. Differences between means reaching a minimal confidence level of 95% were considered as being statistically significant. All data were analysed with the use of Statistica version 8 software (Statsoft Inc. 2007).

The MZmine 2 (version 2.9.1) software package was utilised for the processing, visualisation and analysis of raw LC-MS data (Pluskal et al., 2010). To identify the patterns in the LC-MS data and to highlight the differences and similarities of this data, a principal component analysis (PCA) was performed (Smith, 2002). This technique aims to fit straight lines, known as principal components, to the data points. There are as many principal components as there are variables. The first principal component is the best straight line that can be fitted to the data. The second principal component is the best straight line that can be fitted to the errors of the first principal component and so forth. A PCA-analysis was also performed on the amino acid content of the samples with the use of the LatentiX data analytical software version 2.11 (Latent5, 2012).

4.3 RESULTS

4.3.1 General metabolite analysis

The raw LC-MS data was normalised and aligned using the MZmine 2 software. Centroid mass detection was performed and peaks below detected. The data was processed from a retention time of 2.0 min to 10.0 min and over a mass range of 250 to 1500 Da.

After processing the LC-MS data, an unsupervised multivariate cluster technique (PCA) was used. Pareto scaling was used to enhance the contributory effects of low concentration metabolites without amplifying noise in the metabolite data (Cloarec et al., 2005). The

loadings plot (Figure 4.1b) represents the peaks that are responsible for the separation of the treated samples. The treated samples in the scores plot (Figure 4.1a) that sit in the same vicinity of the compounds in the loadings plot (Figure 4.1b) have high concentrations of the compounds, whereas the treated samples that are positioned further from the compounds contain less of the compounds (Figure 4.1). The PCA model (Figure 4.1) of principal component one and two explains 51.117% of the variation of the metabolite data. There is a difference between the samples treated with NAA and those that were not treated with this auxin (Figure 4.1a). The metabolites that are responsible for the differentiation of the NAA containing samples are the metabolites at the following timepoints and masses; 5.68_697.3, 5.93_869.4, 5.98_737.3, 6.30_699.1, 6.47_697.3 and 6.99_811.4.

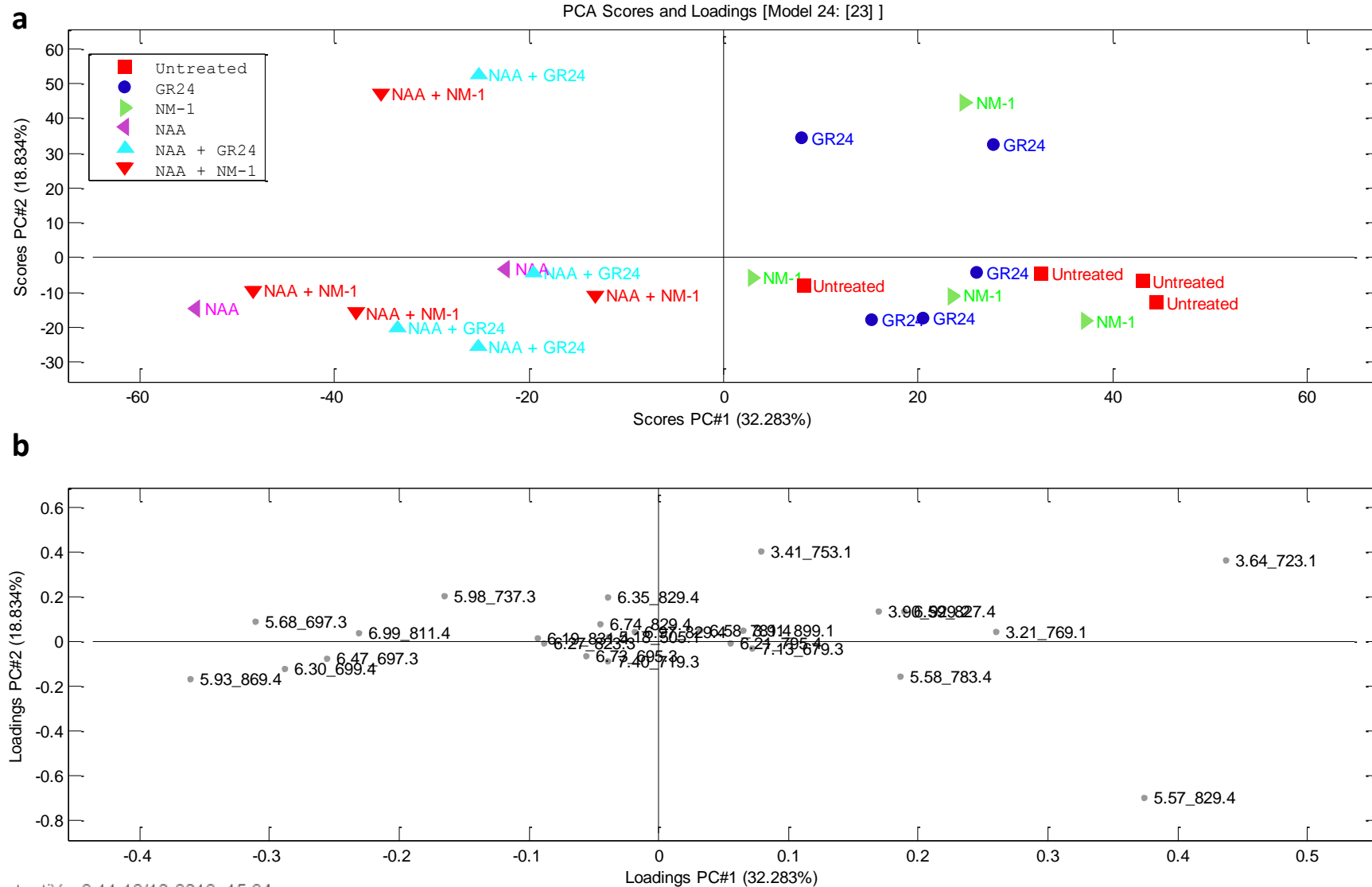


Figure 4.1: Principal component analysis a) scores and b) loadings plot of general metabolites of four week old *S. frutescens* in vitro explant cultures. 32.283% of the variation is explained. NM-1: Nijmegen-1; NAA: Naphthalene acetic acid.

4.3.2 Sutherlandioside B content

Sutherlandioside B (SU1) is an important compound in *S. frutescens* and studies have shown that this compound possesses anti-cancer properties (Van Wyk and Albrecht, 2008; Section 2.1.2.6). The amount of SU1 (μg) in a gram of dry mass leaf and stem material was determined (Figure 4.2a). The GR24 treatment yielded less ($p=0.047159$) SU1 than the control, whereas 1 mg/L NAA with Nijmegen-1 yielded significantly more ($p=0.016299$) SU1 than the control (Figure 4.2a). The amount of SU1 (μg) in an average *in vitro* tissue culture explant was also determined, to establish whether treatment with NAA and Nijmegen-1 is commercially viable (Figure 4.2b). This treatment (3.6 μg SU1 per average explant) yielded twice the amount of SU1 per explant than explants that were not treated with hormones (1.8 μg SU1 per average explant; Figure 4.2b), suggesting that this treatment could potentially be useful to commercially produce this putative anti-cancer compound.

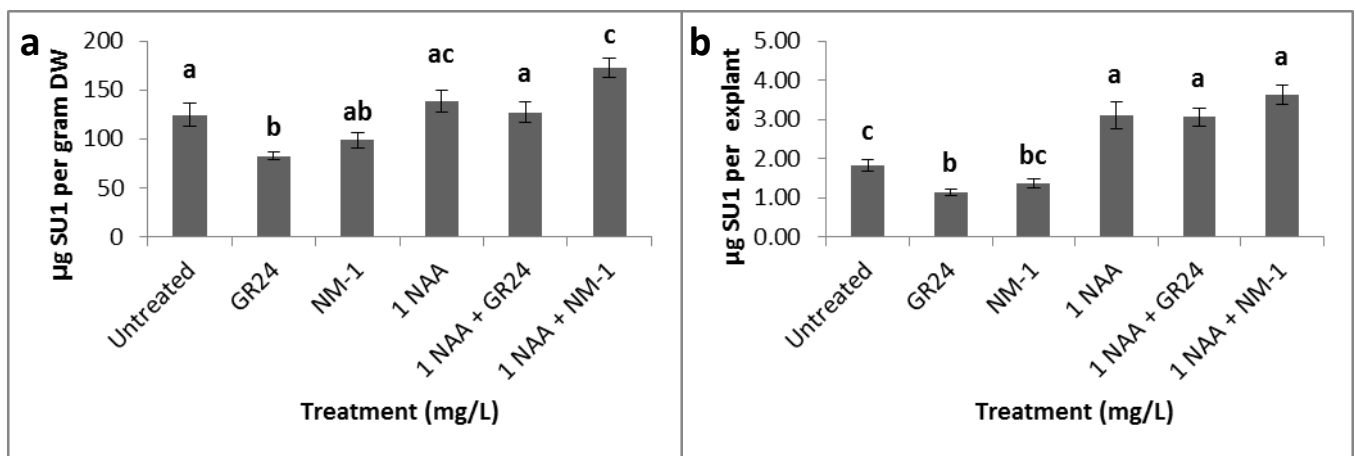


Figure 4.2: Mean amount of Sutherlandioside B (SU1) in micrograms a) per gram of dry mass plant material and b) in a four week old tissue culture explant. Data is means \pm SE; a) $n=5$; b) $N=24$; NM-1: Nijmegen-1; NAA: Naphthalene acetic acid.

4.3.3 Amino acid content

The treatment with NAA and Nijmegen-1 not only doubled the amount of SU1 (Figure 4.2) but also stimulated the production of some amino acids, including histidine, serine, arginine, aspartate, glutamate, threonine, alanine, proline, cysteine, tyrosine, methionine, valine, isoleucine, leucine, phenylalanine and asparagine (Figure 4.3a). An overall amino acid promotion ($p \leq 0.000051$) was observed with the combination of NAA and Nijmegen-1 treatment (Figure 4.3b).

The bioactive amino acids that accumulate in *S. frutescens* are L-arginine, L-asparagine, L-canavanine and GABA (Figure 4.4). These amino are also used in *Sutherlandia* to assess the quality of *Sutherlandia*-based products (Section 2.1.2). Treatment with Nijmegen-1 elevated the amount of asparagine and canavanine significantly when compared to extracts from the untreated explants, whereas treatment including both NAA and Nijmegen-1 increased the amount of asparagine (10.52 mg/g) and arginine (137.64 mg/g) significantly when also compared to untreated explants (Figure 4.4a). The relative percentage of the bioactive compounds; GABA, canavanine, asparagine and arginine; was calculated to determine the percentage at which these important compounds are present in the plant extractions since these metabolites are used for quality control purposes. The relative percentage of arginine was the highest that accumulated in 1 g dry material in all treatments (Figure 4.3b). This amino acid accumulated around 70% in the untreated samples and up to 86% in samples treated with NAA and Nijmegen-1. Gamma amino butyric acid accumulated the least in the plants, at levels of approximately 1% across all the treatments (Figure 4.4b). Canavanine contributed about 18% to the relative amounts of bioactive amino acids in the untreated samples and as little as 6% in the NAA and Nijmegen-1 treated samples (Figure 4.4b). Asparagine made up approximately 10% of the bioactive compounds (Figure 4.4b).

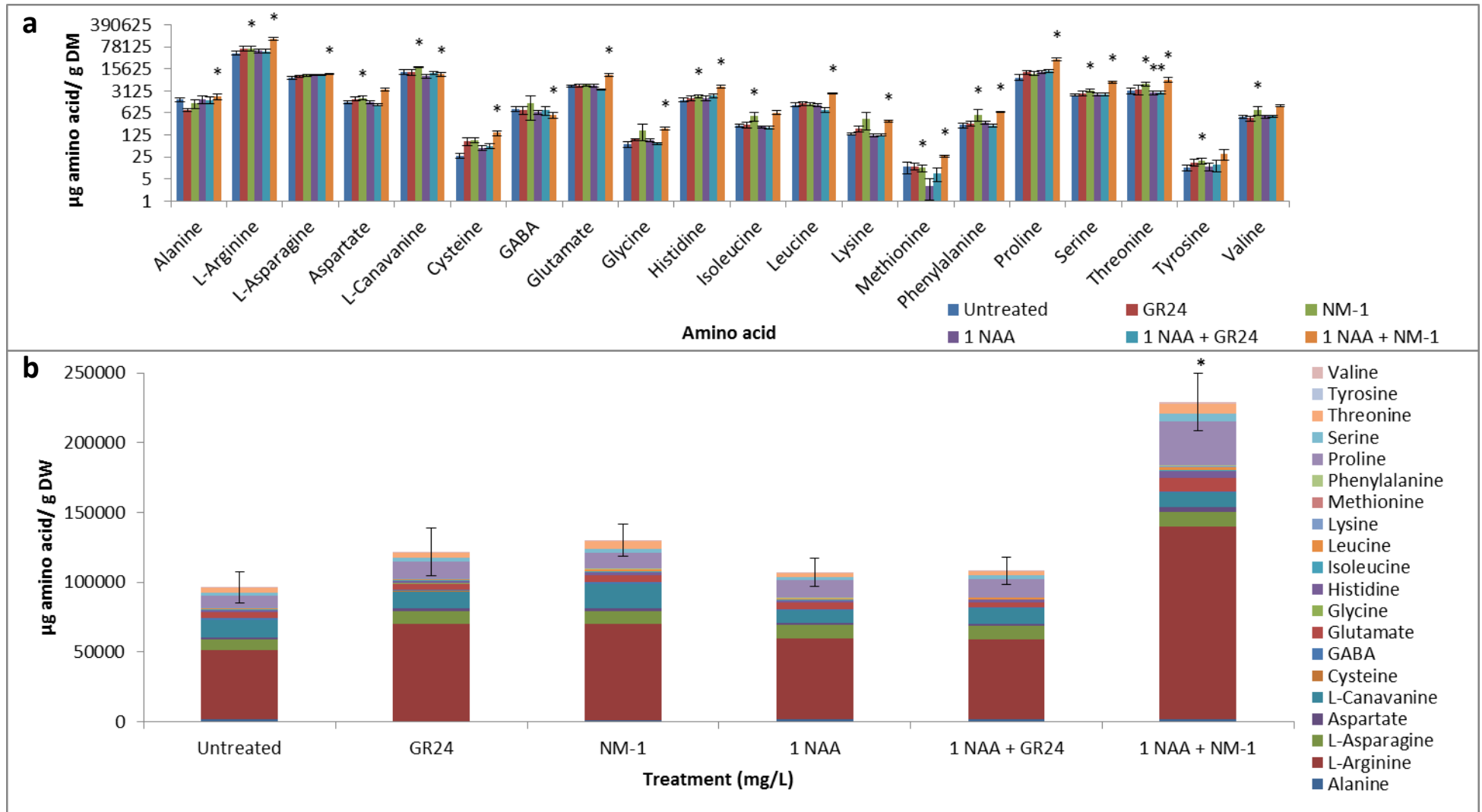


Figure 4.3: Amino acid content a) per gram dry weight of leaf and stem explant material and b) total per treatment. Log scaling is used to view amino acids that are found at low levels. Asterisks indicate significant differences in amino acid content compared to control of specific amino acid. Data is means \pm SE; n=5. NM-1: Nijmegen-1; NAA: Naphthalene acetic acid.

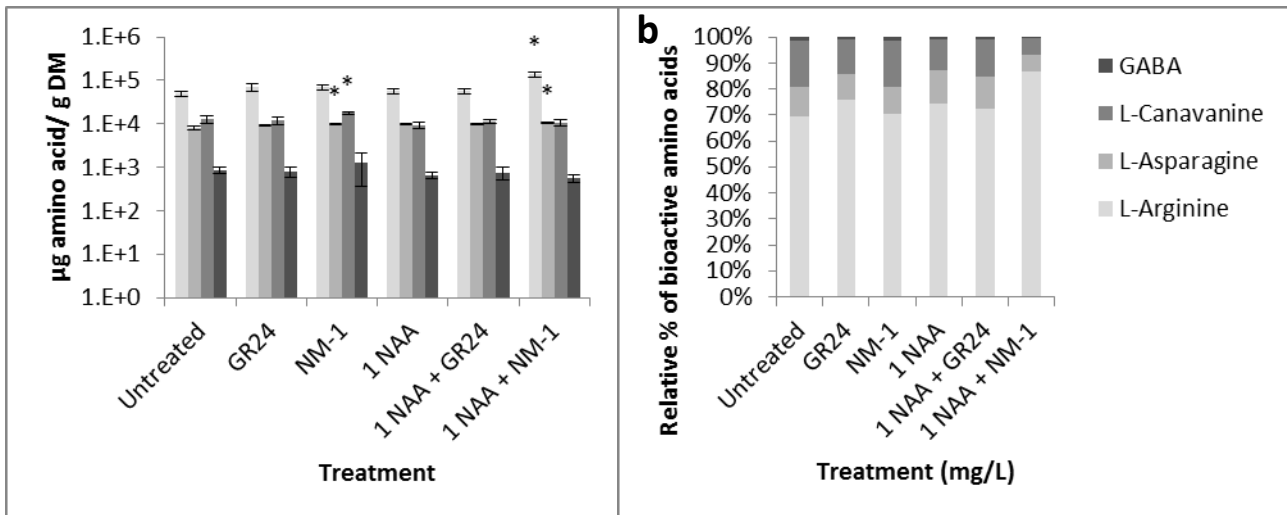


Figure 4.4: a) Micrograms of bioactive amino acids per gram dry mass and b) relative percentage of amino acids per treatment of *S. frutescens* cultures. Asterisks indicate significant differences in amino acid content compared to control of specific amino acid. Data is means \pm SE; n=5. NM-1: Nijmegen-1; NAA: Naphthalene acetic acid.

4.3.4 Polyamine content

The polyamines putrescine and spermidine were detected *via* the LC-MS method used, but spermine was not, possibly due to low amounts. The amounts of putrescine and spermidine did not increase significantly with treatment (Figure 4.5a). The putrescine contributed 70% to 80% of the measured polyamine content, whereas spermidine formed the remaining 20% to 30% (Figure 4.5b).

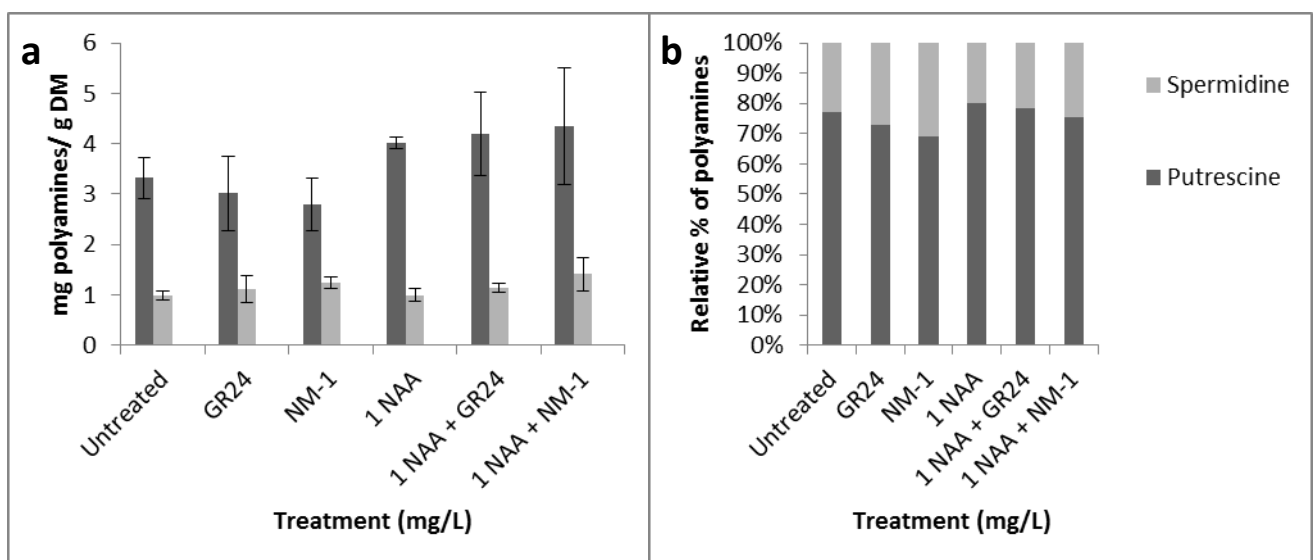


Figure 4.5: a) Milligrams of polyamines per gram dry mass and b) relative percentage of polyamines per treatment of *S. frutescens* cultures. Data is means \pm SE; n=5. NM-1: Nijmegen-1; NAA: Naphthalene acetic acid.

The PCA plot of the amino acids and polyamines (Figure 4.6) indicate that the measured amino acids and polyamines occur in the positive x-axis of the loadings plot (Figure 4.6b). Thus treatments on the scores plot (Figure 4.6a) that lie in the same vicinity as the amino acids contain a high amount of that specific amino acid. This gives a good idea of the amount of amino acids in a treatment compared to the other treatments. The majority of treatments appear on the negative x-axis in the scores plot (Figure 4.6a) and thus contain fewer amino acids than the NAA and Nijmegen-1-treated plants that lie in the positive x-axis.

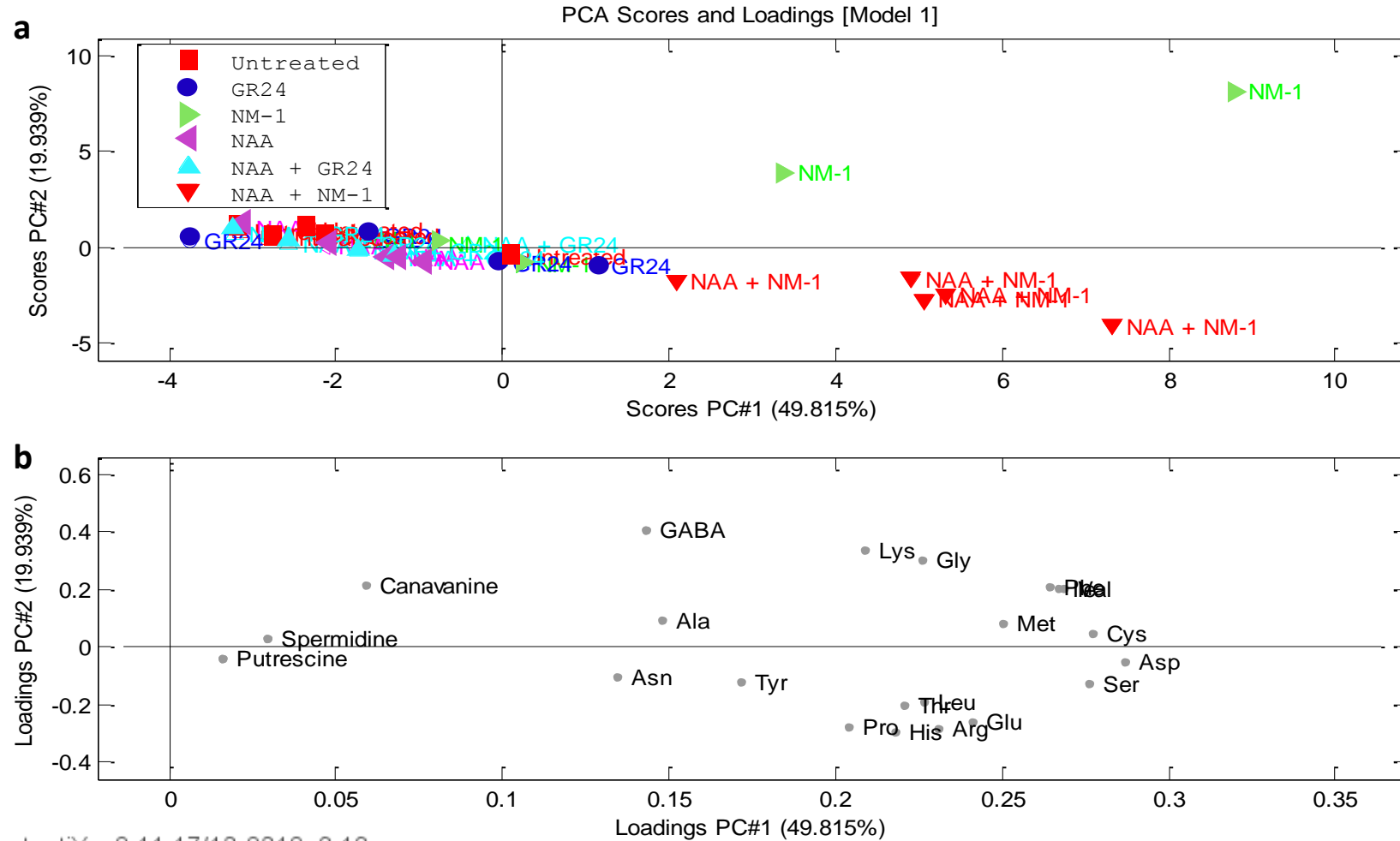


Figure 4.6: Principal component analysis a) scores and b) loadings plot of the amino acid and polyamine content in the treated and untreated samples of four week old *S. frutescens* in vitro explant cultures. 49.815% of the variation is explained. NM-1: Nijmegen-1; NAA: Naphthalene acetic acid.

4.4 DISCUSSION

The general metabolite analysis (Figure 4.1) showed a distinct difference between treatments that contained NAA and those that did not. Further analysis of the SU1 content and amino acids showed that the treatment that contained Nijmegen-1 in conjunction with NAA enhanced the SU1 content (Figure 4.2), the overall amino acids content (Figure 4.3) and some of the bioactive amino acids (Figure 4.4). This *in vitro* treatment is thus the most promising for the development of plants which may be used *ex situ* for phytopharmaceuticals. Since primary metabolites like amino acids are involved in the growth of plants, it is possible that auxin can enhance growth through the up-regulation of the primary metabolites. The use of NAA in plant tissue culture systems to enhance plant growth is well studied (Campanoni and Nick, 2005; Kollárová et al., 2005). The application of NAA on cultured tobacco protoplast-derived cells reduced the cytotoxicity of certain amino acids (Marion-Poll and Caboche, 1984). Thus the threshold of amino acids can be increased without being lethal to the plant. The added effect of Nijmegen-1 to NAA enhanced the already observed result of NAA on the metabolism of *S. frutescens*. This could be due to strigolactones being second messengers of auxin to regulate growth and thus affect metabolism. Strigolactones may therefore stimulate the expression of genes involved in metabolite synthesis due to auxin signalling. Hypothetically, auxin lessens the amino acid toxicity threshold (Marion-Poll and Caboche, 1984) and strigolactones actually promote the synthesis of the amino acids. Although speculating, this might also explain why these hormones alone are not as effective as when combined. It is also possible that the genes involved in amino acid synthesis are stimulated by both auxin and strigolactones and that the result is observed as an additive effect.

The secondary metabolites found in *Sutherlandia* include sutherlandins (flavonoid glycosides; Fu et al., 2010) and sutherlandiosides (cycloartane glycosides; Fu et al., 2008). Sutherlandins are classified under the flavonoids group, which are commonly found in medicinal plants. Some flavonoids inhibit enzyme systems such as hydrolases, ATPases,

kinases and transferases. This inhibition makes these compounds useful in anti-allergic, anti-inflammatory, anti-hepatotoxic, anti-microbial and anti-viral treatments. Consequently, up-regulation of these compounds is important for the potential development of new drugs (Phillipson, 1990). Sutherlandiosides, on the other hand, are part of the terpenoid group which are the most abundant natural compounds in plants (Waller, 1970). Terpenoids are found in a number of chemical variations and include important compounds used in drugs, such as bisabolol, vernolepin, artemisinin and taxol (Phillipson, 1990).

The sutherlandiosides, particularly sutherlandioside B, and sutherlandins are the main compounds that are proposed to inhibit cancer cell growth (Van Wyk and Albrecht, 2008). Sutherlandioside B (SU1) content in *S. frutescens* could be quantified in four week old explants grown in tissue culture on different treatments. The field grown plants contained 27.5 mg/g dry mass SU1 (Avula et al., 2010), whereas the four week *in vitro* untreated plants had a SU1 content of 0.124 mg/g dry mass (Figure 4.2a). This difference may be due to the tissue cultured plants being at a young age, since a full-grown *Sutherlandia* plant is about one year of age.

Treatment with GR24 resulted in a significantly ($p=0.047159$) lower amount of SU1 being produced than in the control. With the application of a combination of GR24 and NAA, the amount of SU1 was reduced slightly when compared with samples treated with NAA only (Figure 4.2a). This suggests that GR24 might have a negative effect on the formation of SU1. The GR24 and NAA treatment did not differ from the untreated samples and this may show that NAA and GR24 have opposite effects on SU1 synthesis. Nijmegen-1, in contrast, caused a significant elevation in the SU1 content when used with NAA ($p=0.016299$), compared with the untreated samples, although treatment with Nijmegen-1 alone resulted in a slight decline. This might suggest that Nijmegen-1 enhances the promotory effects of NAA on the SU1 content (Figure 4.2a). As with the growth results (Section 3.3), a difference in GR24 and Nijmegen-1 activity was perceived. The GR24 was a greater inhibitor of both growth (Section 3.3) and of SU1 synthesis (Figure 4.2a) than Nijmegen-1. This inhibitory

effect of GR24 on growth may have altered the metabolism of the plants and thus also a reduction in the synthesis of SU1 is evident.

To evaluate the commercial viability of NAA treatment, the amount of SU1 produced in an average four week old tissue culture plant was determined (Figure 4.2b). These treatments generated almost twice the amount of SU1 in an average plant than in the control plants (Figure 4.2b). This increase is not only due to an elevation in the SU1 content per gram of dry mass (Figure 4.2a) but also due to the improvement in growth (dry mass; Figure 3.1d).

The hormone treatments not only promoted the synthesis of SU1 but also other important medicinal compounds. Asparagine (Lea et al., 2006), arginine and canavanine (Slocum, 2005) are nitrogen storage and transport compounds. These are also part of the key metabolites found in *Sutherlandia* and are furthermore the compounds used as quality control standards. In this study, the *in vitro* growth of *S. frutescens* resulted in a high arginine to asparagine ratio. The accumulation of arginine in untreated plants yielded 49.46 mg/g and even more in treated plants (Figure 4.4a). The application of NAA and Nijmegen-1 resulted in a significant accumulation of arginine and asparagine ($p=0.000005$ and $p=0.001144$ respectively) when compared with the untreated explants (Figure 4.4a).

Both arginine and canavanine are synthesised from the same precursor (Figure 2.2), making canavanine an arginine antagonist (Rosenthal, 1977). The increase in arginine and not in canavanine after treatment with NAA and Nijmegen-1 can therefore be explained since they compete for the same precursor. Arginine and canavanine can then be degraded to the polyamine putrescine, which can be converted to spermidine and spermine (Figure 2.2). No spermine could be detected in any of the samples. The untreated *in vitro* plants contained approximately 77% (3.33 mg/g) putrescine and only 23% (0.99 mg/g) spermidine (Figure 4.5). It can be expected that an even lower amount of spermine would have accumulated and could thus not be detected. Normally plants contain spermine, since it is the final product in polyamine synthesis, but it is not necessary for plantlet survival. Spermine

mutants in *Arabidopsis* showed that plants deficient in spermine had reduced stem growth, but that it was not essential for survival (Imai et al., 2004). The lack of spermine, not only in the treated samples but also in the untreated plants, shows that *S. frutescens* does not produce detectable levels of spermine. The possibility exists that *S. frutescens* produces thermospermine, which is a structural isomer of spermine (Takano et al., 2012). This chemical has been found in leguminous plants such as pea and alfalfa (Bagga et al., 1997).

Aspartate is a direct or indirect precursor to a few amino acids; including lysine, methionine, threonine, isoleucine and asparagine. Asparagine is used for both nitrogen storage and transport in the plant (Lea et al., 2006) and is also involved in plant stress (Stewart and Larher, 1980). Glutamate also plays a central role in plant nitrogen metabolism and is possibly involved in nitrogen signalling (Forde and Lea, 2007). Glutamate is furthermore a direct or indirect precursor to amino acids mainly involved in plant stress protection; including proline, ornithine, arginine, canavanine, glutamine, GABA (Rai, 2002) and the polyamines also involved in plant stress (Edreva, 1996; Kuznetsov and Shevyakova, 2007).

Treatment with NAA and Nijmegen-1 caused the greatest shift in amino acid metabolism by increasing histidine, serine, arginine, aspartate, glutamate, threonine, alanine, proline, cysteine, tyrosine, methionine, valine, isoleucine, leucine, phenylalanine and asparagine (Figure 4.3a). The amino acids proline, arginine, aspartate, alanine, asparagine, glutamate and serine have been shown to accumulate at higher levels under various plant stresses (Rai, 2002). This treatment induces amino acid changes resembling a stress response, which indirectly gives an explanation of the improved levels of the secondary metabolite SU1 (Figure 4.2a), since secondary metabolites are mainly accumulated under stress conditions (Ramakrishna and Ravishnakar, 2011).

The other amino acids that were stimulated after treatment with NAA and Nijmegen-1 are histidine, methionine, valine, isoleucine, leucine and phenylalanine, which form part of the essential amino acids, as well as cysteine and tyrosine, which are semi-essential amino

acids required for human health (Gold, 2009). The combined amino acid content of natural *Sutherlandia* populations contributed to between 10% and 15% (m/v) of dried plant material, according to a recent study by Mncwangi and Viljoen (2012). Their study also gave a good representation of the average amounts of amino acids in *S. frutescens* populations. A comparison between the wild populations from Mncwangi and Viljoen (2012) and the *in vitro* cultures from this study gives an indication that *in vitro* growth of *S. frutescens* elevates the levels of certain amino acids. These include alanine, arginine, asparagine, canavanine, GABA, glutamate, histidine, leucine, proline, serine and threonine. Thus, *in vitro* culture growth is beneficial for the up-regulation of the bioactive amino acids, arginine, asparagine, canavanine and GABA, and further hormonal treatment stimulated the production of these amino acids even further.

4.5 CONCLUSION

The use of NAA to promote the synthesis of important compounds in *S. frutescens* seems promising. When used in combination with Nijmegen-1, the positive effects of NAA were enhanced. This combination is favourable for commercialisation when higher yields of specific amino acids and SU1 content are desirable. Further studies on the metabolism, proteins and genes are needed in order to complete the metabolite map of *S. frutescens*. Genomics and proteomics will aid in discovering the important genes and proteins involved in metabolite synthesis. This study forms an important part in metabolite discovery and investigation of medicinal products produced by *S. frutescens*.

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CHAPTER 5:

Influence of growth factors on *in vitro* hairy root cultures

5.1 INTRODUCTION

Hairy root cultures are a type of plant culture which is formed when plant organs (stems, leaves or roots) are infected with the soil bacterium *Agrobacterium rhizogenes*. This bacterium transfers DNA (known as T-DNA) from root inducing (Ri) plasmids into the plant genome, which results in the abnormal growth of adventitious roots at the site of infection (Gelvin, 1988, Tzfira and Citovsky, 2006). Through genetic engineering the plasmid in *Agrobacterium* can be modified to contain a gene of interest which can then be transformed into the plant (Kumar et al., 2006).

5.1.1 *Agrobacterium*-mediated transformation

The Ri plasmid contains genes encoding proteins that are used for the production and transport of the T-DNA. Located on this plasmid is a region which is flanked by 25 base pair direct repeats defined as the left (TL region) and right (TR region) T-DNA borders which are ultimately translocated with the T-DNA to the plant genome after a successful transformation event. This process is controlled by a set of proteins encoded by a set of bacterial chromosomal (*chv*) and Ri plasmid virulence (*vir*) genes (Gelvin, 2000; Tzfira and Citovsky, 2006). The *vir* region encodes most of the virulence (Vir) proteins used to produce T-DNA and to deliver it into the host cell. The first step in the transformation process is the

attachment of the bacterium to the host plant after the perception of plant phenolics, such as acetosyringone, which are secreted by the wounded plant. The VirA/VirG signal-transduction system perceives the plant phenolics and in turn stimulate the transcription of the *vir* region (Gelvin, 2000). Most of the Vir proteins are directly involved in the processing and transfer of the T-DNA from the Ri-plasmid to the plant cell. These molecules assist to protect and stabilise the T-DNA for its travel into the host cell nucleus (Ward and Zambryski, 2001). Once inside the nucleus, the escorting proteins are stripped off and the T-DNA is integrated into the host genome (Tzfira and Citovsky, 2006). The hairy root syndrome is associated with the expression of the T-DNA genes.

The TR region contains genes encoding for proteins involved in the biosynthesis of the opines which serve as a food source for the bacterium after the infection of the plants. This region also contains two genes involved in auxin biosynthesis, *aux1* and *aux2*, alternatively referred to as *tms1* and *tms2* (Morris, 1986; Nemoto et al., 2009). Transformed tobacco cells are unable to multiply without the *aux* genes (Nemoto et al., 2009), suggesting that these genes are necessary for auxin-autotrophic⁹ cell division. The importance of these genes in the formation of hairy root cultures is still under dispute. Insertions into the *aux* loci have resulted in the *A. rhizogenes* strains being avirulent (White et al., 1985), while other studies indicated that the formation of hairy roots was possible without the transfer of these genes into the plant's genome (Offringa et al., 1986). The TL region carries approximately 18 potential genes (Slightom et al., 1986), of which four genes, *rol A – D* (White et al., 1985) are necessary for the induction of hairy roots (Cardarelli et al., 1987; Spina et al., 1987). These *rol* genes are involved in the development and maintenance of the hairy root phenotype (Schmülling et al., 1988).

⁹ Production of auxin *via* photosynthesis or chemosynthesis

5.1.2 Properties of transformed hairy root cultures

The advantages of using hairy root cultures for the study of metabolites include the genetic and biochemical stability of these cultures when compared to other transformed cultures (Aird et al., 1988a; b). These plants also exhibit rapid growth in hormone-free media when compared to normal roots (Hilton et al., 1988). The biosynthetic capacity of these cultures is equivalent to the parent plant (Parr and Hamill 1987). Commercially, hairy roots can be exploited in bioreactors without the loss of their biosynthetic capacity, rapid growth and genetic and biosynthetic stability (Kim et al., 2002). Another advantage is the ease with which *Agrobacterium* can be genetically manipulated, making the production of certain compounds possible *via* the insertion of specific DNA. Some hairy roots have been found to secrete metabolites into the growth medium, which is of great advantage for the recovery of metabolites. Previously, a disadvantage of hairy root cultures were that the machinery used for the synthesis of metabolites had to be found in the roots of plants (Rhodes et al., 1990), but now it is possible to synthesise the metabolites found in the green parts of the plant with green hairy roots because these contain the machinery needed for photosynthesis (Sauerwein et al., 1992).

Hairy roots exhibit a typical phenotype in that these roots lack geotropism and that they have a high occurrence of lateral root branching (Shen et al., 1988). Several genes are involved in the growth of hairy roots. The *rolA* gene is responsible for the lack in gravitropism since plants infected with mutant *rolA* T-DNA grew straight out of the infected wound of the plant without bending downwards in response to gravitational pull, whereas *rolB* T-DNA mutants were avirulent, indicating a central role for this gene. Mutations in *rolC* resulted in attenuated root growth and mutations in *rolD* resulted in the formation of callus, which retarded root growth (White et al., 1985).

5.1.3 Effect of hormone treatment on hairy root metabolism

The exogenous application of growth regulators on transformed root cultures results in the alteration of secondary metabolite production (Rhodes et al., 1994; Jeong et al., 2007). Consequently, the application of hormones can be used to alter metabolite synthesis in culture. Hairy roots have the ability to grow in the absence of exogenously-applied hormones (Rhodes et al., 1994; Bais et al., 2001), but the application of hormones could have a positive effect on their metabolism. Hairy root cultures of *Hyoscyamus muticus* produce almost three times more ethylene than normal roots. Application of auxin (IAA, IBA and NAA) lowered the ethylene content of the hairy roots when applied at a concentration of 2.5 μM (Biondi et al., 1997). Increasing concentrations of auxins also resulted in the decreased production of secondary metabolites of *Cichorium intybus* hairy root cultures, whereas gibberellic acid stimulated the production of the metabolites (Bais et al., 2001). Thus different growth regulators have a different effect on hairy root growth and metabolism. In this study, the effects of auxin and strigolactones on *S. frutescens* hairy root cultures was investigated.

The hairy root system has been utilised for the study of secondary metabolism as transformed root cultures will produce higher levels of key metabolites. Several studies highlight the usefulness of hairy root cultures as a tool to study metabolism. For example, Staszków et al. (2011) utilised LC-MS techniques to assess accumulation of flavonoid glycoconjugates in the hairy root cultures of *Medicago truncatula*. Yang et al. (2012) assayed for caffeic acid metabolites in hairy root cultures of *Salvia miltiorrhiza* and *S. castanea* and included a gene expression analysis study using cDNA-AFLP techniques where cultures were treated with yeast extract to encourage the accumulation of secondary bioactives. An NMR-based profiling metabolite study after establishment of hairy root culture lines using *Agrobacterium* transformation from leaf explants of *Gynura procumbens* was conducted by Saiman et al. (2012). These cultures were utilized to study the effects of several culture parameters, including the strength of the medium, concentration of sucrose, medium pH and presence or absence of light. Sharma et al. (2013) discussed a variety of applications related

to the use of hairy root cultures as a tool to study secondary metabolism. These authors reviewed the use of elicitation, radioisotope tracers, inhibitors blocking enzyme steps, mutant selection, heterologous gene overexpression, RNA interference technologies, and high-throughput -omics methods that are used to resolve secondary metabolic pathways using hairy root cultures.

5.1.4 Aim of chapter

The growth of hairy root cultures was evaluated after the addition of different plant growth promoting substances. The effects that strigolactones had on primary root and lateral root growth was also inspected in order to learn more about their impact on root development. Through the treatment of *S. frutescens* hairy root cultures with auxins and strigolactones, the metabolome of *S. frutescens* can be altered. This part of the study also describes the primary and secondary metabolic profiles of hairy root cultures exposed to strigolactones.

5.2 MATERIALS AND METHODS

5.2.1 *In vitro* culture conditions

5.2.1.1 Mass accumulation

Hairy root tips from a continuous *in vitro* *S. frutescens* hairy root culture (C58C1-g) established in 2007 by Ms Janine Colling (refer to Colling, 2009 for the transformation protocol) were placed in liquid half strength Murashige and Skoog (MS; 1962) medium (pH 5.8) containing 3% (m/v) sucrose and 0.1 g/L *myo*-inositol.

Five hairy root tips of approximately 1 cm each were placed in 50 mL half strength liquid MS medium with the treatments described in Table 5.1 in 100 mL Erlenmeyer flasks. All root tips

were placed on an MRC orbital shaker (at 120 rpm) and kept in the dark at $25 \pm 2^\circ\text{C}$. The roots were left to grow for 28 days. After 28 days, hairy roots were removed from treatments, blotted dry with tissue paper, frozen in liquid nitrogen (-196°C) and freeze dried for approximately one day using the VirTis BenchTop K (model 2KBTES) freeze drier. Dry mass was measured for growth. Dried hairy roots were kept at -80°C until further analysis. This experiment was repeated three times.

Table 5.1: The hormonal treatments used for experiments with Murashige and Skoog (1962) medium. The untreated medium contained no added plant growth promoting substances.

Treatment	Untreated	0.1 mg/l IBA ($4.92 \times 10^{-7}\text{M}$)	1 mg/l IBA ($4.92 \times 10^{-6}\text{M}$)	0.1 mg/l NAA ($5.37 \times 10^{-7}\text{M}$)	1 mg/l NAA ($5.37 \times 10^{-6}\text{M}$)
Untreated	X	X	X	X	X
$1 \times 10^{-7}\text{M}$ GR-24	X	X	X	X	X
$1 \times 10^{-7}\text{M}$ Nijmegen-1	X	X	X	X	X

5.2.1.2 Root growth

Three hairy root tips of approximately 1 cm from a continuous *in vitro* *S. frutescens* hairy root culture (C58C1-g) (Colling, 2009) were placed on half strength Murashige and Skoog (1962) medium (pH 5.8) containing 3% (m/v) sucrose and 0.1 g/L *myo*-inositol solidified with 0.8 % (m/v) agar with the treatments described in Table 5.2. All root tips were kept in the dark at $25 \pm 2^\circ\text{C}$. The primary root length was measured and the number of lateral roots that formed from the primary root was counted every two days for a period of 19 days.

Table 5.2: Treatments used for root hair development experiments with solid Murashige and Skoog (1962) medium. The untreated medium contains no added plant growth promoting substances.

Treatment	Untreated	1 mg/l IBA (4.92×10^{-6} M)
Untreated	X	X
1×10^{-7} M GR-24	X	X
1×10^{-7} M Nijmegen-1	X	X

5.2.2 Extraction of hairy root metabolites

After a four week period, the hairy root cultures were harvested from the culture vessels and measured for dry matter accumulation. The selected treatments included untreated, strigolactone-treated, and 0.1 mg/L IBA-treated hairy root cultures. This material was frozen in liquid nitrogen and freeze-dried for a day using the VirTis BenchTop K (model 2KBTES) freeze drier. Material was stored at -80°C until metabolite analysis.

Metabolite extraction from hairy root cultures for LC-MS analysis of general metabolites as well as for amino acid and polyamines were the same as the extraction from leaf and stem material. Please refer to Section 4.2.2 for a detailed description.

5.2.3 Metabolite analysis

5.2.3.1 LC-MS analysis for general metabolites

The hairy root extractions were subjected to the same treatment as in Section 4.2.3.1 for the analysis of general metabolites. The analysis was performed on the same spectrometer utilising the same setting.

5.2.3.2 LC-MS analysis for amino acids and polyamines

The samples were subjected to the same preparation and analysis as in Section 4.2.3.2 for the analysis of amino acids and polyamines. The settings were also kept unchanged.

5.2.4 Data and statistical analysis

5.2.4.1 Hairy root growth analysis

Quantitative analysis is represented as mean values and the reproducibility of the results is expressed as standard error. The normality of the data was determined using a Shapiro Wilk's W test. The data was normally distributed and a *Post Hoc* Fisher LSD test was performed to identify differences between treatments. The differences between means reaching a minimal confidence level of 95% were considered as being statistically significant. All data was analysed with the use of Statistica Release 8 program (StatSoft Inc. 2007).

5.2.4.2 Chemometric analysis

The MZmine 2 (version 2.9.1) software package was utilised for the processing, visualisation and analysis of raw LC-MS data (Pluskal et al., 2010). To identify patterns in the LC-MS data

and to highlight the differences and similarities of this data a principal component analysis (PCA) was performed (Smith, 2002). This analysis was also performed on the amino acid content of the samples with the use of the LatentIX data analytical software version 2.11 (Latent5, 2012).

For the metabolite analysis, five samples were used to measure the general and amino acid content in the hairy root cultures. In order to determine the amount of L-Arginine and GABA in an average four week old *in vitro* grown hairy root culture, the average amount of the amino acid per gram of dry mass was determined in five hairy root cultures. The mean amount of the amino acid in a four week old hairy root culture is represented in Figure 5.10. Quantitative analysis is represented as mean values. The normality of the data was determined using a Shapiro Wilk's W test prior to further analysis depending on the normal distribution of the data using either a *Post Hoc* Fisher LSD test for normally distributed data or a Kruskal-Wallis test for non-normally distributed data. The differences between means reaching a minimal confidence level of 95% were considered as being statistically significant. All data was analysed with the use of Statistica version 8 software (Statsoft Inc. 2007).

5.3 RESULTS

5.3.1 Hairy root growth analysis

All hormonal treatments resulted in reduced accumulation of dry mass of the root cultures (Figure 5.1). Of the two synthetic strigolactones, Nijmegen-1 treatment accumulated significantly less dry mass ($p=0.019328$), whilst the effect of all the auxin treatments was even more detrimental ($p\leq 0.000000006$) when compared to the untreated control. Interestingly, the addition of Nijmegen-1 to 1mg/L IBA and either GR24 or Nijmegen-1 to 0.1 mg/L NAA slightly ameliorated the reduction in mass accumulation caused by these auxins (Figure 5.1).

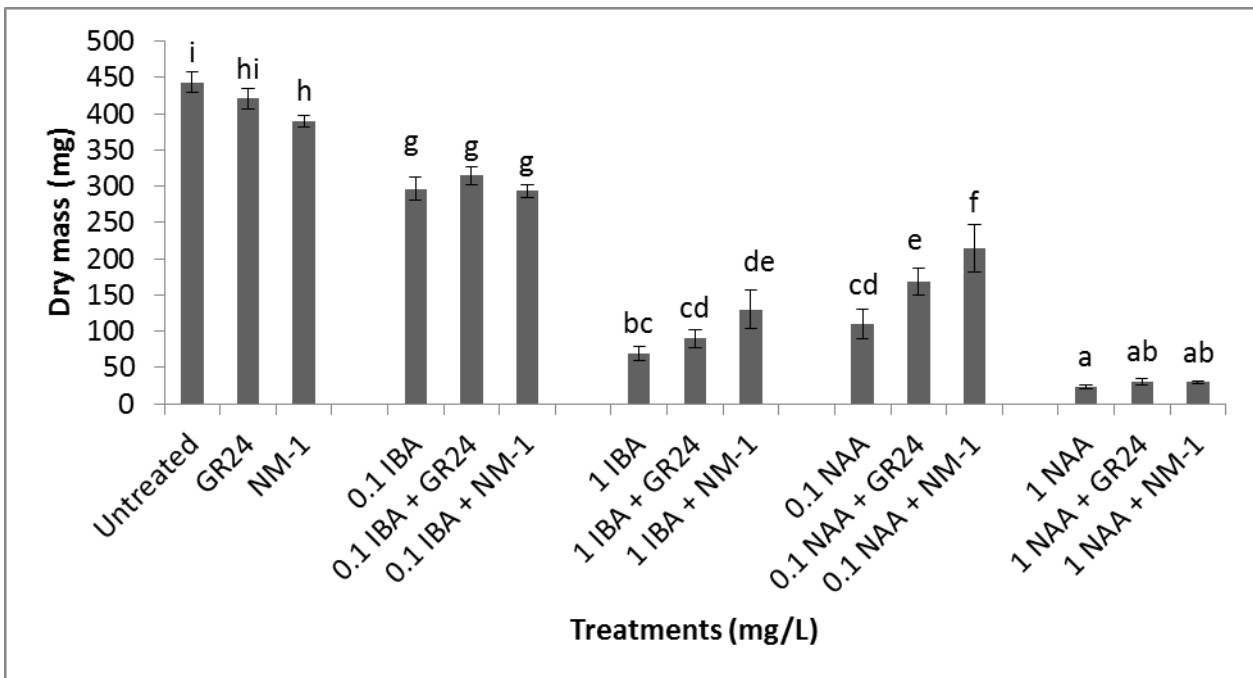


Figure 5.1: Dry mass of *S. frutescens* in vitro hairy root cultures after four weeks of growth in liquid MS medium containing strigolactones and auxins. Data is means \pm SE; $n=15$. NM-1: Nijmegen-1; IBA: Indole-3-butyric acid; NAA: Naphthalene acetic acid.

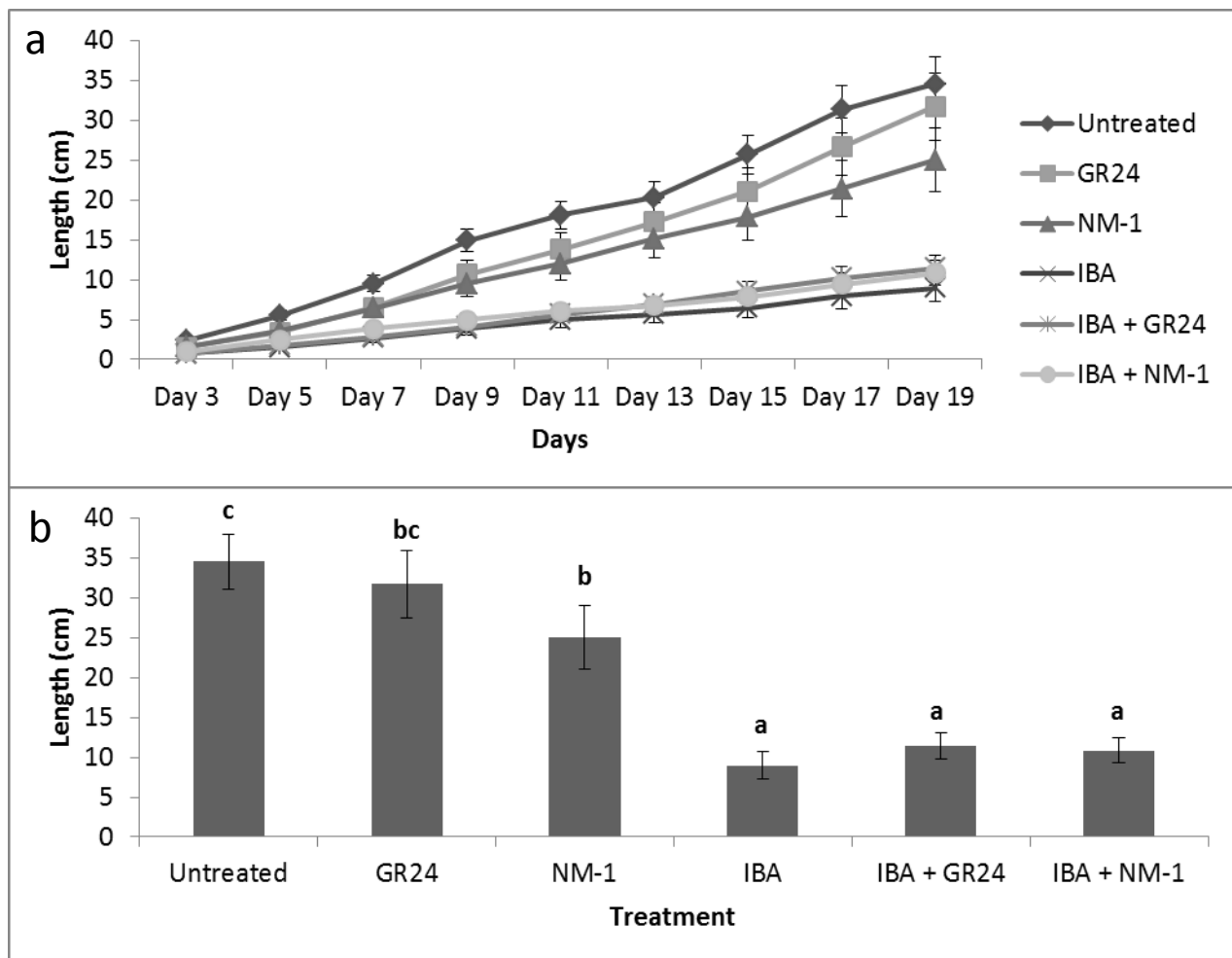


Figure 5.2: Primary root length in centimeters a) over a period of 19 days and b) after 19 days on treatment. Data is means \pm SE; $n=30$. NM-1: Nijmegen-1; IBA: Indole-3-butyric acid.

The addition of strigolactones to 1mg/L IBA slightly ameliorated the reduction in mass accumulation caused by this auxin and thus root elongation and lateral root formation focused on these treatments. The elongation of the primary roots was assessed. Treatments containing IBA resulted in roots that were shorter ($p \leq 0.000269$) than the treatments that did not after 19 days (Figure 5.2). Phenotypically, these roots seemed thicker and more callus-like when compared visually to the untreated roots (Figure 5.3). Thus it seems that IBA treatment results in callus formation rather than root growth and this then results in reduced growth. After 7 days of treatment, the untreated and strigolactone-treated roots were significantly longer ($p \leq 0.021342$) than the treatments containing IBA (Figure 5.2a). The untreated roots were significantly longer ($p \leq 0.023098$) than any of the other treatments after only 5 days on treatment (Figure 5.2a). After 13 days on treatment, GR24-treated roots started to grow faster and had the same growth rate as the untreated roots (Figure 5.2a). All



Figure 5.3: Hairy root plate cultures. Hairy roots containing IBA grew slower. NM-1: Nijmegen-1; IBA: Indole-3-acetic acid.

treatments containing IBA grew at the same rate, which was significantly less than that of the untreated samples (Figure 5.2b).

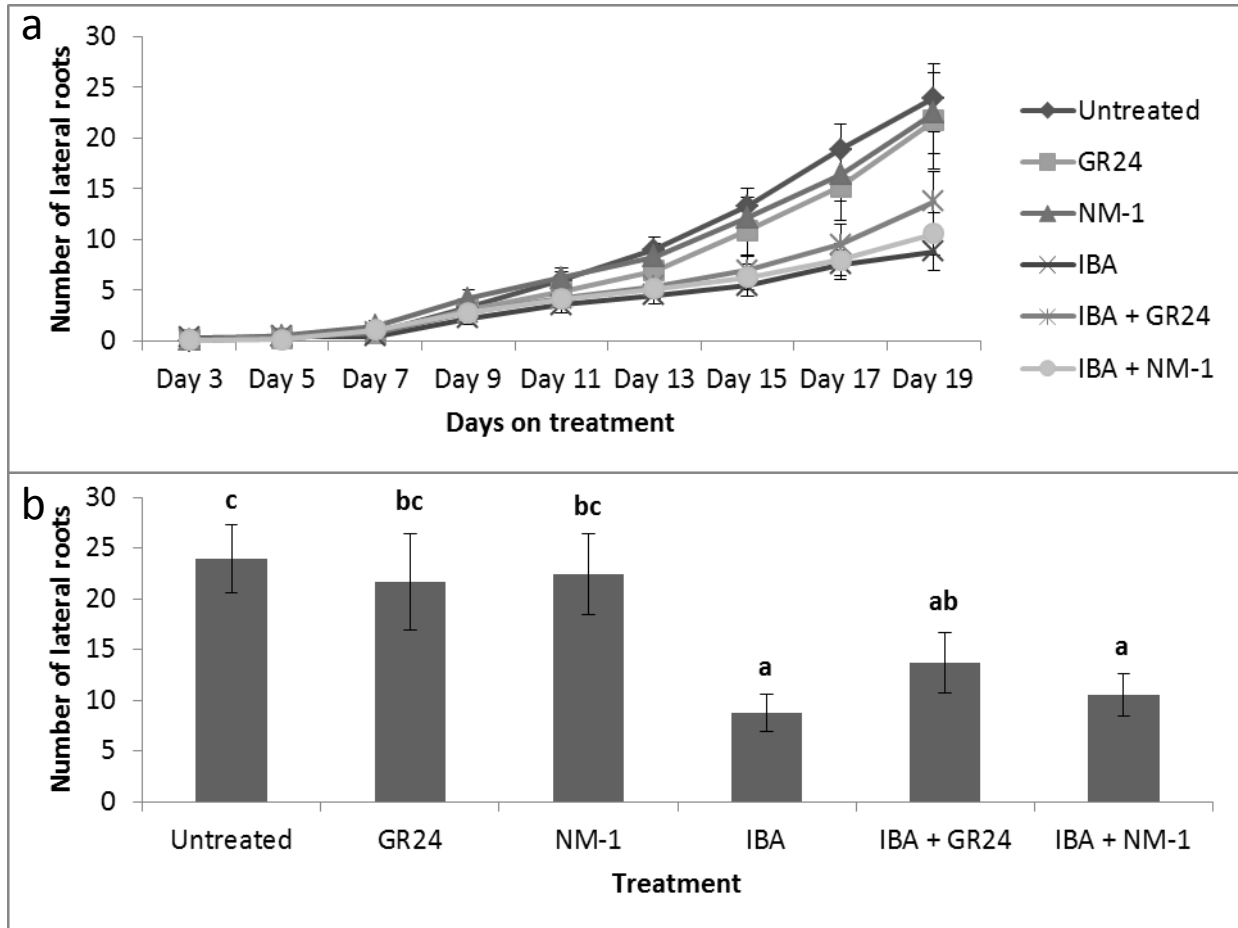


Figure 5.4: Number of lateral roots a) over a period of 19 days and b) after 19 days on treatment. Data is means \pm SE; $n=30$. NM-1: Nijmegen-1; IBA: Indole-3-butyric acid.

The number of lateral roots on different treatments was counted every two days over a period of 19 days (Figure 5.4). From this data, the day on which root growth started to show differences due to treatment could be established. From day 13, the roots from treatments that did not contain IBA had more lateral roots than those that did contain IBA ($p \leq 0.020463$; Figure 5.4a). After 19 days, all treatments containing IBA resulted in the production of significantly fewer lateral roots ($p \leq 0.00648$) than those of untreated and strigolactone-treated cultures (Figure 5.4b). Lateral root development showed similar trends to that observed with the dry mass; strigolactone treatments produced slightly, but not significantly, fewer lateral

roots than the untreated control explants, whilst the addition of strigolactones to IBA slightly ameliorated the negative effects of the IBA although not significantly (Figure 5.1 and Figure 5.4b).

Phenotypically, hairy roots from IBA-containing treatments were shorter and visually thicker than the hairy roots not treated with IBA (Figure 5.2 and Figure 5.3) additionally the liquid cultures treated with IBA were “milky” due to callus formation (Figure 5.5). The untreated and strigolactone-treated samples were visually an orange/dark yellow colour, whereas treatments containing IBA were light yellow (Figure 5.5).

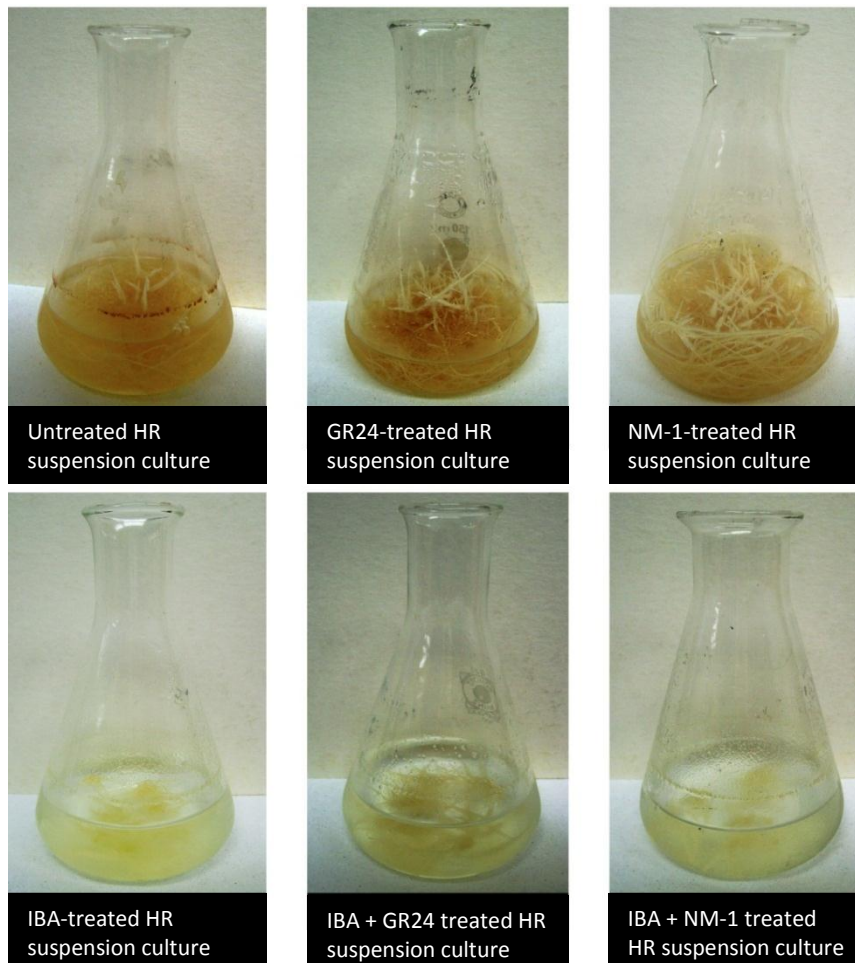


Figure 5.5: Hairy root liquid cultures. Hairy roots containing IBA grew slower and did not clump together as the treatments containing no IBA. NM-1: Nijmegen-1; IBA: Indole-3-acetic acid.

5.3.2 General metabolite analysis

The IBA-treatment differed from the other treatments since it lies farther apart from the other treatments in the PCA plots (Figure 5.6a). The untreated control and strigolactone-treated samples did not differ from each other (Figure 5.6a). The NAA treatment was not used because of the low mass accumulation (Figure 5.1) of the cultures after this treatment, which meant that there was insufficient material available for the analysis.

The distribution of the metabolites is represented by a PCA plot of the scores (treatments; Figure 5.6a) and loadings (metabolites; Figure 5.6b). The scores separated on the plot according to the contribution of the metabolites. Thus the higher concentration of a certain metabolite in a sample, the closer the score (sample) will be to that loading (metabolite). The separation of the scores highlights the differences and similarities between each sample (Trygg et al., 2007).

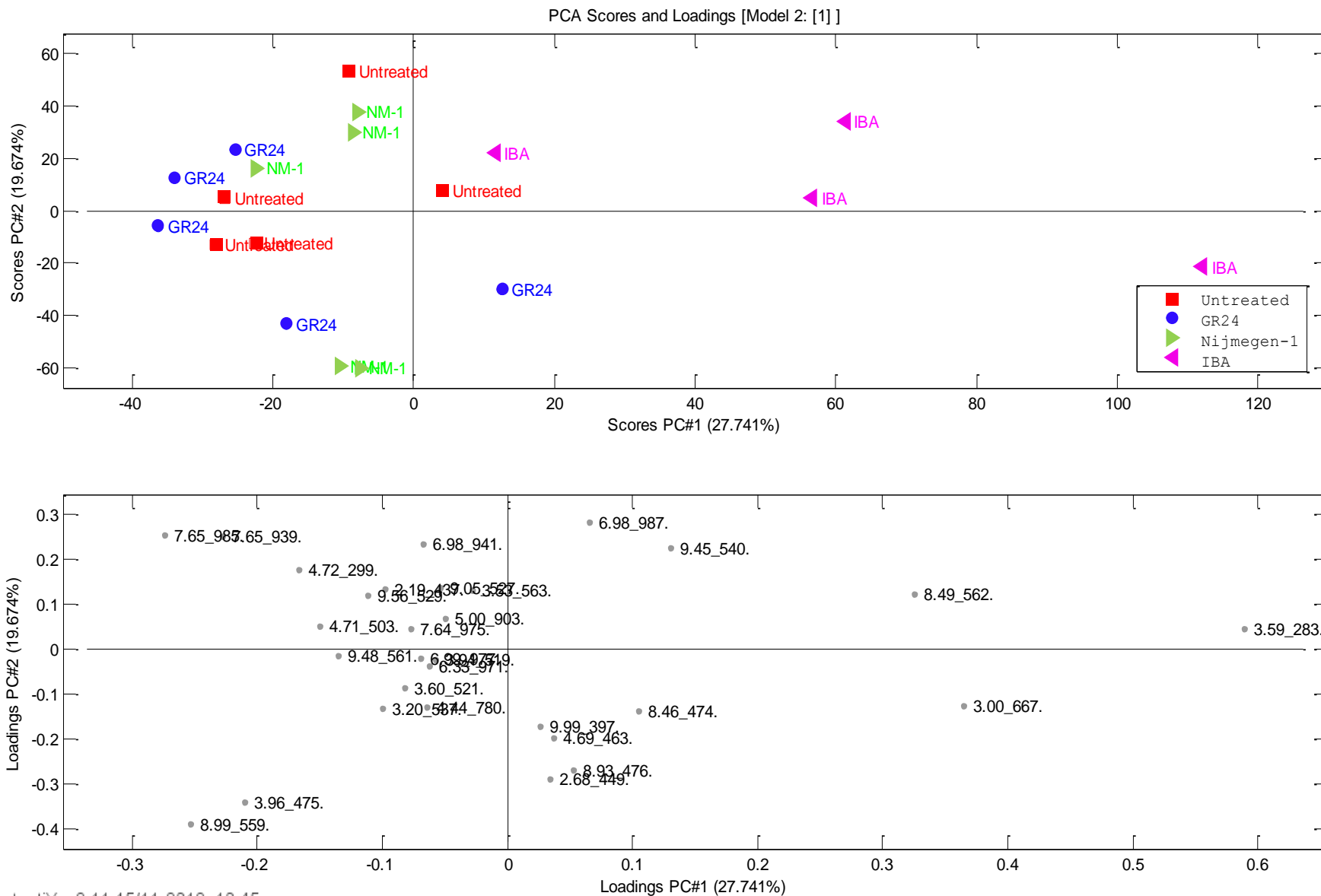


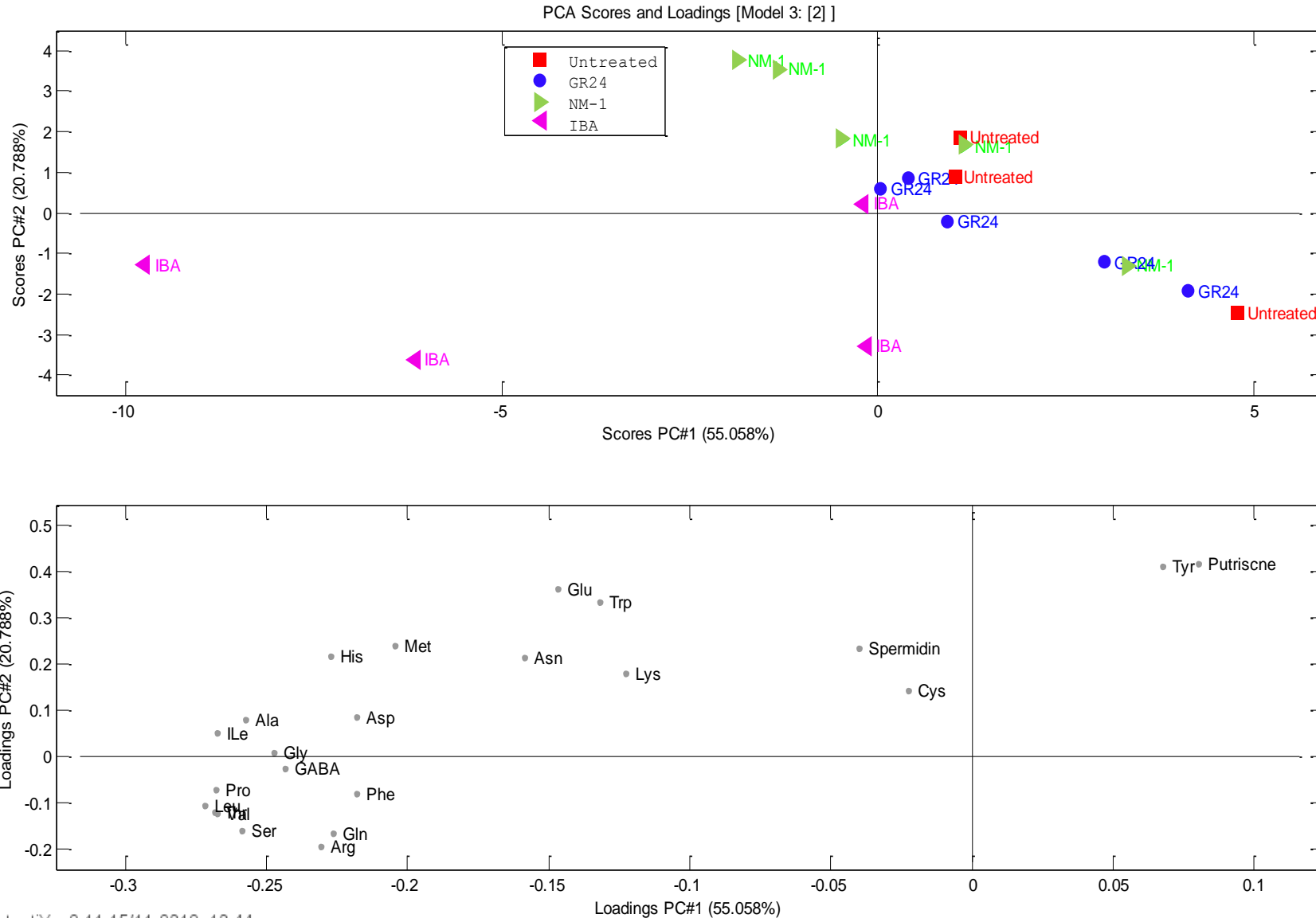
Figure 5.6: Principal component analysis of the a) scores and b) loadings plots of the general metabolite content after treatment with IBA and strigolactones. 27.741% of the variation is explained. NM-1: Nijmegen-1; IBA: Indole-3-butyric acid.

5.3.3 Amino acid and polyamine analysis

The distribution of the amino acids and polyamines are represented by a PCA plot of the scores and loadings. The IBA-treated samples differed from the other treatments based on the amounts of amino acids present in the samples (Figure 5.7a). These samples contained less tyrosine and putrescine and more of the other amino acids. There were no differences in the amino acid composition whether samples were grown in the absence or presence of strigolactones (Figure 5.7a).

To further evaluate the differences noted in the PCA plot, amino acids were quantified and compared for each treatment (Figure 5.8). The IBA-treatment elevated the amounts of serine, arginine, glycine, threonine, proline, valine, leucine, glutamine and GABA and lowered the amount of tyrosine and putrescine when compared with the untreated samples (Figure 5.8a). The total amount of amino acids that accumulated was higher after treatment with IBA, mainly due to the contributions of glutamine, asparagine and arginine (Figure 5.8b). The untreated and strigolactone-treated samples contained similar amounts of amino acids and no significant differences were noted (Figure 5.8a). The total amount of amino acids after GR24-treatment was slightly less than that of the untreated hairy root cultures (Figure 5.8b).

The amounts of the bioactive compounds are important to evaluate because of their role in quality control of *S. frutescens* containing products (Mncwangi and Viljoen, 2012). Gamma-amino butyric acid accumulated at very low levels of only 24 µg/g in untreated hairy roots (Figure 5.9a). Arginine content was 0.23 mg/g, whereas asparagine was very high at 11.22 mg/g in the untreated hairy root cultures (Figure 5.9a). Treatment with IBA significantly increased the arginine and GABA content (Figure 5.9a). Strigolactone-treatment had no effect on the synthesis of the bioactive amino acids (Figure 5.9a). Asparagine contributed to the highest relative amount at 97.7% with arginine at 2% and GABA at only 0.3% in untreated hairy root cultures (Figure 5.9b).



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Figure 5.7: Principal component analysis of the a) scores and b) loadings plot of the amount of amino acids and polyamines in IBA and strigolactone treated hairy root cultures. 55.068% of the variation is explained. NM-1: Nijmegen-1; IBA: Indole-3-butyric acid.

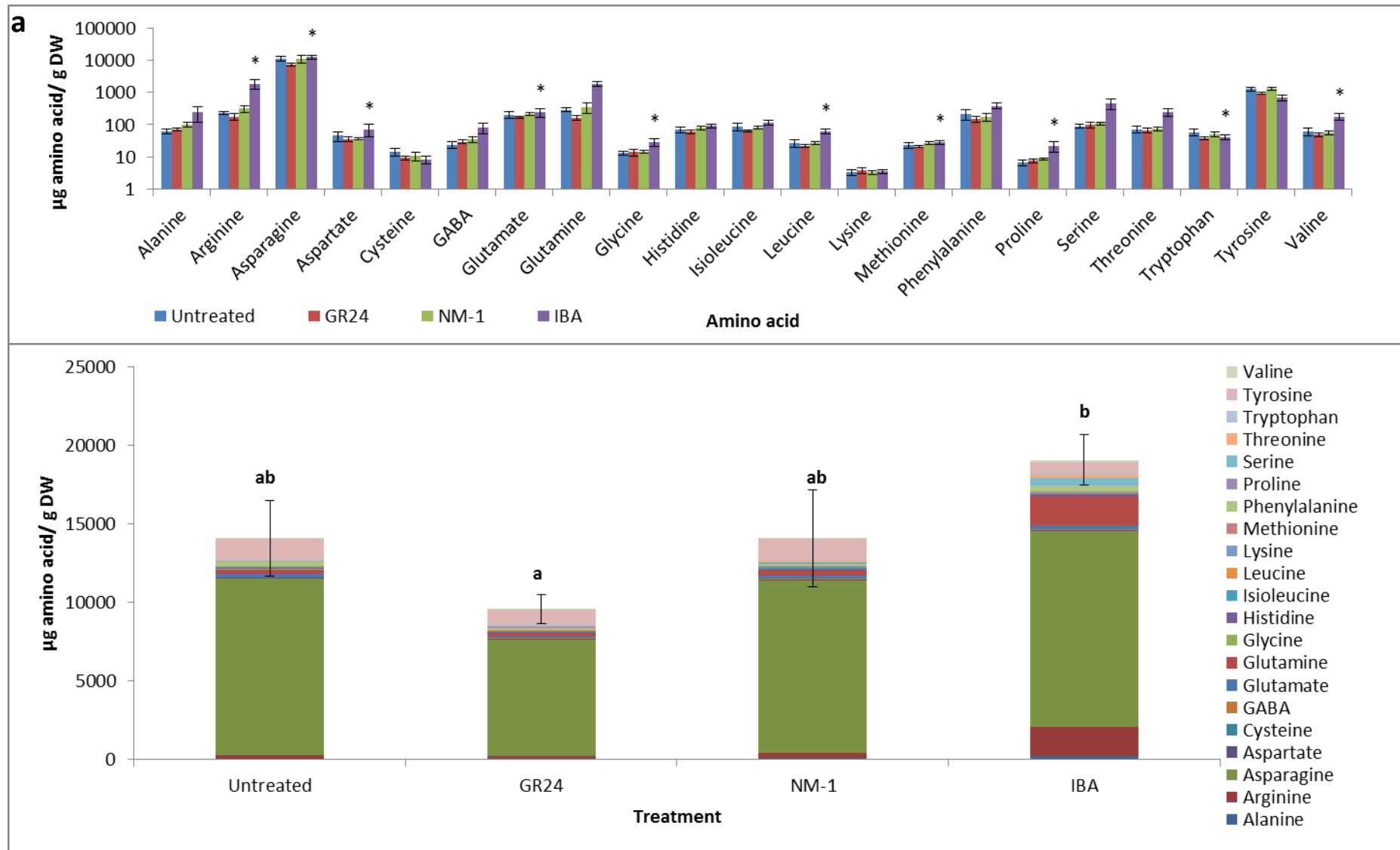


Figure 5.8: Amino acid content a) per gram dry weight of hair root material and b) total per treatment. Log scaling is used to show amino acids that are found at low levels. Asterisks indicate significant differences in amino acid content compared with the control for that specific amino acid. Data is means \pm SE; N=5. NM-1: Nijmegen-1; IBA: Indole-3-butyric acid.

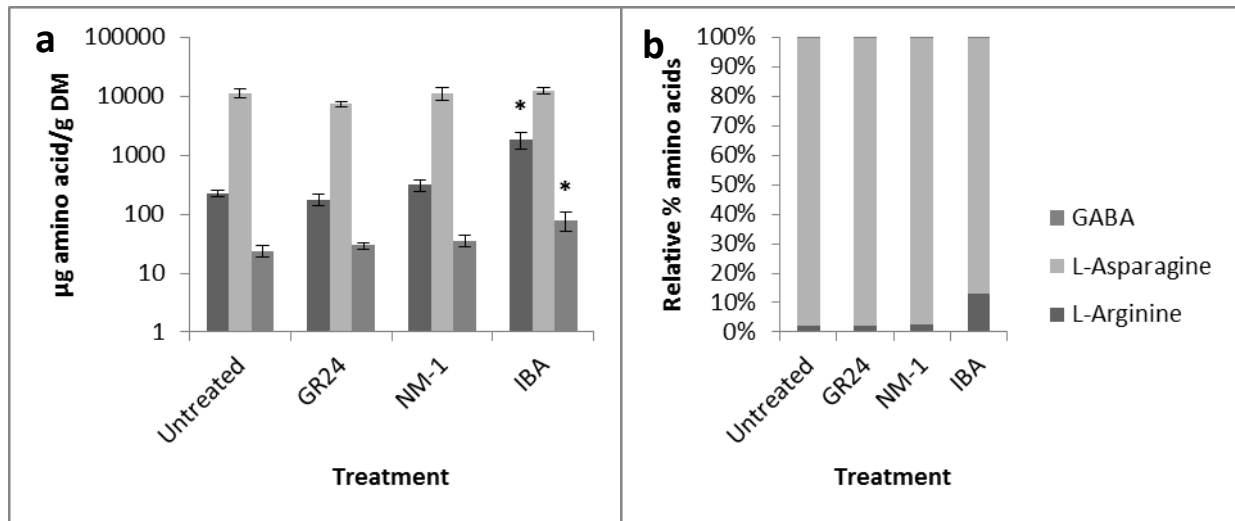


Figure 5.9: Amount of bioactive amino acids in *S. frutescens* hairy root cultures in a) microgram amino acid per gram dry mass and as b) relative percentage of bioactive amino acids. Log scaling is used to display amino acids that are found at low levels. Asterisks indicate significant differences in amino acid content compared with the control for that specific amino acid. Data is means \pm SE; $n=5$. NM-1: Nijmegen-1; IBA: Indole-3-butyric acid.

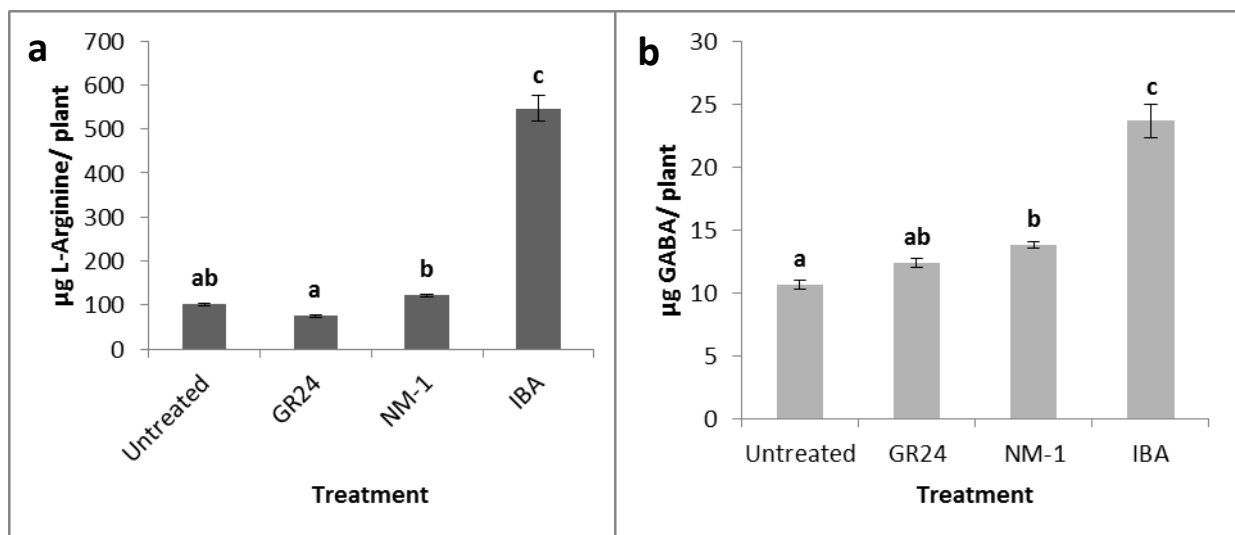


Figure 5.10: Micrograms of a) arginine and b) GABA in a four week old hairy root culture. Data is means \pm SE; $n=5$. GABA: Gamma-amino-butyric acid; NM-1: Nijmegen-1; IBA: Indole-3-butyric acid.

To establish whether or not IBA-treatment is able to up-regulate certain amino acids such as arginine and GABA content even though the growth was significantly lower than the control (Figure 5.1), the amount of these amino acids in an average four week old hairy root culture was determined. The amount of arginine after treatment was 5.4 times higher in an average four week old IBA-treated hairy root culture than in the control (Figure 5.10a). The amount of

GABA was increased 2.2-fold when treated with IBA than in the untreated roots (Figure 5.10b). Thus IBA treatment stimulated the amino acids despite the fact that there was less root growth.

The amounts of the polyamines, putrescine and spermidine, were determined. Putrescine content was significantly reduced after treatment of IBA (Figure 5.11a). The highest amount of putrescine (78.71 mg/g) accumulated in the samples that were treated with Nijmegen-1 (Figure 5.11a). Spermidine content in the hairy root cultures stayed unchanged after treatment and was found in smaller amounts of 2 mg/g or less (Figure 5.11a).

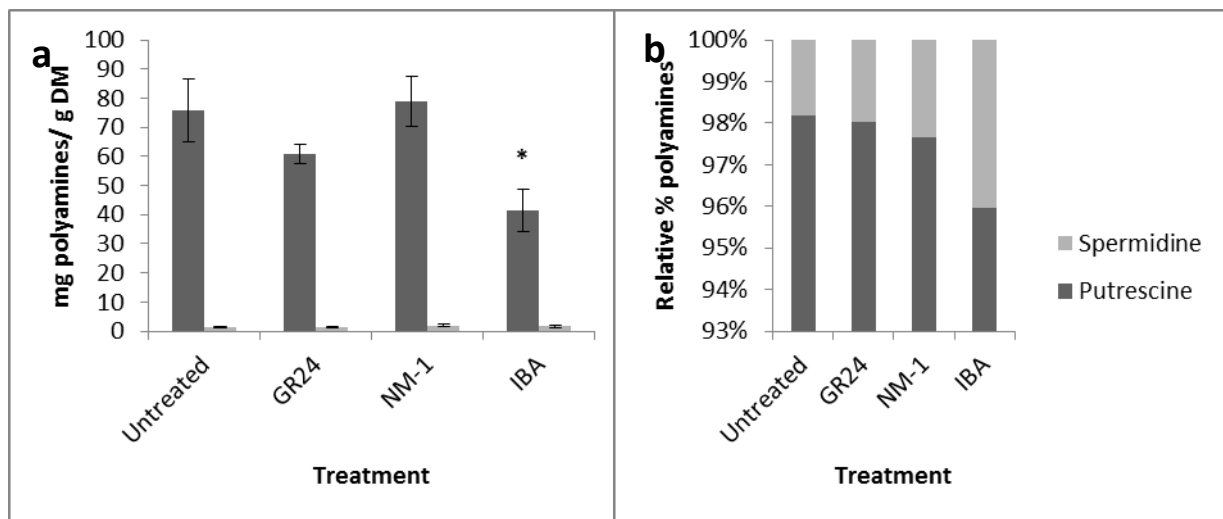


Figure 5.11: Amount of polyamines in treated *S. frutescens* hairy root cultures in a) milligram polyamine per gram dry mass and as b) relative percentage of polyamines. Data is means \pm SE; $n=5$. NM-1: Nijmegen-1; IBA: Indole-3-butyric acid.

5.4 DISCUSSION

The growth of the hairy root cultures was determined by dry mass, the primary root length and the formation of lateral roots per hairy root. Mass accumulation declined slightly in comparison with untreated control hairy roots after treatment with Nijmegen-1, but not GR24. Treatment with either of the two auxins tested, IBA and NAA, resulted in significant reductions of biomass accumulation. The effect was more pronounced for NAA than for IBA.

This auxin-affected reduction in growth of hairy root cultures has been observed previously and it was concluded that hairy roots had a greater sensitivity to auxin than normal root cultures and that this effect was not due to uptake of exogenous auxin (Shen et al., 1988). This sensitivity may be due to the effect of the transgenes situated in the Ri plasmid. These genes, being involved in the auxin biosynthesis (*aux* genes) and in development and metabolism (*rol* genes), could likely affect the sensitivity to exogenous auxins (Morris, 1986; Schmülling et al., 1988; Nemoto et al., 2009). A higher incidence of callus formation associated with the addition of auxin might also be linked to the *rolD* gene. Mutations of this gene resulted in the formation of callus which retarded growth (White et al., 1985). Thus it might be possible that treatment with auxin suppresses the expression of the *rolD* gene which results in callus accumulation.

Interestingly, the addition of strigolactones slightly ameliorated the reduction in mass of the 1 mg/L IBA and 0.1 mg/L NAA treatments. This seems counter-intuitive, since Nijmegen-1 treatment alone also hindered mass accumulation, but when added to 1 mg/L IBA or to 0.1 mg/L NAA it improved the mass when compared to the auxin-only control. It is well known that a link between strigolactone and auxin signalling exists (Beveridge and Kyozyuka, 2010). The addition of strigolactones to auxin can stimulate growth by decreasing uptake of auxin (Ruyter-Spira et al., 2011) or by stimulating secretion of auxin (Koltai et al., 2010). The uptake and secretion of auxin is mainly regulated by auxin flux carriers and it has been suggested that strigolactones affect both the influx and efflux auxin carriers. This is because auxin is able to stunt growth at both too high and too low concentrations. Thus when strigolactones are present in the auxin media, the strigolactones control the auxin flux carriers, resulting in slight ameliorated growth when compared to the only auxin treatments.

To determine whether the number of lateral roots correlated with the dry mass of the suspension cultures, the primary root length and number of lateral roots that grew from the primary root were measured every two days over a period of 19 days. This time-course experiment also facilitated in establishing the time point when the differences in growth

occur. Unfortunately, due to the fast growth of hairy roots, accurate results of lateral root number could only be monitored for 19 days and not a full 28 days of growth. This still gives a good representation whether or not faster growth is the determining factor for mass accumulation.

Application of GR24 to *Arabidopsis* seedlings at a concentration of 10 μM under normal conditions resulted in shorter primary roots (Ruyter-Spira et al., 2011). When 10 μM of GR24 was applied to rice seedlings under normal conditions, the crown root length was promoted (Arite et al., 2012). This indicates that GR24 affects root growth in different species at different concentrations. In this experiment with *S. frutescens* hairy root cultures, the 0.1 μM GR24-treated roots were slightly shorter and those treated with Nijmegen-1 significantly shorter ($p=0.026998$) than the untreated control (Figure 5.2b) after 19 days of treatment. Under phosphate-limiting conditions, the crown roots of wild-type and not strigolactone mutant rice plants were longer (Arite et al., 2012), implying that strigolactone biosynthesis is stimulated under low phosphate conditions. This indicates that the effects of strigolactones on root growth are dependent on the conditions. The shorter primary root length might also be due to the transgenes present in the hairy root cultures or it could possibly be species related.

A previous study showed that treatment with GR24 reduced lateral root formation and promoted root hair length of *Arabidopsis* plants (Kapulnik et al., 2011a; 2011b). A slight decline in the formation of lateral roots was noted with the use of GR24 alone but this was not statistically significant. Treatment with auxin, however, resulted in fewer lateral roots being produced. Interestingly, the addition of GR24 to IBA resulted in a slightly higher number of lateral roots when compared with IBA treatment alone after 19 days (Figure 5.3b) which correlates to previous results on *Arabidopsis* plants when NAA was exogenously added to GR24 (Ruyter-Spira et al., 2011). Due to the hairy roots' sensitivity to auxin (Shen et al., 1988) the number of lateral roots after auxin and strigolactone application was not significantly higher than the untreated. This nonetheless indicates that strigolactone

application to auxin treatment reduces the inhibitory effect of the auxin. Ruyter-Spira et al. (2011) suggested that strigolactones are modulators of auxin flux and that, at low auxin concentrations strigolactones inhibit the import of auxin into the roots, resulting in low lateral root formation. At high auxin concentrations the strigolactone-mediated reduction would aid in achieving the optimum auxin levels, resulting in lateral root formation. Although highly speculate, it is possible that strigolactones may also affect auxin receptors or the expression of *rol* or *aux* genes in hairy root cultures.

Auxin-treatment had a positive effect on the metabolism of the hairy root cultures. The general metabolite analysis established that IBA-treatment differed from the untreated and strigolactone-treated hairy root cultures (Figure 5.6). The amino acids and polyamines could be quantified in the hairy root cultures (Figure 5.10) and again it was observed that IBA-treated hairy roots differed from the other treatments in terms of their amino acid contents (Figure 5.7). The IBA-treatment resulted in a significant increase in the important medicinal metabolites arginine ($p=0.002316$) and GABA ($p=0.030975$) when compared with the control (Figure 5.10a). In contrast to *in vitro* shoots (Section 4.3.3), hairy root cultures do not accumulate any detectable canavanine. This is because canavanine is primarily produced in photosynthetic tissues (Rosenthal, 1972). Colling (2009) suggested that it may be possible that canavanine was present at very low levels and that it was beyond the detection limit of the LC-MS technique used. Canavanine synthesis could also have been down-regulated due to random insertions of the *rol* genes into the plant genome. Another option is that canavanine could have been excreted into the medium and thus not detected in the hairy root culture.

The growth of hairy roots treated with 0.1mg/L IBA was considerably less than that of the untreated samples (Figure 5.1) but even so, IBA-treated cultures still yielded more arginine and GABA (Figure 5.10) than those growing without this plant growth regulator. The IBA-treatment stimulated both GABA ($p=0.030975$) and proline ($p=0.027427$) production, which gives an indication of environmental stress. Proline accumulation has been found to rise

under various conditions including osmotic stress (Yoshida et al., 1995), drought (Choudhary et al., 2005), oxidative stress (Yang et al., 2009), and UV radiation (Saradhi et al., 1995). The addition of auxin has also been shown to stimulate the formation of proline-rich proteins (Meyer et al., 1984). Since the accumulation of proline was not that high (21.60 µg/g), it is more likely to be due to the formation of proline-rich proteins and not due to plant stress. Gamma amino butyric acid (GABA) also accumulates in response to osmotic (Akçay et al., 2012) and pH stress. This amino acid is also involved in C:N balance (Akçay et al., 2012) and is important in plant signalling, including rooting (Roberts, 2007). Exogenously applied GABA resulted in root length inhibition in *Arabidopsis* plants (Roberts, 2007). It might be possible that auxin stimulated GABA production, thus causing a reduction in root elongation. Fortunately, however, treatment with 0.1 mg/L IBA is sufficient to provide a method to improve the yields of medicinally important amino acids such as GABA and arginine, even though it decreased the growth of the hairy root cultures (Figure 5.10).

Sutherlandia frutescens is an adaptogen and thus the accumulation of various amino acids can be anticipated when environmental changes, such as hormonal treatments, are applied. Elevated levels of serine, glycine, proline and glutamine also give an indication of plant stress (Rai, 2002) after treatment with 0.1 mg/L IBA (Figure 5.8a). In human health, these amino acids are non-essential, but supplementation is needed in some circumstances. Other amino acids up-regulated by IBA-treatment are threonine, valine and leucine. Valine and leucine are both branched-chain amino acids that form part of the essential amino acids (Mathews et al., 2000). These amino acids work together with isoleucine, also an essential branched-chain amino acid, for muscle recovery after exercise (Mero, 1999). Threonine was also increased after IBA application and is an essential amino acid, which has been shown to help in the production of antibodies and may be useful to lessen the symptoms of Amyotrophic Lateral Sclerosis (ALS; Blin et al., 1992) and Multiple Sclerosis (MS; Hauser et al., 1992). *Sutherlandia* can be potentially used for the therapeutic relief of ALS, MS and an extensive range of other illnesses.

The potential use of *Sutherlandia* as a phytopharmaceutical is wide spread and application of hormones, specifically IBA, elevated the production of 10 of the 21 amino acids measured. Amino acids are essential for normal human health, while the supplementation of other non-essential amino acids is needed for the relief of some ailments.

5.5 CONCLUSION

Treatment with both strigolactones and auxins, alone or in combination, did not improve the growth of *S. frutescens* hairy root cultures. Rather, strigolactones slightly inhibited, whilst auxins strongly inhibited, primary root elongation. Interestingly, however, the use of both auxin and strigolactones together reduced the reduction of biomass accumulation and lateral root outgrowth caused by auxin alone. This again indicates an involvement of strigolactones in auxin signalling. The ameliorated effect on growth strigolactones exhibited when used with auxin indicates the possible involvement of strigolactones in stress-reduction. Application of strigolactones to *S. frutescens* hairy root cultures did not alter the metabolism of these plants significantly, but the application of 0.1 mg/L IBA had a significant effect. Thus the use of IBA for the up-regulation of metabolites could be commercially viable, since the promotion of metabolites more than compensates for the poorer growth. Further investigation is needed to establish growth in bioreactors and whether or not hairy roots must be treated for the full duration of four weeks to accumulate higher levels of metabolites.

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CHAPTER 6:

Conclusions and future prospects

6.1 CONCLUSIONS

Sutherlandia frutescens has been used in the treatment of cancer, diabetes, HIV/AIDS, inflammation, wound-healing, and as an anti-oxidant. The statements on the use of *S. frutescens* for the treatment of HIV/AIDS (amongst others) have ignited a current interest in the pharmacological activity of *S. frutescens* extracts. This newfound commercial interest has placed *S. frutescens* in high demand. This provided a good incentive for the use of chemicals that act as growth promoters which have the potential to improve plant growth, plant biomass and/or stimulate production of certain metabolites.

This study aimed to improve total metabolite content of plants either by increasing growth or metabolite levels. Using *in vitro* studies on *S. frutescens*, growth promoting chemicals were used to encourage growth and metabolism under controlled conditions. The growth hormones were effective in altering the metabolite profiles of cultured material and this makes it possible to use these chemicals as an application to stimulate the production of secondary metabolites. The application of various treatments to the *in vitro* cultures also helped to establish a combination favourable for commercialisation in that these plants yielded higher growth and metabolite content than untreated plants. The treatments that resulted in the greatest increase in dry mass of nodal explant cultures were those that contained 1mg/l NAA. The addition of strigolactones to this treatment did not alter the growth significantly and it is suggested that NAA treatment alone is sufficient in obtaining higher yields in dry mass than untreated plants. These NAA containing plants also had elevated metabolite levels and here the addition of the synthetic strigolactone Nijmegen-1 to 1mg/l

NAA had a significant positive effect on the SU1 levels and overall amino acid content per gram dry mass.

The strigolactone treatments had no significant effect on the growth of hairy root cultures and the application of auxin inhibited hairy root growth. The addition of IBA to the medium resulted in significantly lower dry mass accumulation compared to the untreated roots, but promoted the production of important amino acids such as GABA and arginine per gram dry mass. The up-regulation of these metabolites makes these treatments valuable for the production of phytopharmaceuticals, since these metabolites are of importance in medicinal products.

6.2 FUTURE PROSPECTS

The current lack of knowledge on strigolactone function in plants makes the study of strigolactone function in non-model plants difficult. Future work on model plants, such as *Arabidopsis thaliana*, *Medicago truncatula* or *Pisum sativum*, may enable work on a protein, metabolite and genetic level. For instance, the use of mutants may aid in clarifying the interactions between strigolactones and auxins. The effects of auxin on the metabolism of most model plant species is known and thus the addition of strigolactones to the plants would be valuable from a metabolite point of view. This will help to discover how strigolactones affect the metabolism of plants. Gene expression of certain auxin transporters after the application of strigolactones could also be investigated in order to understand how strigolactones influence these transporters, similarly to the study of Lazar and Goodman (2006). Future studies should thus include the evaluation of these genes and the use of mutants would be valuable to better explain the mode of action of strigolactones and the effect these chemicals have on auxin function and transport.

Since the growth of *S. frutescens* hairy roots in the presence of IBA seemed to have a positive effect on growth and metabolism, future work should include the development of hairy root growth in bioreactors (Kim, 2002) for commercial purposes. This should include the optimisation of hairy root growth and also large-scale extraction of the metabolites of interest. In addition, future studies should test the medium for metabolites to establish whether or not metabolites are excreted into the medium.

A thorough metabolomics study is urgently needed to identify the secondary metabolites of *S. frutescens* using phytochemical techniques such as GC-MS (Fiehn et al., 2000) and NMR (Mitchell and Costisella, 2007). Our partial knowledge with regards to the metabolite pool of *S. frutescens* is limiting.

This study thus contributed to the knowledge of the metabolites found in *S. frutescens* and methods on how to successfully promote the production of some of the important metabolites found in this medicinal plant. The promotion of *S. frutescens* growth was also achieved by simple hormone applications. The aim to up-regulate growth and metabolite concentrations through the application of plant growth regulators was achieved.

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ADDENDUM A

Table A1: Investigation of the p-value correlation between treatments of the stem length of in vitro grown nodal explants.

Stem length	Untreated	GR24	NM-1	0.1 IBA	0.1 IBA + GR24	0.1 IBA + NM-1	1 IBA	1 IBA + GR24	1 IBA + NM-1	0.1 NAA	0.1 NAA + GR24	0.1 NAA + NM-1	1 NAA	1 NAA + GR24	1 NAA + NM-1
Untreated		0.5039	0.5996	0.3049	0.0913	0.8395	0.0047	0.2010	0.3605	0.0450	0.9746	0.9662	0.0022	0.0020	0.0190
GR24	0.5039		0.8689	0.0956	0.3204	0.3905	0.0007	0.5664	0.1216	0.0094	0.4921	0.5430	0.0003	0.0003	0.0035
NM-1	0.5996	0.8689		0.1179	0.2318	0.4690	0.0008	0.4435	0.1496	0.0114	0.5845	0.6410	0.0003	0.0003	0.0041
0.1 IBA	0.3049	0.0956	0.1179		0.0072	0.4167	0.0638	0.0209	0.9252	0.3077	0.3302	0.2994	0.0366	0.0340	0.1683
0.1 IBA + GR24	0.0913	0.3204	0.2318	0.0072		0.0617	0.0000	0.6530	0.0107	0.0003	0.0914	0.1096	0.0000	0.0000	0.0001
0.1 IBA + NM-1	0.8395	0.3905	0.4690	0.4167	0.0617		0.0092	0.1420	0.4805	0.0736	0.8673	0.8114	0.0046	0.0042	0.0331
1 IBA	0.0047	0.0007	0.0008	0.0638	0.0000	0.0092		0.0001	0.0559	0.4157	0.0061	0.0054	0.8160	0.7935	0.6556
1 IBA + GR24	0.2010	0.5664	0.4435	0.0209	0.6530	0.1420	0.0001		0.0296	0.0012	0.1985	0.2308	0.0000	0.0000	0.0004
1 IBA + NM-1	0.3605	0.1216	0.1496	0.9252	0.0107	0.4805	0.0559	0.0296		0.2743	0.3868	0.3526	0.0320	0.0297	0.1488
0.1 NAA	0.0450	0.0094	0.0114	0.3077	0.0003	0.0736	0.4157	0.0012	0.2743		0.0528	0.0468	0.2955	0.2823	0.7190
0.1 NAA + GR24	0.9746	0.4921	0.5845	0.3302	0.0914	0.8673	0.0061	0.1985	0.3868	0.0528		0.9422	0.0030	0.0027	0.0230
0.1 NAA + NM-1	0.9662	0.5430	0.6410	0.2994	0.1096	0.8114	0.0054	0.2308	0.3526	0.0468	0.9422		0.0026	0.0024	0.0203
1 NAA	0.0022	0.0003	0.0003	0.0366	0.0000	0.0046	0.8160	0.0000	0.0320	0.2955	0.0030	0.0026		0.9768	0.4989
1 NAA + GR24	0.0020	0.0003	0.0003	0.0340	0.0000	0.0042	0.7935	0.0000	0.0297	0.2823	0.0027	0.0024	0.9768		0.4809
1 NAA + NM-1	0.0190	0.0035	0.0041	0.1683	0.0001	0.0331	0.6556	0.0004	0.1488	0.7190	0.0230	0.0203	0.4989	0.4809	

Table A2: Investigation of the p-value correlation between treatments of the bud outgrowths of in vitro grown nodal explants.

Bud outgrowths	Untreated	GR24	NM-1	0.1 IBA	0.1 IBA + GR24	0.1 IBA + NM-1	1 IBA	1 IBA + GR24	1 IBA + NM-1	0.1 NAA	0.1 NAA + GR24	0.1 NAA + NM-1	1 NAA	1 NAA + GR24	1 NAA + NM-1
Untreated		0.0156	0.2287	0.5391	0.0018	0.1846	0.3814	0.0022	0.7565	0.8596	0.0319	0.0884	0.2210	0.1466	0.2687
GR24	0.0156		0.2011	0.0025	0.4841	0.2646	0.0013	0.5754	0.0359	0.0110	0.7701	0.4841	0.0004	0.3319	0.1999
NM-1	0.2287	0.2011		0.0670	0.0454	0.8833	0.0395	0.0577	0.3789	0.1745	0.3224	0.5801	0.0161	0.7754	0.9586
0.1 IBA	0.5391	0.0025	0.0670		0.0002	0.0520	0.7786	0.0002	0.3572	0.6718	0.0058	0.0209	0.5265	0.0391	0.0875
0.1 IBA + GR24	0.0018	0.4841	0.0454	0.0002		0.0678	0.0001	0.8728	0.0051	0.0012	0.3180	0.1621	0.0000	0.0940	0.0479
0.1 IBA + NM-1	0.1846	0.2646	0.8833	0.0520	0.0678		0.0305	0.0857	0.3135	0.1400	0.4067	0.6872	0.0122	0.8906	0.8476
1 IBA	0.3814	0.0013	0.0395	0.7786	0.0001	0.0305		0.0001	0.2417	0.4930	0.0031	0.0119	0.7317	0.0227	0.0533
1 IBA + GR24	0.0022	0.5754	0.0577	0.0002	0.8728	0.0857	0.0001		0.0064	0.0015	0.3861	0.2009	0.0000	0.1180	0.0606
1 IBA + NM-1	0.7565	0.0359	0.3789	0.3572	0.0051	0.3135	0.2417	0.0064		0.6317	0.0680	0.1648	0.1296	0.2562	0.4262
0.1 NAA	0.8596	0.0110	0.1745	0.6718	0.0012	0.1400	0.4930	0.0015	0.6317		0.0227	0.0653	0.3040	0.1102	0.2087
0.1 NAA + GR24	0.0319	0.7701	0.3224	0.0058	0.3180	0.4067	0.0031	0.3861	0.0680	0.0227		0.6783	0.0010	0.4930	0.3157
0.1 NAA + NM-1	0.0884	0.4841	0.5801	0.0209	0.1621	0.6872	0.0119	0.2009	0.1648	0.0653	0.6783		0.0044	0.7918	0.5598
1 NAA	0.2210	0.0004	0.0161	0.5265	0.0000	0.0122	0.7317	0.0000	0.1296	0.3040	0.0010	0.0044		0.0089	0.0233
1 NAA + GR24	0.1466	0.3319	0.7754	0.0391	0.0940	0.8906	0.0227	0.1180	0.2562	0.1102	0.4930	0.7918	0.0089		0.7450
1 NAA + NM-1	0.2687	0.1999	0.9586	0.0875	0.0479	0.8476	0.0533	0.0606	0.4262	0.2087	0.3157	0.5598	0.0233	0.7450	

Table A3: Investigation of the p-value correlation between treatments of the fresh mass of in vitro grown nodal explants.

Fresh mass	Untreated	GR24	NM-1	0.1 IBA	0.1 IBA + GR24	0.1 IBA + NM-1	1 IBA	1 IBA + GR24	1 IBA + NM-1	0.1 NAA	0.1 NAA + GR24	0.1 NAA + NM-1	1 NAA	1 NAA + GR24	1 NAA + NM-1
Untreated		0.8325	0.7273	0.4371	0.2198	0.8765	0.0279	0.3373	0.8078	0.1686	0.1638	0.7271	0.0000	0.0000	0.0003
GR24	0.8325		0.9007	0.3338	0.3234	0.7184	0.0192	0.4717	0.6557	0.1226	0.1189	0.5860	0.0000	0.0000	0.0002
NM-1	0.7273	0.9007		0.2562	0.3680	0.6156	0.0106	0.5353	0.5547	0.0838	0.0810	0.4903	0.0000	0.0000	0.0001
0.1 IBA	0.4371	0.3338	0.2562		0.0469	0.5400	0.1439	0.0814	0.6001	0.5297	0.5194	0.6879	0.0000	0.0000	0.0037
0.1 IBA + GR24	0.2198	0.3234	0.3680	0.0469		0.1718	0.0009	0.7666	0.1466	0.0113	0.0108	0.1259	0.0000	0.0000	0.0000
0.1 IBA + NM-1	0.8765	0.7184	0.6156	0.5400	0.1718		0.0425	0.2689	0.9306	0.2254	0.2195	0.8454	0.0000	0.0000	0.0006
1 IBA	0.0279	0.0192	0.0106	0.1439	0.0009	0.0425		0.0018	0.0521	0.4168	0.4257	0.0724	0.0002	0.0000	0.1519
1 IBA + GR24	0.3373	0.4717	0.5353	0.0814	0.7666	0.2689	0.0018		0.2328	0.0208	0.0199	0.2009	0.0000	0.0000	0.0000
1 IBA + NM-1	0.8078	0.6557	0.5547	0.6001	0.1466	0.9306	0.0521	0.2328		0.2600	0.2535	0.9127	0.0000	0.0000	0.0008
0.1 NAA	0.1686	0.1226	0.0838	0.5297	0.0113	0.2254	0.4168	0.0208	0.2600		0.9877	0.3194	0.0000	0.0000	0.0257
0.1 NAA + GR24	0.1638	0.1189	0.0810	0.5194	0.0108	0.2195	0.4257	0.0199	0.2535	0.9877		0.3120	0.0000	0.0000	0.0267
0.1 NAA + NM-1	0.7271	0.5860	0.4903	0.6879	0.1259	0.8454	0.0724	0.2009	0.9127	0.3194	0.3120		0.0000	0.0000	0.0015
1 NAA	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0002	0.0000	0.0000	0.0000	0.0000	0.0000		0.1234	0.0227
1 NAA + GR24	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.1234		0.0002
1 NAA + NM-1	0.0003	0.0002	0.0001	0.0037	0.0000	0.0006	0.1519	0.0000	0.0008	0.0257	0.0267	0.0015	0.0227	0.0002	

Table A4: Investigation of the *p*-value correlation between treatments of the dry mass of in vitro grown nodal explants.

Dry mass	Untreated	GR24	NM-1	0.1 IBA	0.1 IBA + GR24	0.1 IBA + NM-1	1 IBA	1 IBA + GR24	1 IBA + NM-1	0.1 NAA	0.1 NAA + GR24	0.1 NAA + NM-1	1 NAA	1 NAA + GR24	1 NAA + NM-1
Untreated		0.6933	0.6344	0.2986	0.3165	0.3721	0.0245	0.3262	0.3169	0.0556	0.1120	0.5148	0.0001	0.0000	0.0024
GR24	0.6933		0.9494	0.1602	0.5548	0.2090	0.0103	0.5765	0.1734	0.0249	0.0538	0.3097	0.0000	0.0000	0.0009
NM-1	0.6344	0.9494		0.1267	0.5827	0.1706	0.0064	0.6061	0.1391	0.0167	0.0388	0.2638	0.0000	0.0000	0.0004
0.1 IBA	0.2986	0.1602	0.1267		0.0440	0.8972	0.2095	0.0428	0.9842	0.3598	0.5579	0.7253	0.0045	0.0003	0.0386
0.1 IBA + GR24	0.3165	0.5548	0.5827	0.0440		0.0635	0.0016	0.9608	0.0500	0.0046	0.0118	0.1086	0.0000	0.0000	0.0001
0.1 IBA + NM-1	0.3721	0.2090	0.1706	0.8972	0.0635		0.1745	0.0626	0.9144	0.3054	0.4832	0.8250	0.0036	0.0002	0.0311
1 IBA	0.0245	0.0103	0.0064	0.2095	0.0016	0.1745		0.0014	0.2106	0.7409	0.5146	0.1214	0.1209	0.0192	0.4185
1 IBA + GR24	0.3262	0.5765	0.6061	0.0428	0.9608	0.0626	0.0014		0.0489	0.0042	0.0110	0.1092	0.0000	0.0000	0.0001
1 IBA + NM-1	0.3169	0.1734	0.1391	0.9842	0.0500	0.9144	0.2106	0.0489		0.3582	0.5519	0.7442	0.0050	0.0003	0.0402
0.1 NAA	0.0556	0.0249	0.0167	0.3598	0.0046	0.3054	0.7409	0.0042	0.3582		0.7481	0.2213	0.0601	0.0076	0.2561
0.1 NAA + GR24	0.1120	0.0538	0.0388	0.5579	0.0118	0.4832	0.5146	0.0110	0.5519	0.7481		0.3649	0.0279	0.0029	0.1464
0.1 NAA + NM-1	0.5148	0.3097	0.2638	0.7253	0.1086	0.8250	0.1214	0.1092	0.7442	0.2213	0.3649		0.0021	0.0001	0.0199
1 NAA	0.0001	0.0000	0.0000	0.0045	0.0000	0.0036	0.1209	0.0000	0.0050	0.0601	0.0279	0.0021		0.4262	0.4666
1 NAA + GR24	0.0000	0.0000	0.0000	0.0003	0.0000	0.0002	0.0192	0.0000	0.0003	0.0076	0.0029	0.0001	0.4262		0.1301
1 NAA + NM-1	0.0024	0.0009	0.0004	0.0386	0.0001	0.0311	0.4185	0.0001	0.0402	0.2561	0.1464	0.0199	0.4666	0.1301	

Table A5: Summary of the SU1 content of in vitro grown nodal explants

Treatment	No. of samples	Minimum (µg/g)	Maximum (µg/g)	Mean (µg/g)	Standard deviation
Untreated	5	90.39216	151.6000	124.5086	25.65155
GR24	5	71.13208	89.40000	82.65269	7.693615
NM-1	5	75.80000	114.9020	98.72769	17.92431
NAA	5	103.4000	166.5385	138.1610	24.55666
NAA + GR24	5	102.7451	164.6000	127.1588	23.86622
NAA + NM-1	5	145.4000	199.2157	172.7224	22.10304

Table A6: Investigation of the p-value correlation between treatments of the SU1 content of in vitro grown nodal explants.

Treatment	Untreated	GR24	NM-1	NAA	NAA + GR24	NAA + NM-1
Untreated		0.047159	0.414037	0.907482	0.999955	0.016299
GR24	0.047159		0.833401	0.004535	0.030558	0.000143
NM-1	0.414037	0.833401		0.069162	0.311276	0.000272
NAA	0.907482	0.004535	0.069162		0.960929	0.142210
NAA + GR24	0.999955	0.030558	0.311276	0.960929		0.025596
NAA + NM-1	0.016299	0.000143	0.000272	0.142210	0.025596	

Table A7: Investigation of the p-value correlation between treatments of the L-Arginine content of in vitro grown nodal explants.

L-Arginine	Untreated	GR24	NM-1	NAA	NAA + GR24	NAA + NM-1
Untreated		0.1901	0.2380	0.5823	0.6116	0.0000
GR24	0.1901		0.9514	0.4362	0.4121	0.0001
NM-1	0.2380	0.9514		0.4999	0.4751	0.0002
NAA	0.5823	0.4362	0.4999		0.9660	0.0000
NAA + GR24	0.6116	0.4121	0.4751	0.9660		0.0000
NAA + NM-1	0.0000	0.0001	0.0002	0.0000	0.0000	

Table A8: Investigation of the p-value correlation between treatments of the L-Asparagine content of in vitro grown nodal explants.

L-Asparagine	Untreated	GR24	NM-1	NAA	NAA + GR24	NAA + NM-1
Untreated		0.1174	0.0292	0.0147	0.0083	0.0011
GR24	0.1174		0.4360	0.3221	0.2194	0.0482
NM-1	0.0292	0.4360		0.8734	0.6947	0.2523
NAA	0.0147	0.3221	0.8734		0.8043	0.2937
NAA + GR24	0.0083	0.2194	0.6947	0.8043		0.4185
NAA + NM-1	0.0011	0.0482	0.2523	0.2937	0.4185	

Table A9: Investigation of the p-value correlation between treatments of the L-Canavanine content of in vitro grown nodal explants.

L-Canavanine	Untreated	GR24	NM-1	NAA	NAA + GR24	NAA + NM-1
Untreated		0.8571	0.0907	0.1935	0.5876	0.3734
GR24	0.8571		0.0651	0.2590	0.7163	0.4753
NM-1	0.0907	0.0651		0.0060	0.0319	0.0152
NAA	0.1935	0.2590	0.0060		0.4380	0.6701
NAA + GR24	0.5876	0.7163	0.0319	0.4380		0.7237
NAA + NM-1	0.3734	0.4753	0.0152	0.6701	0.7237	

Table A10: Investigation of the p-value correlation between treatments of the GABA content of in vitro grown nodal explants.

GABA	Untreated	GR24	NM-1	NAA	NAA + GR24	NAA + NM-1
Untreated		0.9077	0.4215	0.6839	0.8671	0.5325
GR24	0.9077		0.3626	0.7705	0.9589	0.6104
NM-1	0.4215	0.3626		0.2396	0.3382	0.1702
NAA	0.6839	0.7705	0.2396		0.8101	0.8268
NAA + GR24	0.8671	0.9589	0.3382	0.8101		0.6467
NAA + NM-1	0.5325	0.6104	0.1702	0.8268	0.6467	

Table A11: Investigation of the p-value correlation between treatments of the putrescine content of in vitro grown nodal explants.

Putrescine	Untreated	GR24	NM-1	NAA	NAA + GR24	NAA + NM-1
Untreated		0.7607	0.6234	0.5045	0.3970	0.3201
GR24	0.7607		0.8377	0.3342	0.2535	0.1984
NM-1	0.6234	0.8377		0.2672	0.2026	0.1589
NAA	0.5045	0.3342	0.2672		0.8549	0.7384
NAA + GR24	0.3970	0.2535	0.2026	0.8549		0.8796
NAA + NM-1	0.3201	0.1984	0.1589	0.7384	0.8796	

Table A12: Investigation of the p-value correlation between treatments of the spermidine content of in vitro grown nodal explants.

Spermidine	Untreated	GR24	NM-1	NAA	NAA + GR24	NAA + NM-1
Untreated		0.6352	0.3776	0.9828	0.5754	0.1310
GR24	0.6352		0.6595	0.6506	0.9311	0.2890
NM-1	0.3776	0.6595		0.3884	0.7192	0.5696
NAA	0.9828	0.6506	0.3884		0.5901	0.1362
NAA + GR24	0.5754	0.9311	0.7192	0.5901		0.3287
NAA + NM-1	0.1310	0.2890	0.5696	0.1362	0.3287	

Table A13: Investigation of the *p*-value correlation between untreated and strigolactone treatments of the amino acid and polyamine content of in vitro grown nodal explants.

Amino acid or polyamine	Untreated					GR24					Nijmegen-1				
	No. of samples	Min (µg/g)	Max (µg/g)	Mean (µg/g)	Standard deviation	No. of samples	Min (µg/g)	Max (µg/g)	Mean (µg/g)	Standard deviation	No. of samples	Min (µg/g)	Max (µg/g)	Mean (µg/g)	Standard deviation
Alanine	5	715.19	2234.13	1674.94	586.57	5	572.53	1077.4	782.48	189.07	4	741.75	2392.79	1223.23	789.64
L-Arginine	5	33236.29	73057.54	49457.19	15521.57	5	38746.62	107228.0	69617.66	26978.25	4	48842.72	98495.03	68642.52	21137.54
L-Asparagine	5	5710.00	9840.00	8034.00	1507.36	5	7480.00	10460.0	9124.00	1095.18	4	8960.00	10550.00	9687.50	779.55
Aspartate	5	867.45	1740.08	1336.85	313.22	5	750.48	2119.2	1709.48	551.48	4	1337.86	2421.25	1839.55	501.51
L-Canavanine	5	8250.00	22390.00	12742.00	5580.38	5	6110.00	20180.0	12272.00	5605.10	4	15060.00	19120.00	17577.50	1782.96
Cysteine	5	14.77	39.68	27.11	9.95	5	32.88	154.8	80.47	51.43	4	56.31	119.54	84.86	32.40
GABA	5	370.00	1200.00	840.00	307.73	5	250.00	1490.0	786.00	496.87	4	310.00	3870.00	1240.00	1753.74
Glutamate	5	3614.04	5315.48	4427.60	627.49	5	3017.41	5830.5	4670.16	1049.64	4	4254.37	5383.70	4738.21	500.55
Glycine	5	31.19	109.13	63.65	28.71	5	65.76	105.4	88.44	18.23	4	53.68	447.82	173.06	185.77
Histidine	5	989.45	2257.94	1617.52	508.30	5	1042.55	3020.9	1841.63	818.63	4	1714.56	2850.89	2130.38	496.14
Isoleucine	5	175.44	301.59	245.67	52.91	5	79.30	408.7	262.01	128.31	4	339.81	931.69	494.32	291.78
Leucine	5	662.77	1557.54	1137.67	351.56	5	442.94	1561.5	1247.13	461.66	4	936.38	1398.48	1160.57	224.98
Lysine	5	101.36	172.62	134.33	25.61	5	75.44	295.0	192.22	86.64	4	147.61	1087.29	404.54	455.67
Methionine	5	2.04	29.76	11.97	10.49	5	3.86	19.9	12.44	6.93	4	5.96	18.98	11.30	5.81

Phenylalanine	5	161.79	408.73	256.40	99.40	5	147.00	443.5	284.59	123.13	4	297.09	1220.11	555.31	444.53
Proline	5	5553.61	15023.81	8324.99	3862.35	5	8137.07	16374.5	12147.05	3360.86	4	7139.81	13508.54	10898.66	3111.18
Putrescine	5	2530.00	4660.00	3326.00	902.46	5	1400.00	5550.0	3016.00	1637.54	4	1850.00	3940.00	2795.00	1035.71
Serine	5	1910.33	2777.78	2265.54	316.60	5	1069.63	4012.6	2647.90	1142.34	4	2477.67	3731.81	3208.74	575.70
Spermidine	5	710.00	1290.00	988.00	220.27	5	540.00	2080.0	1120.00	588.68	4	960.00	1520.00	1250.00	230.22
Threonine	5	1559.45	5720.24	3224.92	1551.32	5	843.33	6085.8	3468.07	2472.72	4	3555.34	7421.47	5191.39	1618.15
Tyrosine	5	4.07	17.86	11.62	5.57	5	7.74	27.2	16.78	8.35	4	9.71	24.67	18.72	7.01
Valine	5	354.43	587.30	462.70	95.36	5	177.95	590.0	426.47	168.68	4	487.38	1493.36	777.39	479.24

Table A14: Investigation of the p-value correlation between NAA containing treatments of the amino acid and polyamine content of in vitro grown nodal explants.

Amino acid or polyamine	NAA					NAA + GR24					NAA + Nijmegen-1				
	No. of samples	Min (µg/g)	Max (µg/g)	Mean (µg/g)	Standard deviation	No. of samples	Min (µg/g)	Max (µg/g)	Mean (µg/g)	Standard deviation	No. of samples	Min (µg/g)	Max (µg/g)	Mean (µg/g)	Standard deviation
Alanine	5	756.76	3542.00	1688.12	1073.91	5	613.28	3064.91	1617.90	928.36	5	1466.82	3735.0	2072.6	939.72
L-Arginine	5	29503.86	71744.00	57786.74	18177.67	5	36009.77	72699.80	57144.15	15213.57	5	98210.28	190836.9	137646.2	36589.90
L-Asparagine	5	8640.00	10860.00	9802.00	940.76	5	8600.00	10810.00	9970.00	860.67	5	9540.00	11930.0	10522.0	949.35
Aspartate	5	889.96	1828.87	1368.33	386.60	5	933.59	1401.64	1127.08	178.78	5	2594.58	4686.9	3505.3	901.45
L-Canavanine	5	5720.00	13750.00	9284.00	2964.66	5	8560.00	14900.00	11322.00	2741.50	5	5520.00	16070.0	10398.0	3817.70
Cysteine	5	22.00	70.10	48.47	19.02	5	27.34	90.16	55.47	22.55	5	67.08	214.2	144.8	57.15
GABA	5	390.00	930.00	650.00	229.78	5	370.00	1690.00	762.00	531.53	5	270.00	870.0	548.0	262.05
Glutamate	5	3110.04	5738.14	4542.56	981.08	5	3053.54	4184.43	3480.15	462.92	5	8003.16	12683.7	10067.0	1910.52
Glycine	5	60.00	106.30	84.35	19.07	5	53.33	84.02	67.12	11.18	5	144.42	224.5	200.6	34.32
Histidine	5	837.84	2360.82	1829.25	618.00	5	1144.53	3113.59	2176.23	747.18	5	2838.00	5528.4	4291.1	1013.69
Isoleucine	5	176.00	296.91	223.96	45.36	5	171.43	286.89	215.62	45.21	5	387.00	839.9	644.0	164.65
Leucine	5	792.00	1445.36	1079.42	256.03	5	360.00	1159.84	764.34	294.15	5	2345.58	3021.8	2623.1	281.59
Lysine	5	81.08	154.43	121.63	30.09	5	89.84	155.74	123.96	23.80	5	242.52	423.3	339.7	68.49
Methionine	5	0.00	9.42	3.04	4.36	5	0.00	18.44	7.54	7.87	5	19.92	35.7	26.5	5.73
Phenylalanine	5	225.87	387.63	309.26	61.37	5	205.08	366.80	248.34	67.00	5	521.16	775.2	667.6	99.92

Proline	5	8194.98	15336.00	12725.00	2944.71	5	8671.88	16685.60	13251.95	3154.05	5	19631.16	40652.1	31413.1	8219.31
Putrescine	5	3680.00	4300.00	4008.00	252.33	5	2300.00	7280.00	4194.00	1848.07	5	2110.00	8580.0	4348.0	2600.05
Serine	5	1812.74	3096.00	2393.68	509.23	5	2020.95	3313.52	2394.64	521.59	5	4055.76	7041.8	5887.8	1119.14
Spermidine	5	720.00	1410.00	994.00	279.70	5	920.00	1360.00	1144.00	181.88	5	860.00	2590.0	1418.0	733.12
Threonine	5	1559.85	3789.08	2777.34	891.17	5	1874.24	4077.87	2789.10	832.91	5	3384.96	10827.3	7046.5	3031.36
Tyrosine	5	5.79	24.00	12.27	7.07	5	0.00	32.45	14.26	12.64	5	0.00	64.7	31.5	26.28
Valine	5	349.42	642.00	486.96	115.70	5	322.27	598.38	484.59	103.33	5	794.64	1187.8	1045.5	154.35

Table A15: Investigation of the correlation between treatments of the dry mass of in vitro grown hairy roots.

Dry mass	Untreated	GR24	NM-1	0.1 IBA	0.1 IBA + GR24	0.1 IBA + NM-1	1 IBA	1 IBA + GR24	1 IBA + NM-1	0.1 NAA	0.1 NAA + GR24	0.1 NAA + NM-1	1 NAA	1 NAA + GR24	1 NAA + NM-1
Untreated		0.3073	0.0193	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GR24	0.3073		0.1745	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
NM-1	0.0193	0.1745		0.0001	0.0013	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
0.1 IBA	0.0000	0.0000	0.0001		0.4125	0.8977	0.0000	0.0000	0.0000	0.0000	0.0000	0.0006	0.0000	0.0000	0.0000
0.1 IBA + GR24	0.0000	0.0000	0.0013	0.4125		0.3437	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
0.1 IBA + NM-1	0.0000	0.0000	0.0001	0.8977	0.3437		0.0000	0.0000	0.0000	0.0000	0.0000	0.0008	0.0000	0.0000	0.0000
1 IBA	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.3565	0.0083	0.0757	0.0000	0.0000	0.0428	0.0829	0.0795
1 IBA + GR24	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.3565		0.0769	0.3834	0.0008	0.0000	0.0039	0.0092	0.0087
1 IBA + NM-1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0083	0.0769		0.3602	0.0889	0.0003	0.0000	0.0000	0.0000
0.1 NAA	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0757	0.3834	0.3602		0.0102	0.0000	0.0003	0.0007	0.0007
0.1 NAA + GR24	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0008	0.0889	0.0102		0.0432	0.0000	0.0000	0.0000
0.1 NAA + NM-1	0.0000	0.0000	0.0000	0.0006	0.0000	0.0008	0.0000	0.0000	0.0003	0.0000	0.0432		0.0000	0.0000	0.0000
1 NAA	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0428	0.0039	0.0000	0.0003	0.0000	0.0000		0.7608	0.7764
1 NAA + GR24	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0829	0.0092	0.0000	0.0007	0.0000	0.0000	0.7608		0.9836
1 NAA + NM-1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0795	0.0087	0.0000	0.0007	0.0000	0.0000	0.7764	0.9836	

Table A16: Investigation of the correlation between treatments of the primary root length of in vitro grown hairy roots.

Primary root length (19 Days)	Untreated	GR24	NM-1	IBA	IBA + GR24	IBA + NM-1
Untreated		0.5083	0.0270	0.0000	0.0000	0.0000
GR24	0.5083		0.1188	0.0000	0.0000	0.0000
NM-1	0.0270	0.1188		0.0003	0.0018	0.0011
IBA	0.0000	0.0000	0.0003		0.5677	0.6597
IBA + GR24	0.0000	0.0000	0.0018	0.5677		0.8947
IBA + NM-1	0.0000	0.0000	0.0011	0.6597	0.8947	

Table A17: Investigation of the correlation between treatments of the lateral root number of in vitro grown hairy roots.

Lateral root number (19 Days)	Untreated	GR24	NM-1	IBA	IBA + GR24	IBA + NM-1
Untreated		0.6288	0.7490	0.0014	0.0301	0.0047
GR24	0.6288		0.8701	0.0065	0.0906	0.0185
NM-1	0.7490	0.8701		0.0040	0.0638	0.0119
IBA	0.0014	0.0065	0.0040		0.2934	0.7063
IBA + GR24	0.0301	0.0906	0.0638	0.2934		0.4996
IBA + NM-1	0.0047	0.0185	0.0119	0.7063	0.4996	

Table A18: Investigation of the p-value correlation between treatments of the L-Arginine content of in vitro grown hairy roots.

L-Arginine	Untreated	GR24	NM-1	IBA
Untreated		0.909879	0.851143	0.002316
GR24	0.909879		0.749960	0.001146
NM-1	0.851143	0.749960		0.002225
IBA	0.002316	0.001146	0.002225	

Table A19: Investigation of the p-value correlation between treatments of the L-Asparagine content of in vitro grown hairy roots.

L-Asparagine	Untreated	GR24	NM-1	IBA
Untreated		0.189488	0.934809	0.674215
GR24	0.189488		0.190967	0.075093
NM-1	0.934809	0.190967		0.595134
IBA	0.674215	0.075093	0.595134	

Table A20: Investigation of the *p*-value correlation between treatments of the GABA content of in vitro grown hairy roots.

GABA	Untreated	GR24	NM-1	IBA
Untreated		0.820906	0.633738	0.030975
GR24	0.820906		0.789700	0.037601
NM-1	0.633738	0.789700		0.062868
IBA	0.030975	0.037601	0.062868	

Table A21: Investigation of the *p*-value correlation between treatments of the putrescine content of in vitro grown hairy roots.

Putrescine	Untreated	GR24	NM-1	IBA
Untreated		0.201527	0.799809	0.007435
GR24	0.201527		0.111574	0.082170
NM-1	0.799809	0.111574		0.002888
IBA	0.007435	0.082170	0.002888	

Table A22: Investigation of the *p*-value correlation between treatments of the spermidine content of in vitro grown hairy roots.

Spermidine	Untreated	GR24	NM-1	IBA
Untreated		0.747155	0.362128	0.516770
GR24	0.747155		0.198517	0.309192
NM-1	0.362128	0.198517		0.773756
IBA	0.516770	0.309192	0.773756	

Table A23: Investigation of the *p*-value correlation between treatments of the amino acid and polyamine content of in vitro grown hairy roots.

Amino acid or polyamine	Untreated					IBA				
	No. of samples	Minimum (µg/g)	Maximum (µg/g)	Mean (µg/g)	Standard deviation	No. of samples	Minimum (µg/g)	Maximum (µg/g)	Mean (µg/g)	Standard deviation
Alanine	4	32.00	86.0	61.85	22.36	5	73.80	722.00	242.84	272.19
L-Arginine	4	161.00	294.8	228.60	55.40	5	801.80	3949.60	1845.56	1260.67
L-Asparagine	4	5509.80	15451.4	11221.55	4149.26	5	9002.80	17166.40	12414.60	3510.67
Aspartate	4	22.20	89.6	44.90	30.32	5	30.20	184.20	71.12	65.16
Cysteine	4	4.60	21.6	14.35	7.11	5	3.00	14.00	8.20	4.43
GABA	4	12.00	39.0	24.00	11.22	5	30.00	189.00	79.80	64.12
Glutamate	4	92.20	316.8	204.40	91.97	5	119.80	494.60	238.24	148.66
Glutamine	4	172.80	371.8	286.65	82.96	5	1180.20	2760.40	1827.60	593.25
Glycine	4	9.00	16.2	12.95	2.98	5	13.60	56.80	28.64	17.70
Histidine	4	44.00	110.0	69.40	28.54	5	59.60	132.20	89.96	27.69
Isoleucine	4	34.60	151.6	89.30	48.18	5	66.60	167.80	113.12	41.05
Leucine	4	12.20	44.0	26.30	13.15	5	32.00	85.00	60.64	24.33
Lysine	4	1.80	5.2	3.30	1.41	5	2.20	4.80	3.56	1.11
Methionine	4	12.60	34.2	23.20	8.82	5	17.20	38.80	27.80	8.06
Phenylalanine	4	54.20	429.8	216.65	164.19	5	167.20	559.00	379.76	174.90
Proline	4	3.60	9.8	6.50	2.61	5	7.20	50.20	21.60	17.43
Putrescine	4	52912.00	104146.0	75848.00	21288.77	5	30110.00	70094.00	41502.00	16230.08
Serine	4	61.00	122.0	88.60	25.29	5	116.00	1118.60	466.72	390.71
Spermidine	4	1140.00	2004.0	1400.50	405.31	5	1152.00	3602.00	1741.20	1044.77
Threonine	4	36.20	113.0	72.60	31.58	5	86.80	455.60	242.00	147.98
Tryptophan	4	24.80	92.4	58.15	28.27	5	25.60	59.80	40.36	13.68
Tyrosine	4	860.60	1718.6	1264.30	352.39	5	486.80	1155.60	678.56	272.73
Valine	4	23.60	104.2	62.35	33.34	5	87.20	294.20	176.16	88.77
Amino acid or polyamine	GR24					Nijmegen-1				
	No. of samples	Minimum (µg/g)	Maximum (µg/g)	Mean (µg/g)	Standard deviation	No. of samples	Minimum (µg/g)	Maximum (µg/g)	Mean (µg/g)	Standard deviation
Alanine	5	51.80	95.20	71.64	17.750	5	57.20	158.80	102.48	37.86
L-Arginine	5	126.60	327.20	177.76	84.548	5	154.00	490.80	312.92	163.15
L-Asparagine	5	4724.80	9317.40	7396.76	1738.910	5	4577.60	20602.40	10990.08	6022.52
Aspartate	5	17.80	54.60	36.32	13.847	5	26.80	45.80	36.12	7.29
Cysteine	5	5.60	11.80	9.16	2.488	5	0.60	18.80	10.60	7.03
GABA	5	18.00	39.00	29.40	8.325	5	21.00	63.00	35.40	17.42
Glutamate	5	133.20	198.80	169.52	27.810	5	156.00	255.20	212.48	41.74
Glutamine	5	104.80	273.00	165.60	64.718	5	116.80	836.80	354.92	290.24
Glycine	5	9.00	25.80	13.96	7.076	5	10.20	19.80	14.40	3.90
Histidine	5	44.20	82.00	59.92	15.197	5	46.60	102.00	78.72	21.13
Isoleucine	5	45.00	76.20	64.20	12.472	5	59.20	94.80	80.04	18.16

Leucine	5	14.00	27.00	21.48	5.686	5	14.80	34.00	26.76	7.66
Lysine	5	1.80	7.00	3.80	1.980	5	2.60	5.20	3.36	1.05
Methionine	5	17.00	25.80	20.60	3.453	5	20.00	31.80	26.96	4.88
Phenylalanine	5	85.00	256.20	147.24	64.376	5	53.80	340.60	173.08	107.86
Proline	5	5.00	11.60	7.60	2.596	5	6.60	10.80	8.56	1.65
Putrescine	5	54186.00	72104.00	61010.80	7370.807	5	53074.00	97734.00	78715.20	19246.88
Serine	5	59.20	149.20	97.84	39.438	5	81.20	149.80	110.40	25.36
Spermidine	5	788.00	1896.00	1232.00	424.855	5	1074.00	3020.00	1882.80	893.72
Threonine	5	39.80	95.20	66.12	22.427	5	39.00	104.80	74.56	24.55
Tryptophan	5	21.80	44.80	37.32	9.111	5	24.20	71.80	51.16	18.72
Tyrosine	5	816.20	1148.20	937.00	138.167	5	877.80	1727.20	1300.36	355.72
Valine	5	30.20	59.20	48.04	12.949	5	32.00	69.20	55.68	15.66

ADDENDUM B

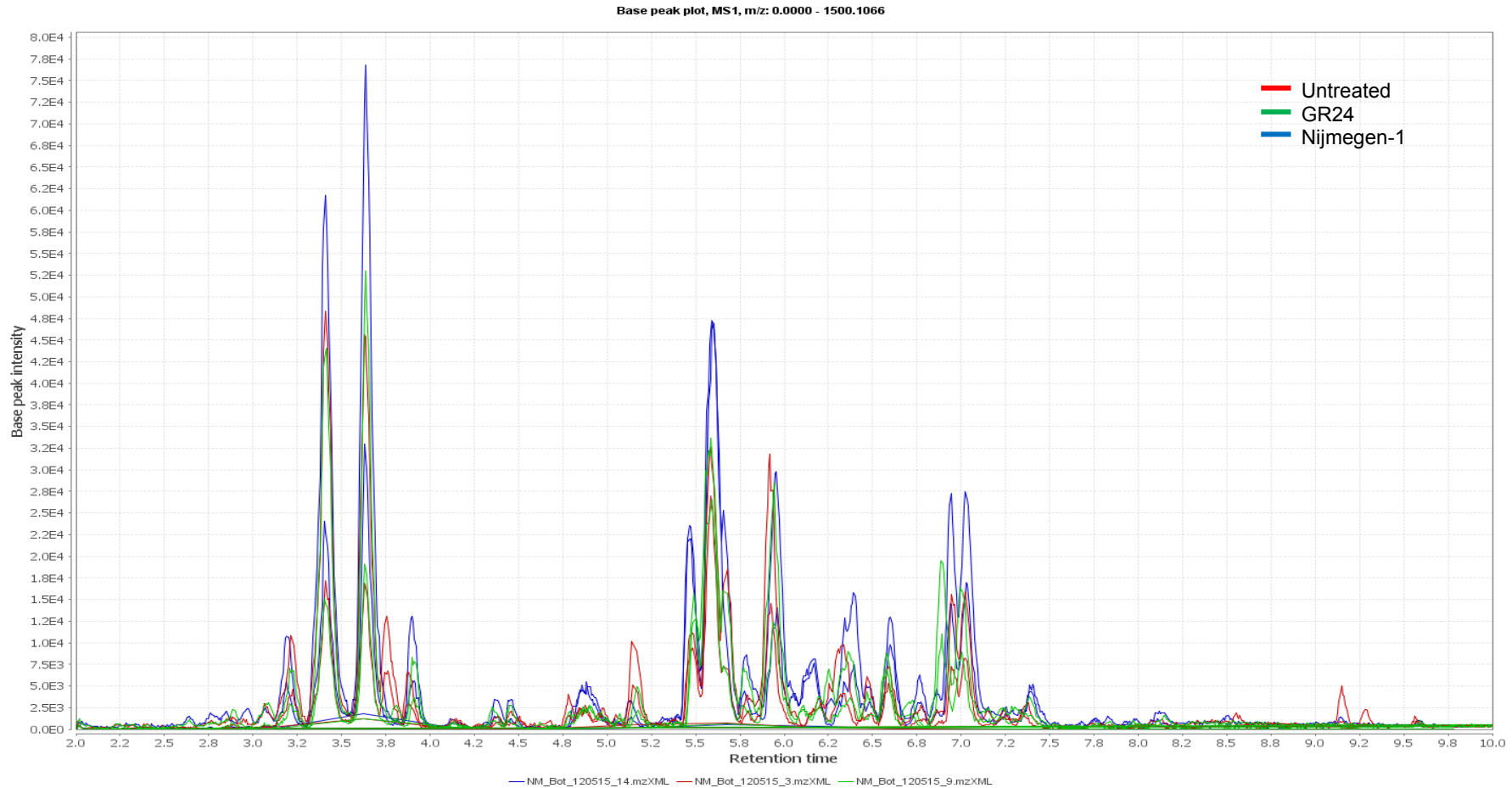


Figure B1: Stacked LC-MS chromatogram of untreated and strigolactone treated *in vitro* nodal explants. m/z: 0.0000 – 1500.1066

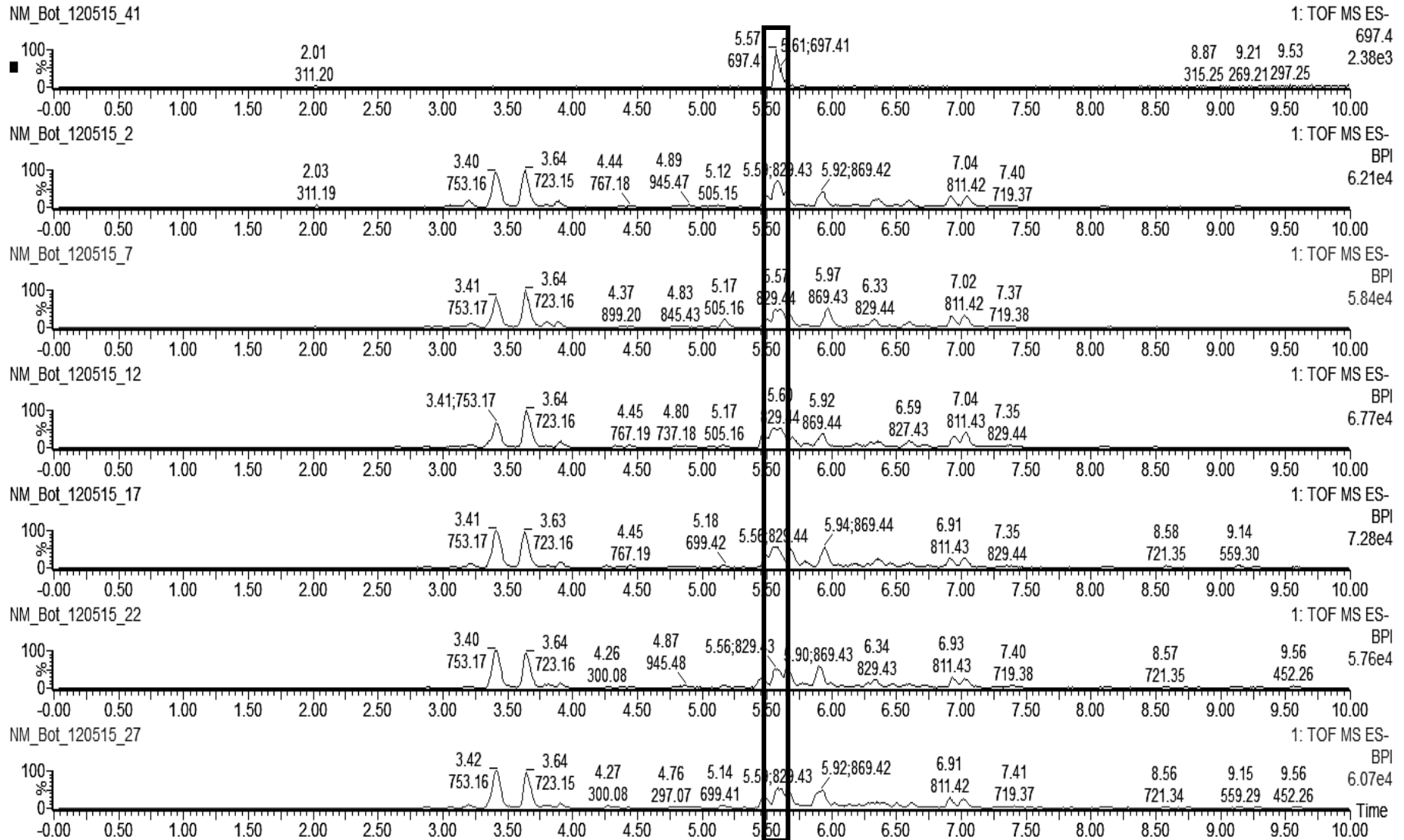


Figure B2: LC-MS chromatogram for treated in vitro grown plants. a) Sutherlandioside B, b) untreated, c) GR24-treated, d) Nijmegen-1-treated, e) NAA-treated, f) NAA and GR24-treated, g) NAA and Nijmegen-1-treated. The Sutherlandioside B is highlighted in the treatments.